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ORIGINAL PAPER

Characterization of recombinant glutathione reductase from the psychrophilic Antarctic bacterium *Colwellia psychrerythraea*

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Abstract Glutathione reductases catalyze the reduction of oxidized glutathione (glutathione disulfide, GSSG) using NADPH as the substrate to produce reduced glutathione (GSH), which is an important antioxidant molecule that helps maintain the proper reducing environment of the cell. A recombinant form of glutathione reductase from *Colwellia psychrerythraea*, a marine psychrophilic bacterium, has been biochemically characterized to determine its molecular and enzymatic properties. *C. psychrerythraea* glutathione reductase was shown to be a homodimer with a molecular weight of 48.7 kDa using SDS-PAGE, MALDI-TOF mass spectrometry and gel filtration. The *C. psychrerythraea* glutathione reductase sequence shows significant homology to that of *Escherichia coli* glutathione reductase (66 % identity), and it possesses the FAD and NADPH binding motifs, as well as absorption spectrum features which are characteristic of flavoenzymes such as glutathione reductase. The psychrophilic *C. psychrerythraea* glutathione reductase exhibits higher k_{cat} and k_{cat}/K_m at lower temperatures (4 °C) compared to mesophilic Baker's yeast glutathione reductase. However, *C. psychrerythraea* glutathione reductase was able to complement an *E. coli* glutathione reductase deletion strain in oxidative stress growth assays, demonstrating the functionality of *C. psychrerythraea* glutathione reductase over a broad temperature range, which suggests its potential utility as an antioxidant enzyme in heterologous systems.

Keywords *Colwellia psychrerythraea* · Glutathione reductase · Psychrophile · Oxidative stress · Antioxidant enzyme

Abbreviations

C. psychrerythraea	*Colwellia psychrerythraea*
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione

Introduction

Colwellia psychrerythraea belongs to a group of strictly psychrophilic gamma proteobacteria, which thrive at temperatures below 5 °C (D'Aoust and Kushner 1972; Huston et al. 2000; Deming et al. 1988). It has been isolated from stably cold marine environments, including deep sea and Arctic and Antarctic sea ice (Junge et al. 2002). Brine channels within the sea ice provide a unique ice-associated life habitat for *C. psychrerythraea* and some other bacteria as well as some viruses, algae, protists, flatworms, and small crustaceans (Mock and Thomas 2005). Organisms living in or on sea ice face several extreme environments: concentrated salinity, low temperatures, high UV light, and dissolved gases (Thomas and Dieckmann 2002). *C. psychrerythraea* can be exposed to high levels of reactive oxygen species (ROS) through their own metabolism as well as the

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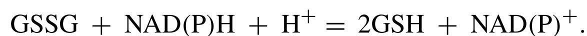
extracellular ROS within the brine channel by exposure to cold temperatures, increased solubility of oxygen, and high UV light (Neftel et al. 1984; King et al. 2005; Cooper and Zika 1983; Mock and Thomas 2005). To enhance membrane fluidity at the low temperatures, the bacterium produces extra polyunsaturated fatty acids and these can undergo lipid peroxidation by ROS (Halliwell and Gutteridge 1984; Methe et al. 2005; Barriere et al. 2001). Several antioxidant enzymes are encoded in the genome of *C. psychrerythraea* including superoxide dismutases (SODs) and catalases, indicating that the bacterium does have methods of coping with the oxygen toxicity (Methe et al. 2005). Since little is understood about oxidative stress in psychrophilic organisms in general, one key antioxidant enzyme produced in *C. psychrerythraea*, glutathione reductase (GR), was the focus of this study.

Reduced glutathione (GSH) is a tripeptide composed of glutamate, cysteine, and glycine that has numerous important functions within cells. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain (Hopkins 1929). Glutathione is ubiquitous in animals, plants, and microorganisms, and, being water soluble, is found mainly in the cell cytosol and other aqueous phases of living systems (Kosower and Kosower 1978; Lomaestro and Malone 1995; Ritz and Beckwith 2001). It is known to protect cells from toxins such as free radicals by serving as a reductant (Grant et al. 1996). The sulfhydryl group of GSH can be used to reduce peroxides. The resulting oxidized form of GSSG consists of two molecules of disulfide bonded together (GSH). Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants (Kehrer and Lund 1994). GSH takes part in many different intracellular processes, including maintenance of reduced thiol groups, protection from oxygen-induced cell damage, and generation of deoxyribonucleotide precursors for DNA synthesis (Holmgren 1976; Kehrer and Lund 1994). GSH is regenerated in an NAD(P)H-dependent reaction catalyzed by glutathione reductase (Grant and Dawes 1996) as indicated:



$$\mathrm{GSSG} + \mathrm{NAD(P)H} + \mathrm{H}^{+} = 2\mathrm{GSH} + \mathrm{NAD(P)}^{+}.$$

Its role in replenishing pools of GSH is critical for maintaining a reducing environment within the cell (Huseby et al. 2009). The active site of GR is a redox-active disulfide bond which receives electrons from the bound FAD cofactor. In *Escherichia coli*, GR is a member of the dimeric FAD-containing thiol reductase family. It shares significant homology with the thioredoxin reductases from higher eukaryotes (Ritz and Beckwith 2001). One interesting aspect of glutathione metabolism in *E. coli* is that the ratio of reduced to oxidized glutathione does not appear to change significantly in mutants that lack glutathione reductase (Tuggle and Fuchs 1985).

There have been a small number of studies characterizing prokaryotic glutathione reductases such as from *E. coli* (Scrutton et al. 1987), *Xanthomonas campestris* (Loprasert et al. 2005), *Streptococcus mutans* (Yamamoto et al. 1999), *Pseudomonas aeruginosa* (Perry et al. 1991), *Rhodospirillum rubrum* (Libreros-Minotta et al. 1992), and the cyanobacterium *Anabaena* (Jiang et al. 1995). In this study, the glutathione reductase from the psychrophillic bacterium, *C. psychrerythraea*, was recombinantly expressed in *E. coli* and was characterized by investigating the biochemical, molecular, catalytic, and kinetic properties of the enzyme. Furthermore, in vivo complementation studies in a glutathione reductase-deficient *E. coli* strain were conducted to validate its functionality in a heterologous system over a broad range of temperatures.

Materials and methods

E. coli glutathione reductase mutant strain construction

To avoid background glutathione reductase (GR) activity, *E. coli* strains, BL21 (λDE3) and JM105, were subjected to disruption of the gene encoding glutathione reductase (*gor*). The *gor* mutant of BL21(λDE3) strain was used for overexpression and purification of *C. psychrerythraea* GR (Killens-Cade et al. 2014), and the g*or* mutant strain of JM105 was generated for the growth studies described in this work. GR was inactivated in the *E. coli* strains using the PCR-based method of one-step inactivation of chromosomal genes described by (Datsenko and Wanner 2000). Plasmid, pKD3, which contains a chloramphenicol resistance gene cassette, was used as the template for generation of PCR fragments encoding the chloramphenicol resistance marker and homologous DNA regions for the target gene (*gor*). The primers consisted of 60 bases, which include 40 that are homologous to the flanking region of the target genes and 20 bases that are complementary to the chloramphenicol resistance cassette (see Online Resource 1). The PCR products were gel purified (Qiagen) for electroporation. pKD46 is a helper plasmid that carries the phage λ Red recombinase that is L-arabinose inducible and can be cured from cells when the incubation temperature is raised to 37 °C. JM105 and BL21 (λDE3) were transformed with pKD46 and plated in LB supplemented with ampicillin (100 µg/ml) and 0.3 % glucose agar and were incubated overnight at 30 °C. Transformants carrying pKD46 were grown in 10 ml SOB medium containing 0.2 % of L-arabinose and ampicillin (100 µg/ml) at 30 °C until OD_{600} reached 0.6. The cultures were spun down at 9500 rpm in a Beckman C1015 rotor for 10 min and washed three times with ice-cold 10 %

Table 1 Bacterial strains used in this study

Strains	Genotype	Source
JM105	endA1 glnV44 sbcB15 rpsL thi-1 Δ(lac-proAB) [F′ traD36 proAB$^+$ lacI^q lacZΔM15] hsdR4($r_K^-m_K^+$)]	ATTC
MJL700	JM105 *gor*:cm^r	This study
MJL701	pTrc99A/MJL700	This study
MJL702	pMLJ40 (*Cpsy* GR in pTrc99)/MJL700	This study
MLJ70	pTrc99A/JM105	This study
BL21(DE)	F-, *omp*T, *hsd*Sβ(rβ-mβ-), *dcm*, *gal*, (DE3) tonA	Stratagene
MLJ600	BL21(DE) *gor*:cm^r	(Killens-Cade et al. 2014)
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′proAB lacIqZΔM15Tn10(Tet^r)]	Stratagene

glycerol to make the cells electrocompetent. The cells carrying pKD46 were suspended in 100 µl of 10 % glycerol and 50 µl aliquots were placed into two separate microfuge tubes. The gel-purified *gor*-specific PCR products (concentration ~1 µg) were added to 50 µl of resuspended cells and no DNA was added to the other 50 µl of cells as a negative control. Electroporation was conducted by using a Gene Pulser (Bio-Rad, Hercules, CA) set at 2.5 V, 200 Ω, and 25 µF according to the manufacturer's instructions. Shocked cells were added to 1 ml of pre-warmed SOC medium and incubated for 1 h at 37 °C. 100 µl of the cell suspension was then plated onto LB agar containing chloramphenicol (35 µg/ml) and incubated at 37 °C overnight. The remainder of the cells was incubated overnight at room temperature before plating the next day. The colonies were screened for mutation by isolating the DNA using the GNOME DNA Kit (Bio 101, Vista, CA), digesting with *Bst*EII, and PCR amplifying with primers specific to the inner and outer region of the gene (see Online Resource 1 for primer sequences). The verified strains were designated MLJ700 for the *gor*-deficient JM105 strain and MLJ600 for the *gor*-inactivated BL21 (λDE3) strain (Table 1).

Cloning and purification of glutathione reductase from *C. psychrerythraea* strain 34H

The *C. psychrerythraea* GR gene was cloned into the pET-21b plasmid and overexpressed in the *gor*-deficient *E. coli* BL21 (λDE3) strain MLJ600 at room temperature, and the GR protein was purified as previously described (Killens-Cade et al. 2014). The specific activities of the recombinant GR at each purification step were determined and are presented in Online Resource 2. Purified recombinant GR was used for characterization.

To investigate the functionality of *C. psychrerythraea* GR in a heterologous system, it was cloned into the pTrc99A vector. The gene encoding *C. psychrerythraea* GR was amplified from genomic DNA using the following primers targeted to *C. psychrerythraea* 34H: forward primer containing an *Nco*I site 5′-CTAATGAGTGAAC<u>CCATGG</u>CACAA-3′ and the reverse primer containing an *Sac*I site 5′-GCAATCAACATT<u>GAGCTC</u>GCTTAA-3′. The following PCR amplification program was used: 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. The *C. psychrerythraea* GR gene was amplified using iProof High-Fidelity DNA polymerase (BioRad). The resulting PCR product was gel extracted and ligated to the pTrc99A plasmid previously digested with *Nco*I and *Sac*I. The ligated construct was transformed into *E. coli* XL-Blue competent cells (Stratagene, Santa Clara, CA). Plasmid DNA was isolated from the transformants using Qiagen Minipreps, and the plasmids were screened for inserts by visualization of DNA agarose gels. The cloned GR gene sequence was verified by sequencing (Eurofins MWG Biotech, High Point, NC).

Molecular weight determination

The molecular weight of the *C. psychrerythraea* GR was calculated using Mac vector software, and the estimation of the molecular weight under denaturing conditions was determined by SDS-PAGE analysis as previously described (Killens-Cade et al. 2014). In this study, a quantitative determination of the molecular weight was conducted using MALDI-TOF mass spectrometry analysis (NC State Mass Spectrometry Facility, Raleigh, NC). The native molecular weight of the recombinant *C. psychrerythraea* GR was determined by gel filtration chromatography using a HiPrep 16/60 Sephacryl S-200 HR column (GE healthcare, Pittsburgh, PA) equilibrated with solution A (0.05 M sodium phosphate buffer with 0.15 M NaCl at pH 7.0). The elution of recombinant *C. psychrerythraea* GR was compared to the elution of gel filtration standards (thyroglobin) (bovine):670 kDa; γ-globulin (bovine):158 kDa; ovalbumin (chicken): 44 kDa; myoglobin (horse): 7 kDa; vitamin B_{12}: 1.35 kDa (Bio-Rad, Hercules, CA).

Enzyme assays, kinetic studies, and protein estimation

GR activity was measured spectrophotometrically with a Shimadzu Spectrophotometer UV2401-PC (Nakagyo-Ku,

Kyoto, Japan) at various temperatures. The GR reaction mix (3 ml) included 75 mM potassium phosphate, 2.6 mM ethylenediaminetetraacetic acid, 1 mM oxidized glutathione, 0.09 mM β-nicotinamide adenine dinucleotide phosphate (reduced form), and 0.13 % (w/v) bovine serum albumin (Mavis and Stellwagen 1968). One unit of enzyme activity was defined as the amount of glutathione reductase that oxidizes 1 μM NADPH per min. K_M and V_{max} values for NADPH were calculated using the UVProbe software (Shimadzu, Japan) for kinetic studies. Activities were determined at 5 different NADPH concentrations (6, 13, 25, 50, 100 μM) in reactions containing 1 mM oxidized glutathione. Baker's yeast glutathione reductase used for the mesophilic enzyme comparison was purchased from Sigma-Aldrich (St. Louis, MO). Protein concentrations were routinely estimated using the Bradford method with bovine serum albumin as the standard (Bradford 1976).

Sequence analysis and UV–visible spectra determination

For gene sequence analysis, orthologs of GR from other organisms were identified by BLAST sequence analysis by using the GeneBank database at the National Center for Biotechnology Information. Predicted amino acids sequences were aligned using the CLUSTAL W program. UV–visible spectra were recorded on a Shimdazu Spectrophotometer UV2401-PC at 25 °C. The reactions were prepared in 100 mM potassium phosphate buffer and 3.4 mM EDTA at pH 7.6. Purified *C. psychrerythraea* GR was reduced with 0.067 mM DTT under anaerobic conditions and GR was oxidized with 1 mM GSSG aerobically.

E. coli in vivo complementation study

The bacterial strains and plasmids used in this study are listed in Table 1. The ability of the *C. psychrerythraea* GR gene to complement an *E. coli gor*-deficient strain was examined by growth in LB media treated with the oxidizing agent diamide, where restoration of growth indicates complementation of *E. coli* GR. The plasmid, pTrc99A, was transformed into the *E. coli* strains JM105 and MLJ700 (JM105:Δ*gor*), and pMLJ40 (*C. psychrerythraea* GR in pTrc99A) was transformed into MLJ700. Cultures grown overnight at 37 °C were used to inoculate 30 ml of LB broth, and they were subsequently grown at either 18, 25 or 37 °C shaking at 200 rpm. When OD_{600} measurements were between 0.15 and 0.25 (at early log phase), IPTG (0.1 mM) was added to every culture for induction. 0.2 mM or 0.3 mM of diamide was also added to some cultures to induce oxidative stress. The cultures were harvested (5 ml of each culture) once the non-diamide-treated cultures reached OD_{600} measurements between 0.8 and 1.0 (at mid to late log phase). The harvested cells were broken with 1.2 ml of B-per (Thermo Scientific, Waltham, MA) with lysozyme (10 mg/ml) and cell debris was pelleted using centrifugation. The cell extracts were assayed for GR activity at the same temperature as the growth studies that had been conducted.

To determine if *C. psychrerythraea* GR would provide protection against hydrogen peroxide or menadione which induces oxidative stress, *E. coli* complemented with *C. psychrerythraea* GR was challenged either with hydrogen peroxide (2 and 4 mM) or menadione (0.05 and 0.08 mM). The culture samples were prepared at room temperature as described above and monitored for growth.

Statistical analysis

Statistical analyses were conducted for the OD 600 nm values of the final time points for the growth study at each temperature using SAS 9.4 version. All estimates of sample variability are expressed as means of standard error ($n = 3$). Student's two-tailed t tests (p value < 0.05) were performed to determine whether the growth responses were significantly affected by the levels of diamide or strain type at the three different temperatures used in the experiment. A two-way ANOVA with replication ($n = 3$) was performed for each temperature to validate whether the responses of the growth with respect to strain type were significantly different from the responses to the amount of diamide cells were exposed to and the possible interaction between these two factors. The analysis was considered significantly different if the strains, the diamide treatments, and the interaction had p values below 0.01. Graphs of marginal means were generated to visualize the characteristics of each factor (strain and diamide effect) and the interaction that may occur between them.

Results

C. psychrerythraea glutathione reductase sequence analysis

Glutathione reductase is a member of the pyridine nucleotide-disulfide oxidoreductase family of flavoenzymes, containing two active site electron acceptors, FAD, and a redox-active disulfide (Meister and Anderson 1983). *C. psychrerythraea* GR was compared with other GR sequences from various sources including bacteria, plant, and human (blood cells) using the Clustal W program (Fig. 1) to identify sequence similarities. *C. psychrerythraea* GR showed a high degree of sequence identity with GR sequences from *E. coli* (66 %) and human blood cells (49 %), but showed less identity to *Arabidopsis* GR (34 %). *C. psychrerythraea*

Fig. 1 Alignment of the amino acid sequence of *C. psychrerythraea* glutathione reductase with other GRs from various organisms. Colwellia psy: *Colwellia psychrerythraea*, E. coli: *Escherichia coli*, Human: human blood cell, Cyanobacterium: *Cyanobacterium anabaena* PCC7120, Xanthomonas cam: *Xanthomonas campestris*, Pseudomonas aer: *Pseudomonas aeruginosa*, Arabidopsis tha: *Arabidopsis thaliana*. The *x marks* indicate the conserved motif for FAD binding, and the *open circles* mark the NADPH binding motif

GR has the conserved FAD binding motif GxGxxG(x_{17})E and NADPH binding motif (GXGYIAX18RX$_5$R) that are typically present in the other GR homologs and are characteristic of flavoenzymes in general (Rescigno and Perham 1994; Jiang et al. 1995; Dym and Eisenberg 2001; Scrutton et al. 1990; Loprasert et al. 2005).

Recombinant *C. psychrerythraea* glutathione reductase molecular weight determination and spectral properties

The purified *C. psychrerythraea* GR enzyme migrated as a single band in SDS-PAGE with an apparent molecular weight of around 50 kDa (Killens-Cade et al. 2014). A molecular weight of 48.7 kDa was determined using MALDI-TOF mass spectrometry, which was similar to Mac Vector software's estimation of 48.9 kDa. The molecular weight of the non-denatured enzyme as determined by gel filtration through a Sephacryl S-200 HR column (GE Health Care, Piscataway, NJ) was 95.24 kDa, suggesting that the recombinant *C. psychrerythraea* GR is a homodimer (Online Resource 3). The color of the purified *C. psychrerythraea* GR enzyme was yellow, and it had an absorption spectrum characteristic of the other flavoproteins (Lopez-Barea and Lee 1979; Garcia-Alfonso et al. 1993; Macheroux 1999), displaying peaks at 376 and 462 nm and a deep trough at 404 nm when it is in the oxidized form (Fig. 2).

C. psychrerythraea glutathione reductase kinetic studies and heat stability

The kinetic parameters of the recombinant *C. psychrerythraea* GR were determined by comparing it with the mesophilic baker's yeast GR using NADPH as a substrate when the enzymes were assayed at 4 and 25 °C (Table 2). The kinetic values of baker's yeast GR assayed at 25 °C in this study are very similar to values reported in previous studies (Mavis and Stellwagen 1968; Massey and Williams 1965). As expected, both psychrophilic and mesophilic GR enzymes tend to exhibit lower K_m values at their preferred environmental temperature (Lonhienne et al. 2001). The temperature dependence of the K_m values allows the fine kinetic optimization of the enzymes to adapt to their environmental condition by modifying the thermodynamic properties of weak interactions for substrate binding (Aghajari et al. 1998; Feller and Gerday 2003). At 4 °C, *C. psychrerythraea* GR had a k_{cat} three times higher and a K_m four times lower compared to the mesophilic baker's yeast GR. These properties of the enzyme described above exhibit a typical psychrophilic behavior (Bentahir et al. 2000). The heat stability of *C. psychrerythraea* GR was also assessed. The thermal half-life of the *C. psychrerythraea* GR was estimated to be 40, 8, and less than 2 min at

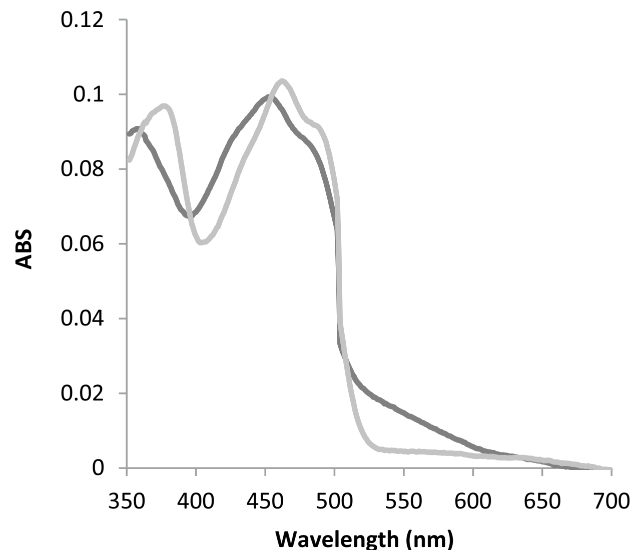


Fig. 2 Absorption spectra of *C. psychrerythraea* glutathione reductase under reduced (*black line*) and oxidized (*gray line*) conditions. 10 uM of purified GR was reduced with 0.067 mM DTT and oxidized with 1 mM of GSSG. The enzyme was dissolved in 100 mM potassium phosphate buffer and 3.4 mM EDTA at pH 7.6

50, 55, and 60 °C, respectively. Also, 75 % of the activity remained after incubation at 45 °C for 3 h, and more than 90 % of the activity remained after incubation at 37 °C for 3 weeks.

Construction and verification of the *E. coli* glutathione reductase mutant strains

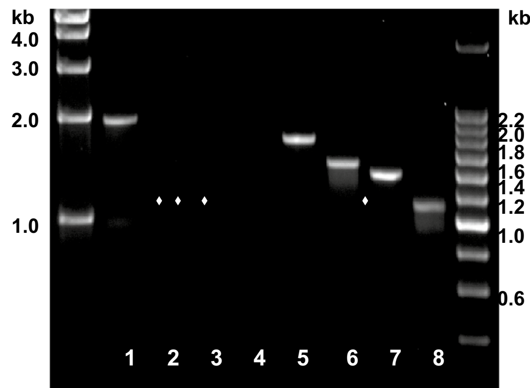
To investigate the ability of the *C. psychrerythraea* GR to function in a heterologous system, the GR gene of *E. coli* strain JM105 was disrupted. The isolated strains (along with the parental type strain) were subjected to PCR analysis for verification of the targeted gene disruption. For the PCR analysis, sheared genomic DNA from each isolate and the control strain were used as the DNA templates and primers that are specific to either the outer region of the genes or located within the disrupted genes were used (see Online Resource 1). The expected sizes of the GR-disrupted DNA were 1.1, 1.35, 1.45, and 1.7 kb when the following primer combinations were used: 5′ *gr*:Cm specific and 3′ *gr*:Cm specific primers, 5′ outer region and 3′ *gr*:Cm specific primers, 5′ *gr*:Cm specific and 3′ outer region primers, and 5′ outer region and 3′ outer region primers, respectively. The expected DNA size when using the parental strain as the template DNA for the 5′ outer region and 3′ outer region primers was about 1.95 kb. PCR analysis of the parental strain (JM105) showed the expected sizes of the DNA fragments, and one of the isolates

Table 2 Comparison of kinetic parameters for recombinant *C. psychrerythraea* glutathione reductase and baker's yeast glutathione reductase at different temperatures

	K_m (μM)[a,b]		K_{cat} (min^{-1})[b]		K_{cat}/K_m (min^{-1} μM^{-1})[b]	
	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C
C. psychrerythraea GR	10.9 ± 0.3	4.5 ± 0.3	$4.2 \times 10^4 \pm 1.1$	$1.3 \times 10^4 \pm 0.5$	$3.8 \times 10^3 \pm 0.5$	$2.8 \times 10^3 \pm 0.9$
Baker's yeast	3.9 ± 0.1	16.3 ± 0.3	$1.5 \times 10^4 \pm 0.6$	$4.0 \times 10^3 \pm 2.2$	$3.7 \times 10^3 \pm 0.6$	$2.1 \times 10^2 \pm 0.5$

[a] Activities were assayed at 5 different NADPH concentrations (6,13, 25, 50, 100 μM) with a fixed concentration of GSSG (1 mM)

[b] All estimates of variability are expressed as means of standard error ($n = 3$)



Lanes	Template used	Primers used	Expected sizes
1	JM105	5' GR outer region 3' GRouter region	1.93kb
2	JM105	5' GR outer region 3' *gor-cm* specific	No DNA
3	JM105	5' *gor-cm* specific 3' GR outer region	No DNA
4	JM105	5' *gor-cm* specific 3' *gor-cm* specific	No DNA
5	MLJ700	5' GR outer region 3' GR outer region	1.7 kb
6	MLJ700	5' GR outer region 3' *gor-cm* specific	1.45 kb
7	MLJ700	5' *gor-cm* specific 3' GR outer region	1.35 kb
8	MLJ700	5' *gor-cm* specific 3' *gor-cm* specific	1.1 kb

Fig. 3 PCR verification of the *E. coli gor* mutant (MLJ700) using different combinations of *gr*:Cm specific or outer region specific primers

did produce PCR fragments that matched the expected sizes (Fig. 3). The verified strain was named MLJ700. GR enzyme assays indicated that negligible GR activity was observed in MLJ700, while the wild-type parental strain, JM105, showed approximately 45-fold greater activity (Table 3).

Table 3 Glutathione reductase activities of the *E. coli* strains used in this study

Strains	GR Activity (μmole/min/mg[a])
JM105	0.218 ± 0.07
MLJ700	0.0048 ± 0.0021
BL21(λDE3)	0.238 ± 0.011
MLS600 [BL21(λDE3)Δgor)]	0.0057 ± 0.00028

[a] Cells were harvested after 8 h of growth at room temperature in LB, and the GR enzyme assays were performed at 25 °C

C. psychrerythraea glutathione reductase can functionally complement a glutathione reductase-deficient *E. coli* strain

To characterize the effect of heterologous expression of *C. psychrerythraea* GR at various temperatures, MLJ70 (JM105 with pTrc99A), and MLJ701 (*gor*-deficient JM105 transformed with pTrc99A), and MLJ702 (*gor*-deficient JM105 transformed with *C. psychrerythraea* GR/pTrc99A) were grown aerobically at three different temperatures (18, 25, and 37 °C) in LB media. Once the OD_{600} reached between 0.15 and 0.25 (early log phase), a final concentration of 0.1 mM IPTG was added for the induction of GR followed by the addition of 0.2 or 0.3 mM diamide as a source of oxidative stress. All cultures for each temperature condition were harvested when MLJ70 cultures (parental strain) that had not been treated with diamide reached mid to late log phase (OD_{600} between 0.8 to 1.0) and enzyme assays were performed on the cell-free extract to determine their GR activity. The results for the growth study presented in Figs. 4 and 5 demonstrated that diamide does affect the growth of all three strains regardless of the temperature, and these factors interact significantly at 25 and 18 °C. (see p values of diamide and strain*diamide in Online Resource 4).

Data were further analyzed in detail to determine if there was a significant difference between strains at a specific diamide concentration or between diamide concentrations for a specific strain (Online Resource 5). The *gor*-deficient strain (MLJ701) did not show a significant inhibition in its

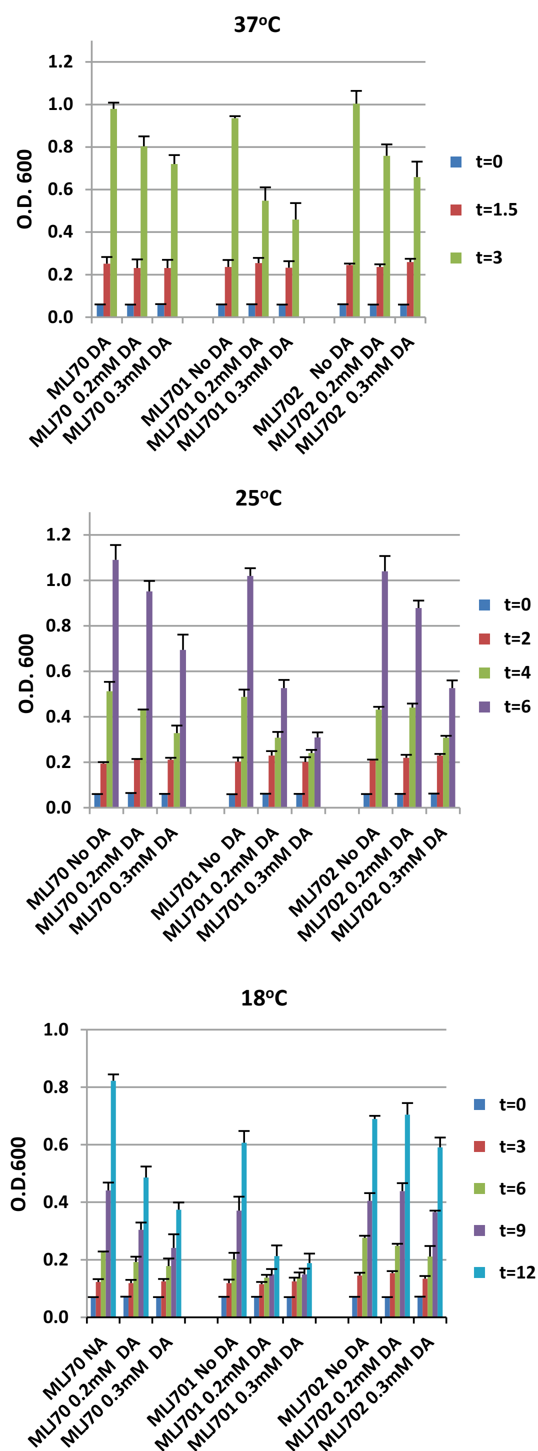


Fig. 4 Demonstration of functional complementation of *E. coli* glutathione reductase mutants with *C. psychrerythraea* glutathione reductase using oxidative stress growth studies. The experimental cultures were inoculated with enough inoculum to give a starting OD_{600} of 0.06. IPTG and diamide were added in the culture when the OD_{600} was between 0.15 and 0.25. Cells were harvested at the end points of the growth to investigate the GR activity (see Table 4). Growth studies were conducted at three different temperatures: 37 °C (**a**), 25 °C (**b**), and 18 °C (**c**). The *error bars* indicate the standard error of triplicate assay repeats. *DA* diamide

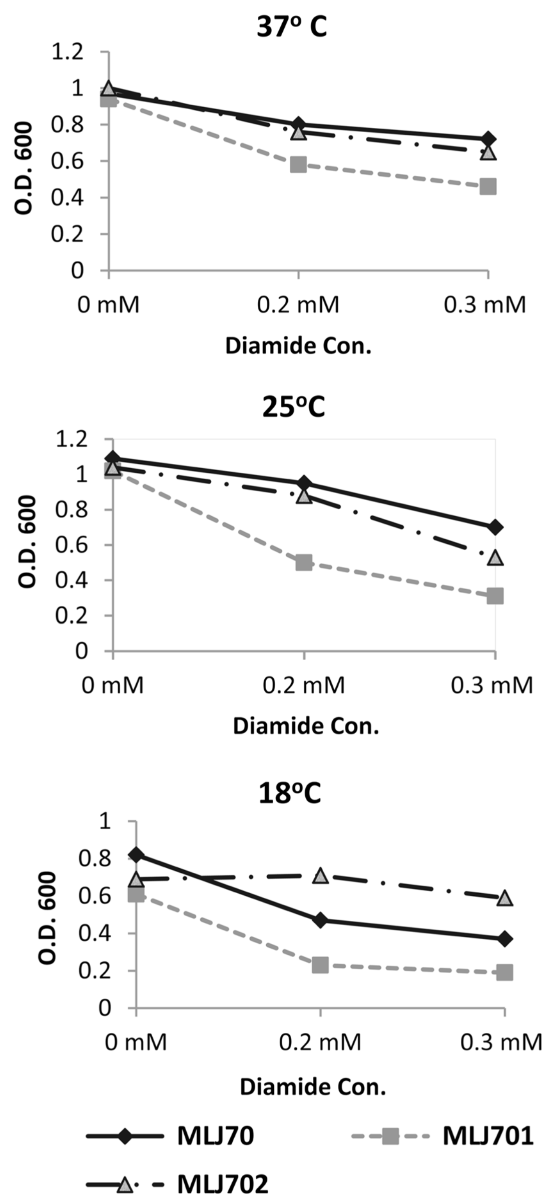


Fig. 5 The graphs of estimated marginal means were generated by averaging the three replications for the OD 600 values of the last time point for the growth study. *Diamonds* indicate the MLJ70 (parental strain), *squares* MLJ701(GR mutant), and *triangles* MLJ702 (overexpressing *C. psychrerythraea* GR in GR mutant) OD values

growth compared to the parental strain (MLJ70) when they were grown at moderate temperatures (25 and 37 °C) under non-oxidative stress conditions (p value > 0.05). However, when grown at low temperature (18 °C), MLJ701 growth was ~30 % lower than MLJ70 (p value < 0.05). In general, for all three temperature conditions, the GR mutant *E. coli* strain expressing *C. psychrerythraea* GR (MLJ702) showed some degree of restored growth in the oxidative stress cultures (diamide-treated cultures), especially at 25 and 18 °C as indicated by the significant p values <0.05.

Table 4 Glutathione reductase activities of *E. coli* strains for complementation growth studies

Strains with diamide treatments	GR activity (μmole/min/mg[a])		
	18 °C	25 °C	37 °C
MLJ70:0 mM	0.029 ± 0.0196	0.036 ± 0.008	0.063 ± 0.0172
MLJ70:0.2 mM	0.018 ± 0.0143	0.029 ± 0.005	0.066 ± 0.0138
MLJ70:0.3 mM	0.024 ± 0.002	0.024 ± 0.012	0.0700 ± 0.0197
MLJ701:0 mM	0.003 ± 0.002	0.003 ± 0.0002	0.003 ± 0.001
MLJ701:0.2 mM	0.004 ± 0.003	0.002 ± 0.0002	0.002 ± 0.0006
MLJ701:0.3 mM	0.004 ± 0.002	0.002 ± 0.0003	0.002 ± 0.0007
MLJ702:0 mM	6.267 ± 1.088	5.977 ± 1.038	0.225 ± 0.039
MLJ702:0.2 mM	7.254 ± 0.709	4.178 ± 0.291	0.273 ± 0.0415
MLJ702:0.3 mM	6.731 ± 1.42	4.921 ± 0.514	0.388 ± 0.1555

[a] GR enzyme assays were performed with cell extracts at the same temperatures that the growth studies were conducted. MLJ70:pTrc99A/JM105, MLJ702: C.psy GR-pTrc99A/JM105(ΔGR), MLJ701:pTrc99A/JM105(ΔGR). Assays were done in triplicate for each sample

Interestingly, at 18 °C, the MLJ702 strain exhibited a high degree of protection from oxidative stress (no significant difference in growth) when treated with 0.2 mM diamide (p value = 0.7131) and growth was only reduced by ~15 % when treated with 0.3 mM diamide (p value < 0.05) compared to the non-diamide-treated condition of the same strain. At the same temperature, the GR mutant strain's (MLJ701) growth was inhibited (~70 %) when treated with 0.2 or 0.3 mM diamide compared to the non-diamide-treated condition (p value < 0.05). Furthermore, when *C. psychrerythraea* GR is expressed in the *E. coli gor* mutant (MLJ702) incubated at 18 °C with diamide treatment, higher growth is observed compared to wild type (MLJ70) (p value < 0.05).

To further analyze the contribution of *C. psychrerythraea* GR activity to growth restoration, GR enzyme assays were conducted on cell-free extracts for each condition previously mentioned (Table 4). Regardless of the temperature or diamide concentration, the overall GR activities of GR-deficient strain MLJ701 were very low, while the GR wild-type strain MLJ70 showed moderate activity. The *C. psychrerythraea* GR expressing strain MLJ702 showed distinguishably higher activity than MLJ70 for 18 and 25 °C incubation conditions, while activity was reduced by ~20-fold for the 37 °C incubation condition.

Similar to the effects of diamide, certain types of ROS are also known to oxidize thiol groups; therefore, *E. coli* heterologously expressing *C. psychrerythraea* GR at room temperature was also treated with hydrogen peroxide and menadione to investigate if the enzyme would provide some protection. However, unlike the diamide treatment, it was determined that no growth restoration was observed for the *gor* mutant heterologously expressing *C. psychrerythraea* GR (data not shown) when the cells were treated with either hydrogen peroxide or menadione.

Discussion

There are several studies that report a high amount of dissolved oxygen within sea ice and a potential for production of ROS caused by UV radiation (Thomas and Dieckmann 2002; Cooper and Zika 1983; McMinn et al. 2005). Damage to cellular components such as proteins, lipids, DNA, and RNA by ROS to the point of cell death is also well documented (Yu 1994; Halliwell and Gutteridge 1984). However, few studies have investigated the antioxidant enzymes utilized by bacteria living in extreme conditions found in brine channels of sea ice. Examination of the properties and activity of glutathione reductase from *C. psychrerythraea* conducted in this study have provided new understanding of oxidative stress defense mechanisms used by psychrophilic bacteria.

The recombinant *C. psychrerythraea* GR exhibits common characteristics of other GRs, such as the use of NADPH (but not NADH) as a substrate, a yellowish color, and peak absorbance at 376 and 462 nm, which are consistent with the characteristics of flavoproteins (Colman and Black 1965; Garcia-Alfonso et al. 1993; Rendón et al. 1986; Lopez-Barea and Lee 1979).

As anticipated, it also has psychrophilic properties. It has more than ten times the catalytic efficiency (k_{cat}/K_m) of baker's yeast GR at 4 °C. Despite catalytic inhibition due to low temperatures, high catalytic efficiency and metabolic flux are achieved by a high k_{cat} and low K_m (Feller 2013). In addition to having high activity at 4 °C, it also has significant stability at 37 °C, retaining activity for 3 weeks. This finding suggests that recombinant *C. psychrerythraea* GR has relatively high thermostability compared to the other reported psychrophilic enzymes (Cavicchioli et al. 2002; Yamanaka et al. 2002; Oikawa et al. 2005). A more specific comparison that demonstrates its high thermostability is

that GR from an Antarctic ice microalga, *Chlamydomonas* sp., retains only about 5 % activity after incubation at 4 °C for the same period of time (Ding et al. 2007).

To verify the activity of recombinant *C. psychrerythraea* GR in vivo, it was heterologously expressed in a *gor*-deficient *E. coli* strain. The results from this study showed that *C. psychrerythraea* GR was able to restore the growth of an *E. coli gor* mutant to some extent when it was cultured with diamide. Diamide is a mild oxidizing compound known to quickly react with and oxidize thiols (Kosower and Kosower 1995). GR protects thiol-containing compounds from oxidation by maintaining a high cellular GSH/GSSG (reduced/oxidized glutathione) ratio (Grant et al. 1996; Carmel-Harel and Storz 2000; Yamamoto et al. 1999). Although the activity of glutathione reductase appears to be essential for maintaining a high glutathione content (Kunert et al. 1990), it was previously shown that *E. coli* GR mutants did not have a significantly different GSH/GSSG ratio suggesting that oxidized glutathione can be reduced independently of GR in *E. coli* (Tuggle and Fuchs 1985; Kunert et al. 1990). Consistent with these previous findings, our results show that when the *gor* mutant and parental strain are incubated at 25 or 37 °C without diamide, there is no significant difference in growth (p value > 0.05). However, there is a detectable difference in growth (~30 %) between these strains when cultured at 18 °C (p value < 0.05). This suggests that the other native thiol protective systems are not as effective at 18 °C. Furthermore, when the *gor* mutant (MLJ 701) and parental strain (MJL70) were tested with 0.2 and 0.3 mM diamide, growth of the *gor* mutant was substantially less than the parental strain at 18, 25, and 37 °C (p values < 0.05), which suggests that GR is responsible for protecting thiols that would be oxidized by diamide. However, when *C. psychrerythraea* glutathione reductase was heterologously expressed in the *E. coli gor* mutant (MLJ702) with diamide treatments at 18 °C, only a minor reduction in growth (~0–15 %) was observed compared to non-diamide treated suggesting that oxidized glutathione was reduced by cold active *C. psychrerythraea* GR, while the other native thiol protective systems in *E. coli* were not as effective at 18 °C. Overall, it was demonstrated that there was some degree of growth restoration of the diamide-treated cultures of MLJ702 compared to MLJ701 at each temperature, even though the growth difference at 37 °C is not considered significant (p value > 0.05). The effect of *C. psychrerythraea* GR expression was most pronounced at 18 °C with diamide treatments (p value < 0.05) (Fig. 5). Complementation of MLJ702 restored and even improved the growth above MLJ70 (parental strain, wild type) levels at 18 °C during the diamide-treated incubation. These observations were also supported by two-way ANOVA analysis (Online Resource 4); the p values of strain*diamide at low temperature incubations (25 and 18 °C) indicated that there was a significant interaction between strains and diamide treatments. This is further evidence that *C. psychrerythraea* GR is an active thiol-protective enzyme when the cells are under cold stress.

Besides demonstrating the ability of *C. psychrerythraea* GR to restore the growth of the *E. coli gor* mutant, the effectiveness of the enzyme in vivo was also supported by enzyme activity assays of cell-free extract. The data presented in Table 4 shows that at 25 °C GR activity of the complemented mutant was more than 15-fold higher than at 37 °C (whether the culture was treated with diamide or not), and at 18 °C GR activity was more than 20-fold higher than at 37 °C. These values when compared to Figs. 4 and 5 show a positive trend between GR activity and growth restoration.

Despite the stability of the purified *C. psychrerythraea* GR, the activity of the cell-free lysate of *E. coli* grown at 37 °C was about 15- to 20-fold lower than for cell lysate from cultures grown at 25 and 18 °C for the *gor* mutant stain with heterologously expressed *C. psychrerythraea* GR (MLJ702). Likely, the *C. psychrerythraea* GR does not fold correctly at 37 °C during expression, but once it is properly folded at lower temperatures it can stably function at 37 °C for weeks. Furthermore, it has been shown that at 37 °C compared to 18 °C, there is less *C. psychrerythraea* GR activity detected (Table 4), suggesting that more inactive protein is produced and turned over than active protein (Feller et al. 1998). Despite lower activity at 37 °C compared to 25 and 18 °C, the growth of the mutant strain was still restored by gor complementation.

Consistent with the overall results of the *C. psychrerythraea* GR complementation study, it was previously shown that in *Streptococcus mutants*, GR provided protection from diamide, but not hydrogen peroxide or menadione (Yamamoto et al. 1999; Becker-Hapak and Eisenstark 1995). As with *S. mutants*, it is presumed that H_2O_2 and menadione can increase intracellular peroxide (O_2^{2-}) or superoxide (O_2^{-}) levels, respectively (Pocsi et al. 2005), but they might further propagate other ROS instead of disrupting the GSH/GSSG level, or ROS may be tightly regulated by other antioxidant enzymes before compromising the thiol redox status (Lushchak 2001).

Since *C. psychrerythraea* GR is a cold-adapted protein and also demonstrated activity in a heterologous *E. coli* system at mesophilic temperatures, there is potential for use of this antioxidant enzyme in biotechnological applications. For instance, when expressed in plants, *C. psychrerythraea* GR may have utility in improving the protection of protein structure and prevention of cellular damage from certain environmental stresses such as exposure to cold temperatures, radiation, and dehydration.

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