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Functional activity of *E. coli* RNase R in the Antarctic *Pseudomonas syringae* Lz4W



Ashaq Hussain^{1*} and Malay Kumar Ray¹

Abstract

Background In Antarctic *P. syringae* RNase R play an essential role in the processing of 16S and 5S rRNA, thereby playing an important role in cold-adapted growth of the bacterium. This study is focused on deciphering the in vivo functional activity of mesophilic exoribonuclease R and its catalytic domain (RNB) in an evolutionary distant psychrophilic bacterium *Pseudomonas syringae* Lz4W.

Results Our results confirm that *E. coli* RNase R complemented the physiological functions of the psychrophilic bacterium *P. syringae* RNase R and rescued the cold-sensitive phenotype of *Pseudomonas syringae* Δrnr mutant. More importantly, the catalytic domain (RNB) of the *E. coli* RNase R is also capable of alleviating the cold-sensitive growth defects of Δrnr mutant as seen with the catalytic domain (RNB) of the *P. syringae* enzyme. The Catalytic domain of *E. coli* RNase R was less efficient than the Catalytic domain of *P. syringae* RNase R in rescuing the cold-sensitive growth of Δrnr mutant at 4°C, as the Δrnr expressing the RNB^{EC} (catalytic domain of *E. coli* RNase R) displayed longer lag phase than the RNB^{Ps} (Catalytic domain of *P. syringae* RNase R) complemented Δrnr mutant at 4°C. Altogether it appears that the *E. coli* RNase R and *P. syringae* RNase R are functionally exchangeable for the growth requirements of *P. syringae* at low temperature (4°C). Our results also confirm that in *P. syringae* the requirement of RNase R for supporting the growth at 4°C is independent of the degradosomal complex.

Conclusion *E. coli* RNase R (RNase R^{Ec}) rescues the cold-sensitive phenotype of the *P. syringae* Δrnr mutant. Similarly, the catalytic domain of *E. coli* RNase R (RNB^{Ec}) is also capable of supporting the growth of Δrnr mutant at low temperatures. These findings have a vast scope in the design and development of low-temperature-based expression systems.

Keywords Psychrophiles, Exoribonuclease R, Cold-adapted enzymes, Degradosome, Functional complementation, RNA processing, Catalytic domain

Background

RNase R is a conserved hydrolytic ribonuclease (3' to 5') that belongs to RNase II family of exoribonucleases. This processive enzyme is capable of degrading the RNA molecules through their complex secondary structures, unlike the exoribonuclease RNase II, which can degrade only single-stranded RNAs [1-4]. The domain

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arrangement of RNase R is similar to that of RNase II and Rrp44, with the CSD1, CSD2, and S1 domains capping the top of the RNB domain [1, 5] (Fig. 1). The crystal structure of *E. coli* RNase R revealed a unique feature that differs from the crystal structures of other reported exoribonucleases is the presence of two open channels that act as important RNA binding sites, i.e., a top channel between the S1 and CSD1 domains and a side channel between the RNB and CSD1 domains [1, 6–8]. Mutational analysis of the enzyme has established that the aspartic acid residues D272, D278, and D280 in the catalytic pocket of the *E. coli* RNase R (RNase R^{EC}) are important for the ribonuclease activity, and D280 is directly



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Fig. 1 Domain organization of RNase R. Schematic representing the domain organization in *E.coli* and *P. syringae* RNase R. Individual domains are shaded in separate colors and numbers represent the amino acids in the primary sequence of protein

involved in the catalysis during the hydrolytic cleavage of terminal nucleotides from the RNA chain. Further, the residue Y324 plays a key role in deciding the final length of end-products. The substrate binding domains (CSD1, CSD2, and S1) bind only to single-stranded 3'-overhang ends of RNA molecules, thereby screening the substrates and regulating the entry of RNA into the catalytic pocket of the enzyme [9]. Structural comparison of *E. coli* RNase R with other RNase II family proteins revealed two open RNA-binding channels in RNase R and suggested a trihelix 'wedge' region in the RNB domain that may induce RNA unwinding. Construction of two tri-helix wedge mutants revealed that mutants, indeed lost their RNA unwinding but not RNA binding or degrading activities. Structural studies revealed that duplex RNA with an overhang is bound in the two RNA-binding channels in RNase R. The 3' overhang is channeled into the active site and duplex RNA is unwound upon reaching the wedge region during RNA degradation [1].

Thus, the RNA binding domains CSD1, CSD2, and S1 play multiple roles in substrate recognition and sensing of 3['] overhangs of RNA molecules, whereas RNB domain plays a critical role in unwinding and processing/degradation of double-stranded RNA molecules [1, 2, 10].

Physiologically, RNase R plays a key role in the RNA metabolism of cell via degradation of polyadenylated RNAs, degradation of mRNA transcripts containing REP (repetitive extragenic palindromes) sequences [3], degradation of defective and non-functional tRNAs, trans-translation and quality control of ribosomes [11], turnover of ribosomal RNAs (e.g., 16S and 23S rRNAs) [8, 12–15] and growth-phase specific (e.g., stationary phase) removal of *ompA* mRNA in *E. coli* [16]. Depletion of RNase R affects tmRNA (transfer-messenger RNA) metabolism as evidenced by the accumulation of tmRNA precursors and tmRNA degradation intermediates [17]. The tmRNA-dependent trans-translation pathway is important for releasing the stalled ribosomes from

truncated or defective mRNAs, tagging the proteins and peptides produced from the truncated mRNAs and facilitating the degradation of both defective RNAs and proteins. Thus, RNase R also plays a role in the maintenance of protein quality in the cells [18].

RNase R is a stress-induced protein that shows increased expression at low temperatures and stationary phase [16, 18]. In E. coli RNase R is encoded by rnr gene, and its levels in the cell are regulated by RNase G, RNase E, and SmpB [19, 20]. In Streptococcus pneumoniae, Aeromonas hydrophila, pathogenic E. coli, and Shigella flexneri, disruption of rnr gene leads to a decrease in virulence [21-23]. RNase R is also required for the low-temperature growth of Legionella pneumophila and A. hydrophila [24]. In Mycoplasma genitalium bearing the smallest bacterial genome, RNase R is the only exoribonuclease that plays a crucial role in all RNA metabolic processes including the processing and degradation of different types of RNA molecules [25-27]. Recent studies have illustrated that methylated ribose in the ribonucleotides acts as stop signals for RNase R-mediated RNA degradation [26]. This indicates that ribose methylation status of RNA might be used as a signaling mechanism by bacteria for the screening of RNA molecules to be sent for degradation or processing and maturation.

In our laboratory, RNase R was found to be a component of the novel RNA degradosomal complex of *P. syringae* Lz4W, in which RNase R is associated with the endoribonuclease RNase E and RNA helicase RhIE [28, 29]. The disruption of *rnr* gene led to a cold-sensitive phenotype of the Antarctic bacterium. Further biochemical investigations revealed that cold-sensitive Δrnr is defective in the 3'-end processing of 16S and 5S rRNAs, as a result of which *rnr* mutants accumulated unprocessed 16S rRNA and 5S rRNA precursor molecules in the cells [30]. Collectively, these studies have established three important things: first, psychrophilic bacteria like *P. syringae* Lz4W possess novel degradosome that has replaced the ss-RNA degrading exoribonuclease PNPase with the ds-RNA degrading enzyme RNase R in the protein complex; second, RNase R plays a crucial role in cold adaptation, and third, remarkably, the exoribonuclease enzyme plays a role in the 3'-end processing of 16S rRNAs which was hitherto unknown, but predicted to be an endoribonuclease mediated process [30].

RNase R is essential for growth of P. syringae at low temperature. *P. syringae* Δrnr mutants display cold sensitive phenotype when grown at 4°C, whereas *E. coli* Δrnr mutants do not display any low temperature associated growth defect. At molecular level, Δrnr mutants of P. syringae and E. coli accumulate rRNA degradation intermediates suggesting their role in rRNA degradation or quality control [28, 31]. However, Δrnr mutant of *P. syrin*gae accumulates 3'-end unprocessed 16S and 5S rRNA precursors at low temperature, while E. coli mutant does not. The *P. syringae* Δrnr mutant is also defective in tmRNA degradation and processing. Thus, there were distinct similarities and differences in the activities and requirements of exoribonucleases (RNase R^{Ps} and RNase R^{Ec}) during growth of the psychrophilic and mesophilic bacteria. Therefore, it was important to know whether E. coli RNase R would be able to complement the coldsensitive growth defect of Δrnr mutant of the Antarctic P. syringae.

The objectives of this study are (i) Will *E. coli* RNase R (RNase R^{Ec}) complement the cold-sensitive phenotype of *P. syringae* Δrnr mutant? (ii) Will the catalytic domain (RNB^{Ec}) of *E. coli* RNase R be able to carry out similar in vivo functions like the catalytic domain (RNB^{Ps}) of *P. syringae* RNase R despite their differences in in vitro activities?

Our study clearly demonstrates that the *E. coli*-specific exoribonuclease R (RNase R^{Ec}) has retained all the functions that are necessary to support the growth of psychrophilic *P. syringae* at 4 °C. This is remarkable as *E. coli* itself does not grow at 4°C, and the enzymes in spite of their evolutionary divergence and alteration in the biochemical properties, have maintained the common essential activities. The individual catalytic domains (RNB^{Ps} and RNB^{Ec}) are also functionally similar, as both of them are capable of supporting the growth of Δrnr mutant at 4°C.

Methods

Growth media and bacterial cultures

The Antarctic *P.syringae* Lz4W was routinely grown at 22°C or 4°C (for optimum and low temperatures respectively) in Antarctic bacterial medium (ABM) composed of 5 g/l peptone and 2.0 g/l yeast extract, as described earlier [32, 33]. *E. coli* strains were cultured at 37°C in Luria–Bertani medium, which contained 10 g/l tryptone,

5 g/l yeast extract, and 10 g/l NaCl [34]. For solid media, 15 g/l bacto-agar (Hi Media) was added to ABM or LB. Both ABM and LB media were supplemented with ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), and tetracycline (10 μ g/ml) as per requirement.

Fresh ABM broth was inoculated with 1% of primary culture and incubated at 22°C or 4°C with constant shaking. The optical density of bacterial culture was measured after different time intervals at 600 nm $[OD_{600}]$ and plotted against time.

Molecular biology methods

General molecular biology techniques including isolation of genomic DNA, polymerase chain reactions (PCR), restriction enzyme digestion and ligation, transformation, etc. were performed as described [32]. All restriction enzymes, T4 DNA ligase, and other enzymes used in this study were purchased from New England Biolabs (NEB). Plasmids were prepared by using a plasmid isolation kit (Qiagen). Oligonucleotides were purchased from a commercial source (Bioserve Biotechnology, India). Gene amplifications were carried out using high-fidelity pfx DNA polymerase (Invitrogen). The conjugal transfer of recombinant plasmids into *P. syringae* was carried out by a biparental mating method using the donor *E. coli* strain S17-1, as described earlier [35].

Construction of plasmids for expression and complementation studies

All gene cloning experiments were performed in *E. coli* DH5 α cells. The detailed methodology has been reported earlier [34, 36, 37]. All plasmids used for protein expression and genetic complementation are listed in Table 1.

Cloning and expression of E. coli RNase R

The amplification and cloning of the RNase R encoding gene (rnr) of *P. syringae* has been reported earlier [30]. The E. coli rnr gene was amplified by using a set of genespecific Forward and Reverse primers with incorporated NdeI and SalI sites, and cloned into pET28a expression vector (Table S 1). The plasmid (pET28arnr^{Ec}) was transformed into E. coli BL-21 (DE3) strain, and expression of RNase R^{Ec} was observed under IPTG induction at various time intervals (Data not shown) [40]. The His-tagged E. coli rnr gene along with vector-specific RBS (Ribosome binding site) was released from pET28arnr^{Ec} plasmid by XbaI and SacI digestion and subcloned into broad host range vector pGL10 [38]. The construct (pGLrnr^{Ec}) was transformed into E. coli S-17 strain and mobilized into P. syringae Δrnr mutant by biparental mating as described earlier [35, 41]. Expression of the RNase R^{Ec} in Δrnr

| Plasmid | Description | Reference/source |
|--------------------------------|--|------------------|
| pET28a | Kan ^r , Expression vector for N-terminal His-tagged proteins, | Novagen |
| pGL10 | Broad-host cloning vector, IncP replicon, <i>mob</i> ⁺ , <i>Kan</i> ^r | [38] |
| pET28a <i>rnr^{Ps}</i> | pET28a plasmid for over-expression of N-terminal His tagged <i>P. syringae</i> RNase R | [30] |
| pET28arnb ^{Ps} | pET28a plasmid for over-expression of N-terminal His tagged RNB domain of <i>P. syringae</i> RNase R | [39] |
| pET28a <i>rnr^{Ec}</i> | pET28a plasmid for over-expression of N-terminal His tagged E. coli RNase R | This study |
| pET28a <i>rnb^{Ec}</i> | pET28a plasmid for over-expression of N-terminal His tagged RNB domain of <i>E. coli</i> RNase R | This study |
| pGL10 <i>rnr^{Ps}</i> | pGL10 expressing His-tagged P. syringae RNase R protein | [28] |
| pGL10 <i>rnb</i> ^{Ps} | pGL10 expressing His tagged RNB domain of <i>P. syringae</i> RNase R protein | [39] |
| pGL10 <i>rnr</i> ^{Ec} | pGL10 expressing His tagged <i>E. coli</i> RNase R protein | This study |
| pGL10 <i>rnb</i> ^{Ec} | pGL10 expressing His-tagged only RNB domain of <i>E. coli</i> RNase R protein | This study |

Table 1 Plasmids used in this study

strain was confirmed by Western analysis using anti-His antibodies (Fig. 2).

Cloning and expression of RNB domain of RNase R^{Ec}

The truncated gene fragment (1239 base pairs) encoding catalytic (RNB) domain (413 amino acids) of *E. coli* RNase R was amplified using gene-specific forward and reverse primers with incorporated *NdeI* and *Bam*HI sites respectively, and cloned into pET28a expression vector. (Table S 1). The resultant plasmid pET28aRNB^{Ec} was transformed into *E. coli* BL-21(DE3) strain and expression of RNB^{Ec} domain under IPTG induction was confirmed by SDS-PAGE analysis (Data not shown). The fragment encoding the His-tagged RNB^{Ec} domain was released from pET28aRNB^{Ec} along with vector-specific RBS (Ribosome binding site) by *XbaI* and *SacI* digestion and subcloned into broad host range plasmid pGL10 [38]. The construct (pGLRNB^{Ec}) was transformed into *E. coli* S-17 strain and mobilized into *P. syringae* Δrnr mutant by bi-parental mating as reported earlier [35, 41]. Expression of the RNB^{Ec} in Δrnr mutant was confirmed by Western analysis using anti-RNase R antibodies (Fig. 3).

Functional complementation studies

Broad host range vectors $pGL10rm^{Ec}$ and $pGL10rm^{bc}$ were mobilized into cold-sensitive Δrm strain, and growth pattern of complemented strains was analyzed at both optimal and low temperatures (22 °C and 4 °C). All bacterial strains used for genetic complementation studies are listed in Table 2.

Results

Bioinformatic analysis of P. syringae rnr gene

Analysis of *rnr* gene (Gene encoding RNase R) sequences from different bacteria revealed that *rnr* locus is highly conserved among the *Pseudomonas*. sp. The operon



Fig. 2 Expression of *E.coli* RNase R. Expression of RNase RPs and RNase REc in Δrnr strain was analyzed by western blotting where cell lysate from Δrnr strains expressing RNase RPs and RNase REc were transferred to a nylon membrane and probed by anti-His antibodies



Fig. 3 Expression of RNB (Catalytic) domain of *E. coli* RNase R. (a) Color-coded schematic diagram showing different domains (color shaded) and position of primers employed for amplification of catalytic RNB domain. The numbers represent the nucleotide base pairs in *rnr* gene. (b) Expression of RNB domain in Δrnr strain complemented by pGLRNBEc was analyzed by western blotting. Cell lysate from wild-type cells (Lane 2), Δrnr pGLRNBEc strain (Lane 1) were loaded on polyacrylamide gel, transferred to a nylon membrane, and probed with anti RNase RPs antibodies

| Bacterial strains | Description | Reference or source |
|-------------------------------|---|---------------------|
| E. coli DH5a | F- φ80 <i>lac</i> ZΔM15 Δ(<i>lac</i> ZYA- <i>arg</i> F) U169 <i>rec</i> A1 <i>end</i> A1 <i>hsd</i> R17 (r _k -, m _k +) <i>pho</i> A <i>sup</i> E44 λ- <i>thi</i> -1 <i>gy</i> rA96 <i>rel</i> A1 used for all gene cloning purpose | [36] |
| <i>E. coli</i> S17-1 | F _ pro recA1 (r_ m_) RP4-2 integrated (Tc::Mu) (Km::Tn7) [Smr Tpr]; used as a donor strain in conjugation | [38] |
| E. coli BL21 (DE3) | F^- ompT gal dcm lon hsdS _B (r _B m _B) λ (DE3 [lacl lacUV5-T7 gene 1 ind1 sam7 nin5]), used for overexpression of proteins under IPTG induction | [40] |
| P. syringae (Lz4W) | Lz4W Amp ^r , wild-type | [29] |
| ∆rnr | <i>rn:: tet^r P. syringae</i> strain with disrupted <i>rnr</i> gene | [30] |
| ∆rnr(pGL10rnr ^{Ps}) | Arnr strain complemented by P. syringae rnr | [30] |
| ∆rnr(pGL10rnb ^{Ps}) | Arnr strain complemented by only RNB domain of P. syringae RNase R | [39] |
| ∆rnr(pGL10rnr ^{Ec}) | Arnr strain complemented by E. coli rnr | This study |
| ∆rnr(pGL10rnb ^{Ec}) | Arnr strain complemented by only RNB domain of E. coli RNase R | This study |

Table 2 Bacterial strains used in this study

consists of *rnr* (RNase R encoding) and *trmH* gene (encoding putative tmRNA or rRNA methyl transferase). Up-stream of the Bi-cistronic operon are two genes that code for tRNA-leucine, whereas downstream of the *rnr* operon is a highly conserved gene (*rpsF*) that codes for S6 ribosomal protein [39].

Sequence alignment studies (T-COFFEE, EMBL-EBI) of *rnr* gene from Antarctic *P. syringae*, *P. aeruginosa*, *P. fluorescens*, and mesophilic representative strains (*E. coli* and *B. subtilis*) revealed that *P. syringae* Lz4W displayed maximum similarity with *P. fluorescens* (88.91%), followed by *P. aeruginosa* (74.97%) (Fig. 4a). The Antarctic bacterium shows only a modest sequence similarity

(53.17%) with *E. coli* and even lesser similarity with *B. subtilis* (37.52%). Sequence alignment results illustrate that there is high similarity among the representative strains from *Pseudomonas* sp., as compared to their sequence similarity with representative strains from mesophiles (*E. coli* and *B. subtilis*).

A gene sequence-based phylogeny of exoribonuclease (*rnr*) genes from *Pseudomonas*. sp., and mesophilic *E. coli, B. subtilis* is shown in Fig. 4b. The analysis indicates that the *rnr* genes belonging to different strains have been clustered into three distinct groups representing, *Pseudomonas*. sp. (*P. syringae* Lz4W, *P. aeruginosa*, and *P. fluorescens*), *E. coli*, and *B. subtilis*. The representative strains

а

b

| | COV | pid | 1 | [| 120 |
|---|--|---|--------------------------|--|-------------------|
| в. | 100.0% | 100.0% | | MEKEAFMEKLLSFMKEEAYKPLTVQELEEMLNITEAEEFKELVKALVALEEKGLIVRTRSDRYGIPEKMNLIKGKISAHAKGFAFLLPEDTSLSDVFISPNE | |
| Ε. | 97.2% | 34.3% | | MSQDPFQEREAEKYANPIPSREFILEHLTK-REKPASRDELAVELHIEGEEQLEGLRRRLRAMERDGQLVFTRRQCYALPERLDLVKGTVIGHRDGYGFLRVEGRK-DDLYLSSEQ | |
| Ρ. | 99.1% | 33.1% | | MADWQTLDPEAAREAEKYENPIPSRELILQHLAE-RGSPAAREQLVEEFGLTTEDQIEALRRRLRAMERDAQLIYTRRGTYAPVDKLDLILGRISGHRDGFGFLIPDDGS-DDLFMSPSQ | |
| P1 | 99.4% | 32.4% | | MADWQNLDPEAAREAEKYENPIPSRELILAHLSE-RGAPATRSQLLDEFGLAGEEPEEALRRRLRAMERDGQLIYTRRGAYAPVDKLDLIRGRISGHRDGFGFLIPDDGS-DDLFLSPTQ | |
| P2 | 99.2% | 32.8% | | MADWQSLDPEAAREAEKYENPIPSRELILAHLAD-RGSPASREQLVEEFGLTTEDQLEALRRRLRAMERDAQLIYT <mark>R</mark> RGTYAPVDKLDLILGRIAGHRDGFGFLIPDDGS-DDLFMSPAQ | |
| | | | 101 | | 240 |
| B | 100 0% | 100 0% | 121 | INTANNEDTVINVELNSOSSESSESSESSESSESSESSESSESSESSESSESSESS | 240 |
| F. | 97 2% | 34 3% | | ANY CHEGON AND CONFERENCE OF THE CONFERENCE OF THE ADVISOR OF THE | |
| P. | 99.1% | 33.1% | | MRLVFDGRALABVSGLDRRGRREGVTVEVVSRAHETTVGRVFEEGGTGFVADNPKT00EVLVTPGRNAGAQVG0FVEVKTHWPTPRE0P0GDVLEVVGNVNAPGMETDTALRTVDIP | |
| P. 1 | 99.4% | 32.4% | | MRLVFDQDRALARVSGHDR8GREGOLVEVTERAHETVVGRVFEESGTGVVVADNPKT00EVLTPPGKAGKARHN0FV0VRTEDWPSTHR0A0GETVEVLGDVNAPGMETEVALRSVDTP | |
| P. 2 | 99.2% | 32.8% | | MRLVFDgDRALARVSGLDRRGRREGVIVEVVSRAHESIVGRYFEEGGIGFVVPDNPKV00EVLITPGRNGAAKVG0FVEVKITHWPTARF0P0GDIVEVVGNYMAPGMEIDVALRTYDIP | |
| | | | | | |
| | cov | pid | 241 | | 360 |
| в. | 100.0% | 100.0% | | GEFPADAMEQASSTPDTIDEKDLKDRRDLRDQVIVTIDGADAKDLDDAVTVTKLDDGSYKLGVHIADVSHYVTENSPIDKEALERGTSVYLVDRVIPMIPHRLSNGICSLNPKV | |
| Ε. | 97.2% | 34.3% | | YIWPQAVEQQVAGLKEEVPEEAKAGRVDLRDLPLVTIDGEDARDFDDAVYCEKKRGGGWRLWVAIADVSYYVRPSTPLDREARNRGTSVYFPSQVIPMLPEVLSNGLCSLNPQV | |
| Ρ. | 99.1% | 33.1% | | HVWPEAVLKEAAKLKPEVEEKDKEKRIDLRHLPFVTIDGEDARDFDDAVYCEARPGKLRLFSGGWTLYVAIADVSSYVKIGSALDAESQVRGNSVYFPERVIPMLPEQLSNGLCSLNPKV | |
| P1 | 99.4% | 32.4% | | HVWPAAVEKEAAKLKPEVAEKOKEKRVDLRQVPFVTIDGEDARDFDDAVYAEAKRGGGWRLFVAIADVSHYVKVGSALDEEAARRGNSVYFPERVIPMLPEVLSNGLCSLNPLV | |
| P2 | 99.2% | 32.8% | | HVWPEAVLKEAAKLKPEVEEKDKEKRIDLRHLPFVTIDGEDARDFDDAVYCEAKPGKLRLFSGGWKLFVAIADVSSYVKIGSALDNEAQVRGNSVYFPERVIPMLPEQLSNGLCSLNPKV | |
| | | | | | 400 |
| P | 100.01 | 100.07 | 361 | PRI TI SCENTTINGAGUTEUETEGUTETTTERNTYCDURYCI UDD | 480 |
| p. | 07 2% | 24 24 | | | |
| D. | 00 1% | 22 19 | | DRIAMVENTVEVENTVEVENTVEVENTVEVENTVEVENTEDUTTE CONTRECTAND DEVANIADULVI VVI VVI VVI ADEUGATEGOATETTECCENTATETTTECCENTATETTTECCENT | |
| P 1 | 99.1% | 32 /% | | DRIAMY ENTISIAGENDINFTERVENSALTINYSTILLEYN ISTLEYN ISTRAUND NAWN NAWN AND ELGENNYN ALTOFE ISTRAUND AND ANN AND ANN AND ANN ANN ANN ANN A | |
| P 2 | 99.4% | 32.4% | | DRIAMY ENTSKTGENTYVOFFANTSORETTYKVSTEENEVSTERENEVSTERENEVSTEREVEVVITALALANDAVARATETEISENEV | |
| | 22120 | 5210/0 | | | |
| | | | | | |
| | cov | pid | 481 | | 600 |
| в. | 100.0% | pid 100.0% | 481 | LVANETVAEHFHWMVVPFIYRIHEEPNAEKLQKFLEFVTTFGYVVKGTAGNIHPRALQSILDAVRDRPEETVISTVMLRSMKQAKYDPQSLGHFGLSTEFYTHFTSPIRRYPDLIVHRLI | 600 |
| В. Е. | 100.0% 97.2% | pid 100.0% 34.3% | 481 | LVANETVALHFHMMVNPFIYRTHEEPNAEKLOKFLEFVTTFGYVVKGTAGNIHPRALQSILDAVRDRPEFTVISTVHLRSVKQAKYDPQSLGHFGLSTEFYTHFTSPIRRYPDLIVHRI ILANISAARFVEKAKEPALFRIHDKPSTEAITSFRSVLAELGLELPG-GNKPEPROYAELLESVADRPDAEHLQTMLLRSVKQAIYDPENRGHFGLALQSVAHFTSPIRRYPDLITHRAI | 600 |
| В. Е. Р. | 100.0% 97.2% 99.1% | pid 100.0% 34.3% 33.1% | 481 | LVANETVAEHHMANDPETVREHEEPNAEKLOKFLEFVTTFGVVKGTAANEHPRALGELDANRORPEETVLSTVILRSIKOAKVOPGSLOHGLISTEFTTHF15PTRAVPDLTWAE LLANESAARVEKAKEPALRKINDOSTEALTSKSVLAEGGELDG-OMPEPROVAELESNARORDADILGTILLSIKKGAKVOPGGLOHGATTOPBINGHGLUGSVATHF15PTRAVPDLTWAE LLANESAARVEKAKEPALRKINDOSTEALTSKSVLAEGGELDG-OMPEPROVAELESNARORDADILGTILLSIKKGAKVOPGGUGHGUTVATHF15PTRAVPDLTWAEJ | 600 |
| B. E. P1 | cov 100.0% 97.2% 99.1% 99.4% | pid 100.0% 34.3% 33.1% 32.4% | 481 | LVANETVAEHFHMINVPETVREHEEMAEKLOKFLEFVITFOYVKGTAONLHPRALOSILDAVRORPEETVISTVILRINKOAKYDPOSLGHFGLISTFYTHFISTRKYPDLIVKG I LANISAARVEKAKEPALREHDKOSTEALTSFKIVLAELGLEUPG-GROKPROVAELESVARDADHUGTILLRINKGAKYDPOSUGGHUTVATHFISTRRYPDLITHAT LANIATAFELUKHETALVRHOOPPOELLINALGGLEGELGLASIGKGAPTPGOVGELLESVARDADHUGTIVILRISUGVYSONGGHUTVATHFISTRRYPDLITHAT LCANATAFELGKHAITALVRHOOPPOELLINALGGLEGELGLEGEGLAGRIGAAPTGOVGELLEKTHRADFRITTIVILRISUGVYSONGHGUTVATHFISTRRYPDLITHAT | 600 |
| B. E. P1 P2 | 100.0% 97.2% 99.1% 99.4% 99.2% | pid 100.0% 34.3% 33.1% 32.4% 32.8% | 481 | LVANETVAENHHMINDETVREHEEPINAELQKE LEFVTTFGVVKGTAGNINPRALQEILDANRDRHETVISTVILKSIKQAKYDPQSLGHGLSTEFYTHFISPIRAPPDLTWREI LLAAISAARVEKAKEPALRENDOSTEALTSFKSVLAELGELD-GANEPHOVAELLESVARDABAUGTILLISSKQAKYDPQSUGHGVGFGVTFATHTSPIRAPPDLTWRAI LAAISAARVEKAKEPALRENDOSTEALTSFKSVLAELGELD-GANEPHOVAELLESVARDABAUGTILLISSKQAKYDQGGUGFGVTFATHTSPIRAPPDLTWRAI LAAISAARVEKAKEPALRENDOSTEALTSFKSVLAELGELD-GANEPHOVAELLESVARDABAUGTILLISSKQAKYDQGGUGFGVTFATHTSPIRAPPDLTWRAI LAAISAARVEKAKEPALRENDOSTEALTSFKSVLAELGELGISGUGAPPOOYGELLESINGRAFGUGGGUGGUGFGVTFATHTSPIRAPPDLTWRAI LAAINATAFLUNKIEPIN LYNDOPPEKLINILGELGISGUGGUFPOOYGELLESINGRAFUNGGUGGUGGUGGUGFGVTFATHTSPIRAPPDLTWRAI LAAINATAFLUNKIEPIN LYNDOPPEKLINILGELGISGUGGUFPOOYGELLESINGRAFUNGGUGGUGGUGGUGGUGGUTFATHTSPIRAPPDLTWRAID | 600 |
| B. E. P1 P2 | 100.0% 97.2% 99.1% 99.4% 99.2% | pid 100.0% 34.3% 33.1% 32.4% 32.8% | 481 | LVANETVAEHFHAMWYPETVREHEEPMAEKLOKFLEFVTFGYVKGTAONLHPRALGSLLDAVRORPEETVLSTWILRSWKOAKYDPOSLGHFGLSTEFYTHFISPIRKYPDLUWRLI ILANISARAFVEKAKEPALERRHONSTEALTSFRSVLALGLELOG-GWKPEPBOYAELLESVARDRORPUTLLRSWKGAKYDPOSUGHGHGUTAATHFISPIRKYPDLUWRL ILANISARFVEKAKEPALERRHONSTEALTSFRSVLALGLELOG-GWKPEPBOYAELLESVARDRORPUTLRSWKGAKYDPOSUGHGUTVATHFISPIRKYPDLUWRLI LCANATAFELGHKITALWRHOOPPOELLWILRFLGELGISLGRGKAAPTPOOYOELLEKTHORADFLIQTWILRSUSGVYSONWHFGUNVATHFISPIRKYPDLUWRAT LCANATAFELGHKITALWRHOOPPOELLWILRFLGELGISLGRGKAAPTPOOYOELLEKTHORADFLIQTWILRSUSGVYSONWHFGUNVATHFISPIRKYPDLUWRAT LAANWATAEFLKIHEIDALVRWHOOPPOELLEKLRAFLGELGISLGRGKAPTPOOYOELLEKTHORADFLIQTWILRSUSGVYSONWHFGUNVATHFISPIRKYPDLUTHRI | 600 720 |
| B. E. P1 P2 | cov 100.0% 97.2% 99.1% 99.4% 99.2% cov | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% | 481 601 | LVANETVALBEHARNVPFLYREIHEERIALELQKELEEVIT FOVVKKT AGNIBIRALGELLDANDRREETVESTVILKSIKGAV7DPGLGHEGLSTEFYTH FUP TRAVPDLTVREI LLANESAARVEGALKEPAL REINOSTEALTSFKSVLALGLEUP- GRUPPROVAELLESVARDGENGUNLLSIKGAV7DPGLGHGUNGAVTHFUP TRAVPDLTWREI LLANEARVEGALKEPAL REINOSTEALTSFKSVLALGLEUP- GRUPPROVAELLESVARDGENGUNLLSIKGAV7DQGHGUNGAVTHFUP TRAVPDLTWREI LLANEARVEGALKEPAL REINOSTEALTSFKSVLALGLEUP- GRUPPROVAELLESVARDGENGUNGAVTDPGNOHGUNGGAVTHFUP TRAVPDLTWREI LLANEARTELGUNTALINGUNGGAVGHGUNGAVTHFUP TRAVPDLTWREI LLANEARTELGUNTALINGUNGGAVGHGUNGAVTHFUP TRAVPDLTWREI LLANEARTELGUNTALINGUNGGAVGHGUNGAVTHFUP TRAVPDLTWREI LLANEARTELGUNTALINGUNGGAVGHGUNGAVTHFUP TRAVPDLTWREI RIVV. INGV/DF | 600 720 |
| B. E. P1 P2 B. E. | cov 100.0% 97.2% 99.1% 99.4% 99.2% cov 100.0% 97.2% | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% 34.3% | 481 601 | UNART VALUERABERANKUPST VERHERNALDER LEVT FOLVAKK TAKING MALOS LLAUKOBERT VIS TIMLE RIKKANDODI, GARGIS STEPT MET DE TEKPOL UNAL I TAKISAART VALUERAL TEKNOSTE DE TEKNISTER VIS LEGUES LINKKKOPPERDVALLASTIKORPOPLI TIMLERIKKANDODI GARGIS LINKKKOPPEND TIMLE LAMVATAE FLANKEDA I TYMORPPELEKIAR GEGUES LINKKKOPPENDVALLASTIKORPOPLI TIVLERIKKANDODI GARGIS LINKKKOPPEND TIMLE LAMVATAE FLANKEDA I TYMORPPELEKIAR GEGUES LINKKKOPPENDVALLASTIKORPOPLI TIVLERIKKANDODI GARGIS TENTENDEL TIMLE LAMVATAE FLANKEDA I TYMORPPELEKIAR GEGUES LINKKKOPPENDVALLASTIKORPOPLI TIVLERIKSANDODI GARGIS TENTENDE TIVLERIK LAMVATAE FLANKEDA I TYMORPPELEKIAR GEGUES LINKKKOPPENDVALIASTIKORPOPLI TIVLERIS SAVI FORMANGEGUNA TINT FOT BERNPOL LINKAS LAMVATAE FLANKED I TYMORPPELEKIAR GEGUES LINKKKOPPENDVALIASTIKORPOPUTZI TIVLERIS SAVI FORMANGEGUNA TINT FOT BERNPOL LINKAS I TYMIN TAKEFU AN TIVLERIKSANDODI GARGIS TAKING TIVLERIKSANDODI GARGIS TIVLERIKSSI TIV FORMANGEGUNA TINT FOT BERNPOL LINKASI TIVLI ANGOLOGIS TIVLERIKSKI TIVLERIKSKI TIVLERIKSKI TIVLERIKSSI SAVI FORMATIKANDODI FOT DIVLERIKSSI SAVI FORMANGEGUNA TIVLERIKSSI TIVLERIKSI TIVLERIKSSI TIVLERIKSSI TIVLERIKSSI TIVLERIKSSI TIVLERIKSSI TIVLERIKSI TIVLERIKSSI TIVLERIKSSI TIVLERIKSI TIVLERIKSSI TIVLERIKSSI TIVLERIKSI TIVLERIKSI TIVLERIKSI TIVL | 600 |
| B. E. P1 P2 B. E. P. | cov 100.0% 97.2% 99.1% 99.4% 99.2% cov 100.0% 97.2% 99.1% | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% 34.3% 33.1% | 601 | LVANETVALEHHAWNVPTIVETHEEMALELQKELEFVTTFGVVKGTAGNIBRALQLILDANDRHETVISTVILKSIKQAKYDPGLGHGLSTEFVTHF19 TRAYPDLTVAL LLANSAANVEGALKEPAL PRIMOSTEALTSFKSVLALGLELP- GANEPROVIAELESVARDADILG (MLLKSIKQLSVDROKGULVFANTH TSTRAYPDLTWAL LLANSAANVEGALKEPAL PRIMOSTEALTSFKSVLALGLELP- GANEPROVIAELESVARDADILG (MLLKSIKQLSVGROKGULVFANTH TSTRAYPDLTWAL LLANSATAFLUHNTDALWNDOPPELELARAFIGGLESUS (MURGOSPSVOQULLASSICARDHL) (MLKSIQVTSONGHGULVFANTH TSTRAYPDLTWAL LLANSATAFLUHNTDALWNDOPPELELARAFIGGLESUS (MURGOSPSVOQULLASSICRAPHILG) (MLKSIQVTSONGHGULVFANTH TSTRAYPDLTWAL MURGOSPSVOQULLASSICRAPHILGSKOW (MURGOSPSVOQULLASSICRAPHILG) (MLKSIQVTSONGHGULVFANTH TSTRAYPDLTWAL MURGOSPSVOQULLASSICRAPHILGSKOW (MURGOSPSVOQULLASSICRAPHILG) (MLKSIQVTSONGHGULVFANTH TSTRAYPDLTWAL MURGOSPSVOQULVCASTAFLASTRAYPONTALISTRAYPONTALISTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTALISTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTALISTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVSSVOTAFGFVLODLETDUKUNSTAFDVOXPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVCASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULVSSVOTASTAFLASTRAYPONTATION (MURGOSPSVOQULVSSVI MURGOSPSVOQULV | 600 720 |
| B. P1 P2 B. E. P. 1 | cov 100.0% 97.2% 99.1% 99.4% 99.2% cov 100.0% 97.2% 99.1% 99.4% | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% 34.3% 33.1% 32.4% | 601 | LUMATE VALUE HARWINGT TO THE FONKLUCKELE KUTT FORVIKKT ARKING HALOS LLAUKEDBETT VIS TIMLE REVORATIONS (LAGHESS FETTINE TO TRAVOLUMENT) LUMATSARFURENE LARKINGTS TATTEFSKULA ELI GLE CARGER BOYANGT LEVANDERA LOS LLAUKEDBETT VIS TIMLE REVORATIONS (LAGHESS FETTINE TO TRAVOLUMENT) LARMATARE FLICHED AL TWHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPPOHLI TYMLE SIGAVISORMIKALUNGANTHE TO FERMORI LARMATARE FLICHETA LIVHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPPOHLI TYMLE SIGAVISORMIKALUNGANTHE TO FERMORI LARMATARE FLICHETA LIVHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPPOHLI TYMLE SIGAVISORMIKALUNGANTHE TO FERMORI LARMATARE FLICHETA LIVHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPPOHLI TYMLE SIGAVISORMIKALUNGANTHE TO FERMORI LANMATARE FLICHETA LIVHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPPOHLI TYMLE SIGAVISORMIKALUNGANTHE TO FERMORI LANMATARE FLICHETA LIVHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPPOHLI TYMLE SIGAVISORMIKALUNGANTHE TO FERMORI LANMATARE FLICHETA LIVHOOPPOELEKIAR GEGLE SLIKKIKOOPPORTUGALLASTICKOPONTUTIOTIKES SUN TIGOFINI DUNG SUN TI | 600 720 |
| B. P1 P2 B. E. P1 P2 | cov 100.0% 97.2% 99.1% 99.2% cov 100.0% 97.2% 99.1% 99.4% 99.2% | pid 100.0% 34.3% 32.4% 32.8% pid 100.0% 34.3% 33.1% 32.4% 32.8% | 601 | LVARETVISEHEAMINPETVERHEEMAELQEKE EFVTT FGVVKKT JACINEMALQEI LDANDEREETVISTVIKEIKKAAVDOGL GHEGISTEPTIHET PTRAVPDLTVHET LAATSAAVERAKEPAL PRINOTSTAATSFKSVALGIELD - OMPERAVAELESVARDADEN QTILLESKARVDOGL GHEGISTEPTIHET PTRAVPDLTVHET LAATSAAVERAKEPAL PRINOTSTAATSFKSVALGIELD - OMPERAVAELESVARDADEN QTILLESKARVDOGL GHEGISTEPTIHET PTRAVPDLTVHET LAATSAAVERAKEPAL PRINOTSTAATSFKSVALGIELD - OMPERAVAELESVARDADEN QTILLESVARDADEN QTILLESKARVDOGL GHEGISTEPTIHET PTRAVPDLTVHET LAATSAAVERAKEPAL PRINOTSTAATSFKSVALGIELD - OMPERAVIAELESVARDADEN QTILLESVARDADEN QTILLESKARVDOGL GHEGISTEPTIHET LAATSFAAVERAKEPAL PRINOTSTAATSFKSVALGIELD - OMPERAVIAELESVARDADEN QTILLESVARDADEN QTILLESKARVDOGL GHEGISTEPTIHET LAANTAAFELKINETPAL VRWEDOPPERLEKLARE GELGISLEKKKDOPTKOVQALLSSIKKROVATQTVILESSAVVSADNQOHGUIVEAVTHFTSPTRAVPDLTVHAT RTVLINKKOP | 600 720 |
| B. P1 P2 B. E. P1 P2 | cov 100.0% 97.2% 99.1% 99.2% cov 100.0% 97.2% 99.1% 99.4% 99.2% | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% 34.3% 33.1% 32.4% 32.8% | 601 | LUMETUALIPHARMINETY ZHALEFUNELUGELE EVIT FOLVIKK TARINDBALOT. LUNKOBERTUS TIMUK BIKAGAMOPOL GARGEST EFVINTE TO TRAVPOL TIMUL TUMISTAARIVEKEENEN TRAVPOLETATIETY TERVIK ALGUE EN GARGERPORVAL LEVIKORDA TOTUL EN KOATOPOL GARGEST EFVINTE LAAMATAE ELIKHETAAL YMHOOPPOELEKLAAL GELGLS LIKKIKOOPPKOVQLLASTICORPOHLI TIMUK SIGAVISONGHGULVANTHET DE TRAVPOL TIMAT LAAMATAE ELIKHETAAL YMHOOPPOELEKLAAL GELGLS LIKKIKOOPPKOVQLLASTICORPOHLI TIMUK SIGAVISONGHGULVANTHET SIDTERVOL TIMAT LAAMATAE ELIKHETAAL YMHOOPPOELEKLAAL GELGLS LIKKIKOOPPKOVQLLASTICORPOHLI TIMUK SIGAVISONGHGULVANTHET SIDTERVOL TIMAT LAAMATAE ELIKHETAAL YMHOOPPOELEKLAAL GELGLS LIKKIKOOPPKOVQLLASTICORPOHLI TIMUK SIGAVISONGHGULVANTHET SIDTERVOL TIMAT LAAMATAE ELIKHETAAL YKHOOPPOELEKLAAL GELGLS LIKKIKOOPPKOVQLLASTICORPOHLI TIMUK SIGAVISONGHGULVANTHET SIDTERVOL LITATI LAAMATAE ELIKHETAAL YMHOOPPOELEKLAAL GELGLS LIKKIKOOPPKOVQLLASTICORPOHVITITIVIK SI SIGAVISONGHGULVANTHET SIDTERVOL LITATI LAAMATAEE LIKHETAAL YKHOOPPOELEKLAAL GELGLS LIKKIKOOPTKOVGULASTICORPOHVITITIVIK SIGAVISONGHGULVANTHET SIDTERVOL LITATI RIVI LINKKOD | 600 720 |
| B. P1 P2 B. E. P1 P2 | cov 100.0% 97.2% 99.1% 99.2% cov 100.0% 97.2% 99.1% 99.4% 99.2% cov | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% 34.3% 33.1% 32.4% 32.8% pid | 481 601 721 | LUMETVALEVALEHMENNY DY DEHEDMELUKELE EVIT EGYVKKI AKUMERALGI. LUMIDBETU IST VILLE VIKAALVOOL, GHELS EFYNET DY BRYND LUMET LAMITARE HUKERAL ISTNOFT STAFFSKI AL LUGE - GAUGREDWORK LEEVARDDEN (DY WILLE NAMA DE HUKERALGY VILLE VIKAALVOOL) LAMITARE HUKERAL ISTNOFT STAFFSKI AL LUGE - GAUGREDWORK LEEVARDDEN (DY WILLE NAMA DE HUKERALGY VIKATON) LAMITARE HUKERAL ISTNOFT STAFFSKI AL LUGE - GAUGREDWORK LUESTICKORDHIL TO VIKA RISAGAV SONGGHELIK SYNTHFOTER VIKA LAMITARE FURHET NA INNOFORE LIKLARE (GELGS LIKKING OFTRAVIO) LAMITARE FURHET NA INNOFORE LIKLARE (GELGS LIKKING OFTRAVIO) LAMITARE FURHET NA INNOFORE LIKLARE (GELGS LIKKING OFTRAVIO) LAMITARE FURHET NA INNOFORE LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVIL NIK KORD RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING RIVING RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING RIVING RIVING RIVING LIKLARE (GELGS LIKKING OFTRAVIO) RIVIL NIK KORD RIVING RIVING RIVING RIVING RIVING RIVING RIVING RIVING LIKLARE (GELGS RIVING R | 600 720 840 |
| B. E. P1 P2 B. E. P1 P2 B. | cov 100.0% 97.2% 99.1% 99.4% 99.2% cov 100.0% 97.2% 99.4% 99.2% cov 100.2% cov | pid 100.0% 34.3% 33.1% 32.4% pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% | 481 601 721 | LUMATEVALEHAMENNET VERHERVIKELURE LEVIT FOLVIKK: TARINEBALOGI LUNIKOBERTUS SINUK BIOGANAPOPOL GARGES STEPTINE DI KAPODI LUMALI LAMASAAFEVANE REINORDE REINOSTETSESSA EL OLI SE CARDER PORVALA LEVINORDA IN UNIK SINUK SINUKAN MARKAN DALA | 600 720 840 |
| B. E. P1 P2 B. E. P1 P2 B. E. E. | cov 100.0% 99.1% 99.4% 99.2% cov 100.0% 99.2% 99.2% 99.2% cov 100.0% 97.2% | pid 100.0% 34.3% 33.1% 32.4% 32.8% pid 100.0% 34.3% 32.4% 32.8% pid 100.0% 34.3% 32.4% | 481 601 721 | LVARETVALEHEANING TVEHEDNIKELUKELERT FEVT KENVIKKI AKUMBALGI. LENKABETU STVILLENKAAN OPGL. GHE STEFTINTET DE RAVPALISHE LLANISAAR FUKKETAL INDROPPERTATIONETSTEFTING LUKELE SKARDBER VALLESTERBOHL STVILLENKAAN OPGLAGUESTEFTINTET DE RAVPALISHE LLANISAAR FUKKETAL INDROPPERTATIONETSTEFTING LUKELE SKARDBER VALLESTERBOHL STVILLENKAAN OPGLAGUESTEFTINTET DE RAVPALISHE LLANISAAR FUKKETAL INDROPPERTATIONETSTEFTING LUKELE SKARDBER VALLESTERBOHL STVILLENKAAN OPGLAGUESTEFTINTET DE RAVPALISHE LLANISAAR FUKKETAL INDROPPERTATIONETSTEFTING LUKELE SKARDBER VALLESTERBOHL STVILLENKAAN OPGLAGUESTEFTINTET DE RAVPALISHE LLANISAAR FUKKETAL INDROPPERTE KULLAGUESTE SLEISKARDBER VALLESTERBOHL STVILLENKAAN FUKKES AN STANDBER SLUKVESTINTET DE RAVPALISHE LLANISAAR FUKKETAL INDROPPERTE KULLAGUESTE SLEISKARDBER VALLESTERBOHL STVILLENKES AN STANDBER SLUKVESTINTET DE RAVPALISHE RIVLINKER FUKKETAL INDROPPERTE KULLAGUESTE SLEIKKERDOFTROVGALLESTERBOHL STVILLENKES AN STANDBER SLUKVESTINTET DE RAVPALISHE RIVLINKER SUNT SVERTIGEN VALLESTERBOHLE SLEIKKERDOFTROVALISTERBOHLESTERBOHLSSTINFOHFVELMT - EGUINVESTINTET DE RAVPALISHER RIVLINKER SUNT SVERTIGEN VALLESTERBOHLE SLEIKKERDOFTROVALISTERBOHLESTERBOHLESSEN VALLESTERBOHLESSEN VALLESSEN VALL | 600 720 840 |
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| | B. Subtilis | E. Coli | P. syringae Lz4W | P. aeruginosa | P. fluorescens |
|---------------------|-------------|---------|---------------------|------------------|-------------------|
| B. subtilis | 100.00 | 37.38 | 38.21 | 37.86 | 37.52 |
| E. coli | 37.38 | 100.00 | 52.29 | 53.28 | 53.17 |
| P. syringae Lz4W | 38.21 | 52.29 | 100.00 | 74.25 | 88.91 |
| P. Aeruginosa | 37.86 | 53.28 | 74.25 | 100.00 | 74.97 |
| P. Fluorescens | 37.52 | 53.17 | 88.91 | 74.97 | 100.00 |





from *Pseudomonas* group display sufficient homology among each other to be clustered as a group and provide a possible explanation for a convergent evolution among the representative strains in *pseudomonas* group or divergence from the mesophilic representatives which have been clustered in two separate groups.

Growth analysis of $\Delta \textit{rnr}$ strain complemented with RNase R^{Ec}

To confirm whether the *E. coli* RNase R^{Ec} is capable of complementing the cold-sensitive growth defect of P. syringae Δrnr mutant, the complemented Δrnr mutant $(\Delta rnr/pGLrnr^{Ec})$ expressing the *E. coli* RNase R was monitored for growth, and the growth profiles were compared with the wild-type and Δrnr mutant. Experimentally, all bacterial strains were grown at optimal (22°C) and low (4°C) temperatures, and OD₆₀₀ values of the cultures were recorded at regular intervals and plotted against time. All strains displayed optimal growth pattern at 22°C (Fig. 5a). As expected, the cold-sensitive Δrnr mutant did not display any measurable growth at 4°C; however, the cold sensitive phenotype of *P. syringae* Δrnr mutant was rescued by *E. coli*-specific RNase R^{Ec} in the complemented mutant ($\Delta rnr/pGLrnr^{Ec}$) (Fig. 5b). The results confirmed that the *E. coli* specific RNase R^{Ec} is active in *P. syringae*, and the enzyme is capable of complementing the functions of *P. syringae* RNase R^{Ps} at low temperature. The activity of the RNase R^{Ec} was comparable to the endogenous RNase R^{Ps}, as the growth profiles of the two complemented mutants ($\Delta rnr/pGLrnr^{Ec}$ and $\Delta rnr/pGLrnr^{Ps}$) were similar.

Complementation of cold-sensitive phenotype of Δrnr mutant by catalytic domain (RNB^{Ec})

To assess the biological activity of the catalytic domain (RNB^{Ec}) of *E. coli*-specific RNase R^{Ec} by complementation analysis, we expressed the RNB^{Ec} domain in P. syringae Δrnr mutant from a broad host range plasmid pGL10. For biological activity, growth profiles of Δrnr strain (Arnr/pGLRNB^{Ec}) expressing the RNB^{Ec} of E. coli RNase R were compared with Δrnr expressing P. svringae RNB ($\Delta rnr/pGLRNB^{Ps}$) and the wild-type. The above strains were grown at 22°C and 4°C, and optical densities of the cultures were measured at OD₆₀₀ at regular intervals and plotted against time. All strains displayed normal growth at 22°C (Fig. 6a). At 4°C, *Arnr* mutant displayed a cold-sensitive phenotype, whereas the complemented mutants *Arnr*/pGLRNB^{Ps} and *Arnr*/pGLRN-B^{Ec} displayed measurable growth to stationary phase but with a long lag time (Fig. 6b). The lag time was longer in $\Delta rnr/pGLRNB^{EC}$ compared to $\Delta rnr/pGLRNB^{PS}$ strain. The results confirmed that catalytic domains of both the mesophilic and psychrophilic exoribonuclease R (RNase R) are physiologically active and sufficient for rescuing



Fig. 5 Mesophilic RNase R complements cold-sensitive phenotype of Δrnr . (a) Growth profile of wild-type, Δrnr , Δrnr pGLrnrPs, and Δrnr pGLrnrEc strains at 22°C and (b) at 4°C confirmed over-expression of RNase R^{Ec} from broad host range plasmid (pGL10) complements cold-sensitive phenotype of *Pseudomonas syringae* Δrnr strain. For measurement of growth, samples were collected from each culture at regular intervals, OD at 600 nm was recorded and plotted against time. Each growth curve was performed at least three times



Fig. 6 Complementation of Δrnr strain by catalytic domain (RNB) of *E. coli* RNase R. (a) Growth analysis of *P. syringae* wild type, Δrnr , $\Delta rnrpGLRNB^{Ps}$, and $\Delta rnrpGLRNB^{Ec}$ strains at 22°C and (b) at 4°C established that complementation of cold-sensitive Δrnr strain by RNB^{Ec} alleviates the cold-sensitive phenotype of mutant strain but with a long Lag phase even longer than with RNB^{Ps}. For measurement of growth in cell cultures at 22°C or 4°C, samples were collected from each culture at regular intervals, and their OD at 600 nm was recorded and plotted against time. Each growth curve was repeated at least three times

the cold-sensitive phenotype of *P. syringae* Δrnr mutant. At low temperatures (4°C), the longer growth lag exhibited by Δrnr mutant complemented by RNB^{EC}, compared to Δrnr mutant expressing the RNB^{Ps} might be related to the physiological efficiency of RNB^{Ps} over the heterologous *E. coli* specific RNB^{EC}.

Discussion

The major focus of the current study was to examine the activity of E. coli RNase R in the cold-adapted Antarctic P. syringae, as RNase R plays an important role in the growth and viability of the *P. syringae*, especially at low temperatures (4°C). RNase R functions inside the cells either as an integral component of a multi-enzyme complex or as a freely soluble enzyme. This is important, as bacteria possess a huge RNA degrading multienzyme complex (degradosome) for efficient processing and degradation of different RNA substrates with variable complexity. The RNA degradosome is generally composed of Endo-ribonuclease E (RNase E), that acts as a scaffold for the assembly of other components like exoribonucleases (e.g., PNPase and RNase R), RNA helicases (e.g., RhlB, RhlE, and Rho factors), regulatory proteins (e.g., metabolic enzymes enolase and aconitase) and many transient proteins (e.g., DnaK, GroEL, GroES, Hfq, poly(A) polymerase [42] and polyphosphate kinase, etc.) that functionally interact with each other for the efficient processing and degradation of the substrates. The degradosome is a highly dynamic structure which undergoes changes in composition under different growth conditions within the cells of a species and in different bacterial species with diverged adaptability to different environmental stresses. This has been achieved by the binding activity of highly variable and intrinsically disordered regions of the C-terminal domain of RNase E. In E. coli degradosome, C-terminal region of RNase E acts not only as a scaffold for the assembly of exoribonuclease PNPase, RNA helicase RhlB, and the glycolytic enzyme enolase [43, 44] but also helps in localizing the complex to plasma membrane using a membrane targeting sequence (MTS) motif on this domain [45]. Since Antarctic *P. syringae* is adapted to grow at low temperatures, the degradosomal components include RNase E that provides a scaffold for the assembly of the ds-RNA degrading exoribonuclease RNase R, and RNA helicase [46]. Disruption of *rnr* gene leads to defects in the processing of rRNA (16S rRNA and 5S rRNA) and consequent cold-sensitive phenotype accompanied by cell death [30]. On the other hand, the disruption of rnr gene in E. coli has no deleterious effect on the processing of RNA or growth, although double mutant of *rnr* and *pnp* is not viable [13]. This study has convincingly proved that in *P. syrin*gae, the important functions of exoribonuclease R in rRNA processing and maintenance of cellular physiology are independent of the degradosomal complex.

Maintaining the rate of enzyme-catalyzed reactions at an acceptable limit to sustain growth by synthesizing cold-active and thermo-labile enzymes in cold environments is the most important adaptation of psychrophiles [46-51]. Lack of strong selective pressure for structural stability in cold environments probably helped in the evolution of cold-active enzymes with increased destabilization and flexibility of active site or whole protein [52]. Reactions catalyzed by cold-active enzymes progress with decreased ΔG and ΔH reflecting that these enzymes are more efficient with high specific activity and are less temperature dependent [53-56]. The active site of these enzymes is less stable and heat-labile [57, 58] whereas these enzymes unfold at relatively lower temperatures than their mesophilic homologs [59]. It has been observed that the majority of cold-adapted enzymes have a half-life of less than 12 min at 50 °C [49, 60, 61]. Coldadapted enzymes are prone to increased error in folding [62] and cold denaturation, most probably caused by the hydration of polar and non-polar groups [59, 63, 64]. Psychrophilic enzymes are also accompanied by structural changes outside the active site that modulate the activity of critical residues at freezing temperatures and enhance the flexibility of loops around the active site. Cold active enzymes have more accessible and large-sized active sites [65, 66], better channeling of the substrate to the active site, enhanced electrostatic potential, and better release of products [53, 65–69].

Recent studies have provided valuable insights into the structure-function relationship of various enzymes including the ribonucleases. These studies have provided the role of structural flexibility, side chain flexibility, and the role of hydrophobic amino acids in the functioning of the protein. Our results have provided an important input that, despite having variable structural adaptability and substrate specificity, conservation of function allowed the mesophilic enzyme to perform its function in an evolutionary distinct cold-adapted species of bacteria. The role of different polar/non-polar amino acids, amino acid side chains, and structural flexibility may be more related to protein thermostability, thermolability, substrate specificity, and catalysis. Our study has comprehensively proved that exoribonuclease R has a flexible structure that allows it to interact even with less specific substrates and perform its function at a physiologically acceptable rate.

In the light of the differences between two types of degradosomal assembly exemplified by the *E. coli* and *P. syringae*, variations in structure–function relationship among cold-adapted and mesophilic enzymes, differences in biochemical properties and divergent in vivo functions of two exoribonucleases, two questions were raised: (i) will mesophilic *E. coli* RNase R (RNase R^{Ec})

be able to complement the cold-sensitive growth defect of *P. syringae* Δrnr mutant? (ii) Despite bearing differences in in vitro activities, will the catalytic domain (RNB^{Ec}) of *E. coli* RNase R be able to carry out similar in vivo functions like the catalytic domain (RNB^{Ps}) of *P. syringae* RNase R?

Our results illustrate that E. coli RNase R (RNase R^{Ec}) is capable of rescuing the cold-sensitive growth defect of *P. syringae* Δrnr mutant. The findings of the current study also illustrate that the catalytic domains of two exoribonucleases from E. coli and P. syringae (RNB^{Ec} and RNB^{Ps}) are also capable of complementing the growth defects of *P. syringae* Δrnr mutant at low temperature (4°C). However, *P. syringae* Δrnr mutant expressing the catalytic domain derived from E. coli RNase R (RNB^{Ec}) displays a longer lag time (~240 hours) as compared to the lag time (~120 hours) displayed by the *P. syringae* Δrnr mutant complemented by catalytic domain derived from the P. syringae RNase R (RNB^{Ps}). The difference in the catalytic efficiencies of RNB^{Ps} and RNB^{Ec} at low temperatures is probably related to the substrate specificity, substrate binding, and structural flexibility associated with the two catalytic domains.

Altogether, the results presented here suggest that *E.* coli RNase R could alleviate the cold-sensitive phenotype of *P. syringae* Δrnr mutant at 4°C. The activities of the conserved catalytic domains (RNB^{Ps} and RNB^{Ec}) are largely intact in the two diverged bacteria (*P. syringae* and *E. coli*) adapted to grow in different temperature ranges. These results also provide valuable insights into the flexibility of protein structure, structure–function relationship, and conservation of function among the exoribonucleases. Since cold-sensitive Δrnr mutant accumulates unprocessed 5S and 16S rRNA at low temperatures, the rescue of cold sensitivity in Δrnr mutant by RNase R^{Ec} is indicative of an essential role being played by *E. coli* RNase R in 16S and 5S rRNA processing [30, 31].

Abbreviations

| RNase R | Exoribonuclease R |
|-----------------------|--|
| RNase R ^{Ec} | E. coli-Specific RNase R |
| RNase R ^{Ps} | P. syringae-Specific RNase R |
| RNB ^{ps} | Catalytic domain of RNase R [P. syringae-Specific] |
| RNB ^{Ec} | Catalytic domain of RNase R [E. coli specific] |
| tmRNA | Transfer-messenger RNA |
| | |

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43141-023-00553-2.

Additional file 1: Table S1. Functional activity of *E. coli* RNase R in the Antarctic *Pseudomonas syringae* Lz4W.

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Authors' contributions

Conception or design of the work: Ashaq Hussain and Malay Kumar Ray. Acquisition and interpretation of data: Ashaq Hussain. Drafted and reviewed the manuscript: Ashaq Hussain. Both authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- 1. Chu L-Y et al (2017) Structural insights into RNA unwinding and degradation by RNase R. Nucleic Acids Res 45(20):12015–12024
- Hossain ST, Malhotra A, Deutscher MP (2016) How RNase R Degrades structured RNA: Role Of The Helicase Activity And The S1 Domain. J Biol Chem 291(15):7877–7887
- Cheng ZF, Deutscher MP (2005) An important role for RNase R in mRNA decay. Mol Cell 17(2):313–318
- Suzuki H et al (2006) Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing. Nucleic Acids Res 34(8):e63
- Matos RG et al (2011) Swapping the domains of exoribonucleases RNase II and RNase R: conferring upon RNase II the ability to degrade ds RNA. Proteins 79(6):1853–1867
- Cairrao F, Arraiano CM (2006) The role of endoribonucleases in the regulation of RNase R. Biochem Biophys Res Commun 343(3):731–737
- Cheng ZF, Deutscher MP (2002) Purification and characterization of the Escherichia coli exoribonuclease RNase R. Comparison with RNase II. J Biol Chem 277(24):21624–9
- Vincent HA, Deutscher MP (2006) Substrate recognition and catalysis by the exoribonuclease RNase R*. J Biol Chem 281(40):29769–29775
- 9. Matos RG, Barbas A, Arraiano CM (2009) RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. Biochem J 423(2):291–301
- Vincent HA, Deutscher MP (2009) The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. J Biol Chem 284(1):486–494
- 11. Domingues S et al (2015) The role of RNase R in trans-translation and ribosomal quality control. Biochimie 114:113–118
- 12. Awano N et al (2010) Escherichia coli RNase R Has Dual Activities, Helicase and RNase. J Bacteriol 192:1344–1352
- Cheng ZF, Deutscher MP (2003) Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. Proc Natl Acad Sci U S A 100(11):6388–6393

- Sulthana S, Deutscher MP (2013) Multiple exoribonucleases catalyze maturation of the 3' terminus of 16S ribosomal RNA (rRNA). J Biol Chem 288(18):12574–12579
- Tejada-Arranz A et al (2021) RNase R is associated in a functional complex with the RhpA DEAD-box RNA helicase in Helicobacter pylori. Nucleic Acids Res 49(9):5249–5264
- Andrade JM, Cairrao F, Arraiano CM (2006) RNase R affects gene expression in stationary phase: regulation of ompA. Mol Microbiol 60(1):219–228
- Venkataraman K, Zafar H, Karzai AW (2014) Distinct tmRNA sequence elements facilitate RNase R engagement on rescued ribosomes for selective nonstop mRNA decay. Nucleic Acids Res 42(17):11192–11202
- Cairrao F et al (2003) Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. Mol Microbiol 50(4):1349–1360
- Liang W, Deutscher MP (2010) A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. J Biol Chem 285(38):29054–29058
- 20. Moreira RN et al (2012) Synergies between RNA degradation and trans-translation in Streptococcus pneumoniae: cross regulation and co-transcription of RNase R and SmpB. BMC Microbiol 12:268
- 21. Erova TE et al (2008) Cold shock exoribonuclease R (VacB) is involved in Aeromonas hydrophila pathogenesis. J Bacteriol 190(10):3467–3474
- Tobe T et al (1992) vacB, a novel chromosomal gene required for expression of virulence genes on the large plasmid of Shigella flexneri. J Bacteriol 174(20):6359–6367
- 23. Bárria C et al (2022) RNase R, a new virulence determinant of Streptococcus pneumoniae. Microorganisms 10(2):317
- 24. Charpentier X et al (2008) Loss of RNase R induces competence development in Legionella pneumophila. J Bacteriol 190(24):8126–8136
- 25. Abula A et al (2021) Molecular mechanism of RNase R substrate sensitivity for RNA ribose methylation. Nucleic Acids Res 49(8):4738–4749
- Lalonde MS et al (2007) Exoribonuclease R in Mycoplasma genitalium can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation. RNA 13(11):1957–1968
- 27. Hutchison CA et al (1999) Global transposon mutagenesis and a minimal Mycoplasma genome. Science 286(5447):2165–2169
- Purusharth RI et al (2005) Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium Pseudomonas syringae Lz4W. J Biol Chem 280(15):14572–14578
- Shivaji S, et al (1989) Isolation and identification of Pseudomonas spp. from Schirmacher Oasis, Antarctica. Appl Environ Microbiol 55(3):767–70
- 30. Purusharth RI, Madhuri B, Ray MK (2007) Exoribonuclease R in Pseudomonas syringae is essential for growth at low temperature and plays a novel role in the 3' end processing of 16 and 5 S ribosomal RNA. J Biol Chem 282(22):16267–16277
- Sulthana S, Basturea GN, Deutscher MP (2016) Elucidation of pathways of ribosomal RNA degradation: an essential role for RNase E. RNA 22(8):1163–1171
- Janiyani KL, Ray MK (2002) Cloning, sequencing, and expression of the cold-inducible hutU gene from the antarctic psychrotrophic bacterium Pseudomonas syringae. Appl Environ Microbiol 68(1):1–10
- Regha K, Satapathy AK, Ray MK (2005) RecD plays an essential function during growth at low temperature in the antarctic bacterium Pseudomonas syringae Lz4W. Genetics 170(4):1473–1484
- Malke H, Sambrock J, Fritsch EF, Maniatis T (1989) Molecular Cloning, A Laboratory Manual (Second Edition), Volumes 1, 2 and 3. 1625 S., zahlreiche Abb. und Tab. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. \$ 115.00. ISBN: 0–87969–309–6. 1990. 30(8): p. 623–623
- 35. Strand TA et al (2014) A new and improved host-independent plasmid system for RK2-based conjugal transfer. PLoS ONE 9(3):e90372
- Liu J et al (2018) An improved method of preparing high efficiency transformation Escherichia coli with both plasmids and larger DNA fragments. Indian J Microbiol 58(4):448–456
- 37. Froger A, Hall JE (2007) Transformation of plasmid DNA into E. coli using the heat shock method. J Vis Exp 6:253
- Bidle KA, Bartlett DH (1999) RecD function is required for high-pressure growth of a deep-sea bacterium. J Bacteriol 181(8):2330–2337
- Sulthana S et al (2011) rnr gene from the antarctic bacterium Pseudomonas syringae Lz4W, encoding a psychrophilic RNase R. Appl Environ Microbiol 77(22):7896–7904

- Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189(1):113–130
- Sinha AK et al (2013) Replication arrest is a major threat to growth at low temperature in Antarctic Pseudomonas syringae Lz4W. Mol Microbiol 89(4):792–810
- 42. Braman J, Papworth C, Greener A (1996) Site-directed mutagenesis using double-stranded plasmid DNA templates. Methods Mol Biol 57:31–44
- Carpousis AJ (2002) The Escherichia coli RNA degradosome: structure, function and relationship in other ribonucleolytic multienzyme complexes. Biochem Soc Trans 30(2):150–155
- 44. Worrall JA et al (2008) Reconstitution and analysis of the multienzyme Escherichia coli RNA degradosome. J Mol Biol 382(4):870–883
- Khemici V et al (2008) The RNase E of Escherichia coli is a membranebinding protein. Mol Microbiol 70(4):799–813
- Ponnada PSk, et al (2011) Cold active enzymes from the marine psychrophiles: biotechnological perspective. Adv Biotech 10:16–20
- Åqvist J, Isaksen GV, Brandsdal BO (2017) Computation of enzyme cold adaptation. Nat Rev Chem 1(7):0051
- Gerday C (2014) Fundamentals of cold-active enzymes, in cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Buzzini P, Margesin R, Editors. Springer Berlin Heidelberg, Berlin, 325–350
- Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. Annu Rev Biochem 75:403–433
- Lonhienne T, Gerday C, Feller G (2000) Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. Biochim Biophys Acta 1543(1):1–10
- Rishi N et al (2022) Enzymatic Behaviour of Cold Adapted Microbes. In: Goel R et al (eds) Survival Strategies in Cold-adapted Microorganisms. Springer Singapore, Singapore, pp 113–131
- 52. Gerday C et al (2000) Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol 18(3):103–107
- Collins T, et al (2008) Fundamentals of Cold-Adapted Enzymes, in Psychrophiles: from Biodiversity to Biotechnology, R. Margesin, et al., Editors. Springer Berlin Heidelberg, Berlin, p. 211–227
- Deniz AA (2018) Enzymes can adapt to cold by wiggling regions far from their active site. Nature 558(7709):195–196
- Peterson ME et al (2007) The dependence of enzyme activity on temperature: determination and validation of parameters. Biochem J 402(2):331–337
- 56. Daniel RM et al (2008) The effect of temperature on enzyme activity: new insights and their implications. Extremophiles 12(1):51–59
- Marx JC et al (2007) Cold-adapted enzymes from marine Antarctic microorganisms. Mar Biotechnol (NY) 9(3):293–304
- Sočan J, Purg M, Åqvist J (2020) Computer simulations explain the anomalous temperature optimum in a cold-adapted enzyme. Nat Commun 11(1):2644
- 59. D'Amico S et al (2003) Activity-stability relationships in extremophilic enzymes. J Biol Chem 278(10):7891–7896
- Chen Y, Tian Q, Wang H, Ma R, Han R, Wang Y et al (2022) A manganesebased metal-organic framework as a cold-adapted nanozyme. Adv Mater e2206421. https://doi.org/10.1002/adma.202206421
- Georlette D et al (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28(1):25–42
- 62. D'Amico S, Gerday C, Feller G (2001) Structural determinants of cold adaptation and stability in a large protein. J Biol Chem 276(28):25791–25796
- Makhatadze GI, Privalov PL (1995) Energetics of protein structure. Adv Protein Chem 47:307–425
- 64. Aurilia V, et al (2009) Structure and dynamics of cold-adapted enzymes as investigated by FT-IR spectroscopy and MD. The Case of an Esterase from Pseudoalteromonas haloplanktis. J Phys Chem B 113(22):7753–7761
- Aghajari N et al (2003) Crystal structures of a psychrophilic metalloprotease reveal new insights into catalysis by cold-adapted proteases. Proteins 50(4):636–647
- Russell RJ et al (1998) Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. Structure 6(3):351–361
- Khan S, Farooq U, Kurnikova M (2016) Exploring protein stability by comparative molecular dynamics simulations of homologous hyperthermophilic, mesophilic, and psychrophilic proteins. J Chem Inf Model 56(11):2129–2139

- Kim SY, et al (1999) Structural basis for cold adaptation. Sequence, biochemical properties, and crystal structure of malate dehydrogenase from a psychrophile Aquaspirillium arcticum. J Biol Chem 274(17):11761–7
- Smalås AO et al (2000) Cold adapted enzymes. Biotechnol Annu Rev 6:1–57

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