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Construction and Characterization of *Escherichia coli*-*Corynebacterium nephridii* Hybrid Thioredoxins

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Abstract: Thioredoxin is a small redox protein with an active-site disulfide/dithiol, and is ubiquitous in bacteria, plants, and animals. To investigate the structure-function relationship of thioredoxin, the genes encoding *Escherichia coli* thioredoxin and *Corynebacterium nephridii* thioredoxin C3 were fused via a common restriction site in the nucleotide sequence coding for the active site of the proteins to generate two chimeric thioredoxins, designated E-C3(N to C-terminal) and C3-E. The hybrid thioredoxin genes were put under the T7 promoter and their productions were confirmed. The two hybrid thioredoxins complemented phenotypes of a thioredoxin-deficient *E. coli* strain. A strain containing the C3-E hybrid thioredoxin supported growth of the T7 phage, whereas a strain expressing the E-C3 hybrid thioredoxin did not. However, both hybrids supported growth of M13 phages. The two hybrid thioredoxins were also characterized in other aspects. Differences in activity between the hybrid thioredoxins were attributed to altered interactions of the N- and C-terminal domains of the molecule, which produced changes in the three-dimensional structure of the active site region.

Key words: Thioredoxin, *Escherichia coli*, *Corynebacterium nephridii*.

Thioredoxin is a small protein with an active site of a redox-active disulfide, which exists in either a reduced form or an oxidized form where half-cystine residues form a 14-membered intramolecular ring. The oxidized form of thioredoxin (thioredoxin-S_2) is generally reduced by thioredoxin reductase (Holmgren, 1985). Thioredoxin serves as a reducing agent for ribonucleotide reductase (Laurent *et al.*, 1964) and methionine sulfoxide reductase (Porque *et al.*, 1970), and functions as a general protein disulfide reductase (Holmgren, 1985). Thioredoxin *in vitro* substrates have been demonstrated for thioredoxin, including insulin (Holmgren, 1979), coagulation factors (Blomback *et al.*, 1974), and the glucocorticoid receptor (Grippo *et al.*, 1985). The amino acid sequence of *E. coli* thioredoxin (Holmgren, 1968) and the nucleotide sequence of the corresponding *trxA* gene (Lim *et al.*, 1985) are known. According to X-ray crystallography at 2.8Å resolution (Holmgren *et al.*, 1975; Katti *et al.*, 1990), *E. coli* thioredoxin is composed of two domains.

In the gram-negative bacterium *Corynebacterium nephridii*, multiple forms of thioredoxin have been identified on a protein and a gene level. An initial thioredoxin (thioredoxin C1) from *C. nephridii* was purified and characterized, and its amino acid sequence was determined (Meng & Hogenkamp, 1981). However, this thioredoxin could not serve as a cofactor for *C. nephridii* ribonucleotide reductase. A gene encoding a second thioredoxin (thioredoxin C2) from *C. nephridii* was isolated and sequenced (Lim *et al.*, 1987). Its amino acid sequence predicted from the nucleotide sequence showed a high degree of homology with other prokaryotic thioredoxins. A gene encoding a third thioredoxin (thioredoxin C3) from *C. nephridii* was isolated by complementation of an *E. coli* thioredoxin mutant strain (Lim & Fuchs, 1994). It was found to consist of 145 amino acid residues predicted from the nucleotide sequence, and it shares a high degree of homology with prokaryotic thioredoxins in the region of the active center. However, it contains 6 cysteine residues, two of which reside in the known active center sequence. The *E. coli trxA* gene and the *C. nephridii* gene encoding thioredoxin C3 contain a common *Ava*II site in the nucleotide sequence coding for the active site sequence -Cys-Gly-Pro-Cys-. Using a common *Ava*II site, two reciprocal hybrid thioredoxin genes (E-C3 and C3-E) were constructed and characterized.

Materials and Methods

Bacterial strains, plasmids, and phages

The *E. coli* strains, plasmids, and bacteriophages used in this study are listed in Table 1. All bacterial strains

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Table 1. *E. coli* strains, bacteriophages, and plasmids used in this study

Strains, bacteriophages, and plasmids	Characteristics (relevant genotypes etc.)	Sources
***E. coli* strains**		
JF521	F′ *supE metE46 srl*::Tn10 *trxA7004 recA*	Our lab.
BH2012	F⁻ *metA46 trxA7004 ilvC*::Tn5	Our lab.
BH5262	F⁻ *gshA trxA7004 srl*::Tn10	Our lab.
DH5αf′	F′ *supE44 Δlac169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Our lab.
MV1190	F′ *thi supE44 proAB lacI^{q}ZδM15* Δ(*srl-recA*)306::Tn10	Our lab.
Bacteriophages		
T7	wild-type T7 bacteriophage	Univ. of Minn.
mutant T7	T7 mutant with broad specificity for thioredoxin	Univ. of Minn.
M13mp18	Filamentous bacteriophage M13	Our lab.
Plasmids		
pCJF5	pUC13 derivative containing *E. coli trxA* gene	Our lab.
pKH9	pUC13 derivative containing a third thioredoxin gene from *Corynebacterium nephridii*	Our lab.
pTZ18R	a T7 promoter vector plasmid	Our lab.
pGP1-2	a vector containing T7 RNA polymerase gene	Our lab.

used were derivatives of *E. coli* K-12. Strain BH2012 was used to test for the growth of bacteriophages T7 and T7*tas*. Strains JF521 and BH5262 were used for complementation tests, and strain BH5262 was used as a recipient for co-transformation of plasmid pGP1-2, and a recombinant plasmid harboring the hybrid thioredoxin gene under the T7 promoter. Derivatives of JF521 and MV1190 were used to test for the growth of filamentous bacteriophage M13. T7 and T7*tas*, obtained from the University of Minnesota, were used as phage sources for growth tests.

Media, enzymes, and chemicals

E. coli strains were grown in Luria-Bertani medium (Miller, 1972) or in Davis-Mingioli minimal medium (Davis & Mingioli, 1950) supplemented with required components. When needed, ampicillin and kanamycin were added at final concentrations of 50 and 40 μg/ml, respectively.

Restriction endonucleases (*Eco*RI, *Hin*dIII, and *Ava*II) and T4 DNA ligase were purchased from Boehringer Mannheim. All enzymes were used as specified by the manufacturer. Agarose, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-β-thiogalactoside (IPTG), ampicillin, and kanamycin were also obtained from Boehringer Mannheim. 5,5′-dithio-2-nitrobenzoic acid (DTNB), NADPH, acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), and ammonium persulfate were obtained from Sigma Chemical Company. L-^{35}S-methionine (1000 Ci/mmol) was purchased from Amersham Corporation. *E. coli* thioredoxin reductase was obtained from J. A. Fuchs, Department of Biochemistry, University of Minnesota.

Isolation of plasmid DNA and restriction fragments

Plasmid DNA was isolated from a fresh overnight culture by the alkaline lysis method (Sambrook *et al.*, 1989). DNA fragments were purified through electroelution using agarose gel (Dretzen *et al.*, 1981). Transformation of *E. coli* cells was performed according to the $CaCl_2$ procedure of Mandel and Higa (1970).

Construction of hybrid thioredoxins

Plasmid pCJF5 (Lim *et al.*, 1985) harbors a 650 bp insert containing the *E. coli* thioredoxin gene, the transcriptional direction of which is opposite to that of the *lac* promoter of the pUC13 vector. Plasmid pKH 9 contains a *C. nephridii* gene encoding thioredoxin C3. The *Eco*RI-*Ava*II fragment of the insert from pCJF 5, which contains the N-terminal coding region for *E. coli* thioredoxin, and the *Ava*II-*Hin*dIII fragment of the insert from pKH9, which contains the C-terminal coding region for *C. nephridii* thioredoxin C3, were isolated by electroelution. The *Eco*RI-*Ava*II and *Ava*II-*Hin*dIII fragments were inserted into the *Eco*RI-*Hin*dIII linker region of plasmid pTZ18R. The resulting plasmid coded for a hybrid thioredoxin having the N-terminal sequence from *E. coli* thioredoxin and the C-terminal sequence from *C. nephridii* thioredoxin C3 (E-C3 thioredoxin). Alternatively, the *Eco*RI-*Ava*II fragment of the insert from pKH9, which contained the N-terminal coding region for *C. nephridii* thioredoxin C3, and the *Ava*II-*Hin*dIII fragment of pCJF5, which contained the C-terminal coding region for *E. coli* thioredoxin, were isolated and inserted into pTZ18R to generate the reciprocal construct (C3-E thioredoxin) (Fig. 1).

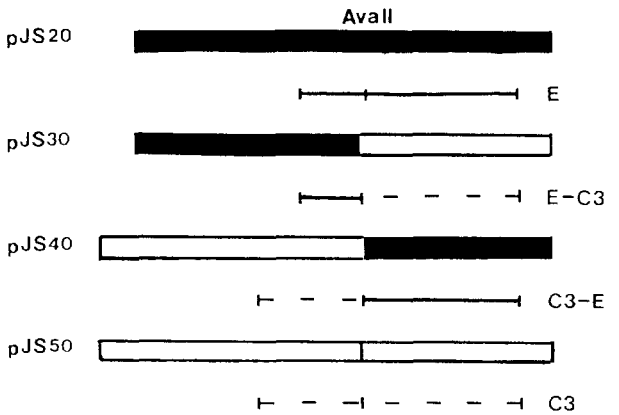

Fig. 1. Construction of hybrid proteins between *E. coli* thioredoxin and *C. nephridii* thioredoxin C3. Plasmid pJS20 contains *E. coli* thioredoxin gene, and pJS50 *C. nephridii* thioredoxin C3 gene. Plasmid pJS30 encodes E-C3 thioredoxin whereas plasmid pJS40 encodes C3-E thioredoxin. The four plasmids were constructed with pTZ18R. The straight line (|——|) indicates the open reading frame of *E. coli* thioredoxin gene, whereas the dotted line (|----|) indicates that of *C. nephridii* thioredoxin C3 gene.

Expression of hybrid thioredoxins using the T7 RNA polymerase/promoter system

The two pTZ18R derivatives, pJS30 and pJS40 (Fig. 1), were introduced into thioredoxin-deficient strain BH 5262 together with plasmid pGP1-2, which carried a gene encoding T7 RNA polymerase. Their expression products were specifically labelled with ^{35}S-methionine by the T7 RNA polymerase/promoter system (Tabor & Richardson, 1985).

Thioredoxin assay

E. coli thioredoxin catalyzes NADPH-dependent reduction of the disulfide bond in DTNB (Holmgren, 1977; Luthman & Holmgren, 1982). The assay mixture contained 100 mM Tris-HCl (pH 8.0), 2 mM DTNB, and 0.24 mM NADPH in a volume of 1.0 ml. A crude extract of 50~100μl was added, and the reaction was started by adding *E. coli* thioredoxin reductase. An increase in absorbance at 412 nm was directly monitored.

The protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% acrylamide gel by the method of Laemmli (1970). Proteins were either stained with Coomassie brilliant blue R-250, or analyzed by autoradiography. After ^{35}S-labeled samples were separated by the use of 12.5% SDS-PAGE, the gel was dried onto a piece of Whatman 3MM paper using a commercial gel dryer. The dried gel was autoradiographed using an intensifying screen.

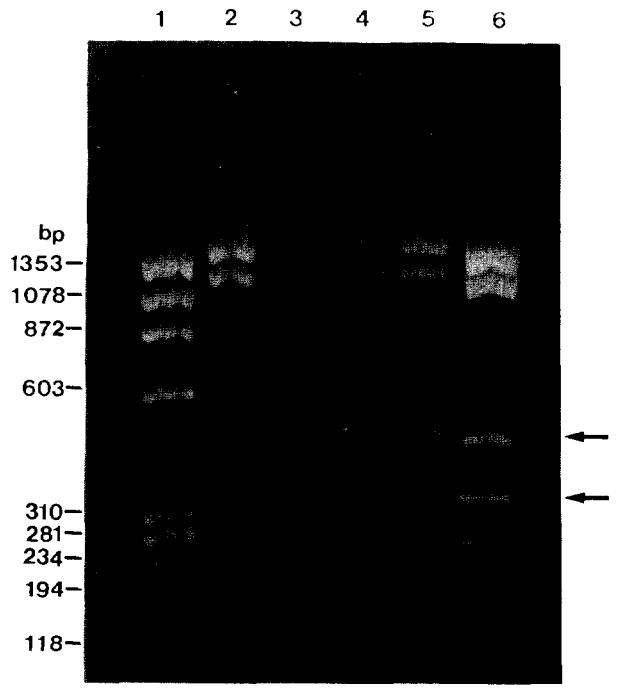

Fig. 2. Agarose gel electrophoretic patterns of plasmids harboring hybrid thioredoxin genes. Plasmid DNAs of pTZ18R, pJS20, pJS30, pJS40, and pJS50 were digested with *Eco*RI, *Ava*II, and *Hind*III. The arrows indicate the positions of switched DNA fragments. Lane 1 : φX174 DNA digested with *Hae*III; lane 2 : pTZ18R; lane 3 : pJS20; lane 4 : pJS30; lane 5 : pJS40; lane 6 : pJS50.

Results and Discussion

Construction of hybrid thioredoxin genes

The two hybrid thioredoxin genes were constructed from the *E. coli* thioredoxin gene and the *C. nephridii* thioredoxin C3 gene. Fig. 2 shows the restriction patterns of plasmids harboring the hybrid genes. Triple digestions with *Eco*RI, *Hind*III, and *Ava*II confirmed that the generated plasmids contained the expected hybrid thioredoxins genes, indicating that they contained switched DNA fragments (Lane 4 and 5), compared with the original plasmids (Lane 3 and 6). The two resultant pTZ18R derivatives were deginated pJS30 and pJS40, which encoded E-C3 and C3-E hybrid thioredoxins, respectively. Since the two reciprocal hybrid thioredoxins carried alternate N- and C-terminal domains of *E. coli* and *C. nephridii* C3 thioredoxins, they were suitable for studying the relationship between the function and the domain of thioredoxin. Estimated from their predicted amino acid sequences, the E-C3 hybrid thioredoxin contained two half cystines and the C3-E hybrid thioredoxin contained six half cystines. The E-C3 hybrid thioredoxin consisted of 113 amino acid residues, whereas the C3-E hybrid thioredoxin consisted of 141 amino acid residues.

Biological characterization of the hybrid thioredoxin

Table 2. Influence of heterologous thioredoxins on the growth of *E. coli trxA* strain in LB broth

Plasmids	Thioredoxin-type	Growth*	
		JF521 (*trxA*)	BH5262 (*trxA gshA*)
pTZ18R	None	−	−
pJS20	E	+	+
pJS30	E-C3	+	+
pJS40	C3-E	+	+
pJS50	C3	+	+

*+ : Good growth, − : Poor growth.

Table 3. Ability of heterologous thioredoxins as a cofactor of *E. coli* methionine sulfoxide reductase

Plasmid	Thioredoxin-type	Doubling time* (min)
pTZ18R	None	−
pJS20	E	82
pJS30	E-C3	95
pJS40	C3-E	114
pJS50	C3	107

*Doubling times were calculated from the growth curves of *E. coli trxA metE* strain JF521 carrying heterologous thioredoxin genes in M9 minimal media supplemented with proline and methionine sulfoxide.

Table 4. Plaque formation of T7 phage on *Escherichia coli trxA* cells harboring hybrid thioredoxins

Phage	Plasmid (thioredoxin-type)*				
	pTZ18R (none)	pJS20 (E)	pJS30 (E-C3)	pJS40 (C3-E)	pJS50 (C3)
T7	−	+	−	+	−
T7*tas*	−	+	+	+	+

*+ : Growth, − : No growth.

Table 5. Plaque formation of filamentous phage M13 carrying hybrid thioredoxin genes on *E. coli trxA* cells

M13 phages	Thioredoxin-type	*E. coli* strains*	
		MV1190 (F′, *trxA*⁺)	JF521 (F′, *trxA*⁻)
M13mp13	None	+	−
M13mp18-20	E	+	+
M13mp18-30	E-C3	+	+
M13mp18-40	C3-E	+	+
M13mp18-50	C3	+	−

*+ : Growth, − : No growth.

genes in *E. coli*

E. coli trxA⁻ mutant strains exhibited reduced colony sizes when plated on an enriched medium. A large colony size was restored in transformants containing plasmid pCJF5, which codes for *E. coli* thioredoxin, or pKH9, which codes for *C. nephridii* thioredoxin C3 (Lim & Fuchs, 1994). In a like manner both hybrid-type plasmids (pJS30 and pJS40) restored normal growth in *E. coli trxA* strain JF521 (Table 2). The plasmids coding for hybrid thioredoxins were also able to complement a *gshA trxA* double mutant BH5262, which cannot produce thioredoxin and γ-glutamylcysteine synthetase (Table 2). This strain otherwise requires glutathione for growth.

E. coli JF521 (*trxA metE*) is unable to use methionine sulfoxide to satisfy its methionine requirement. Strains JF521/pJS30 and JF521/pJS40 could use methionine sulfoxide as a methionine source (Table 3), indicating that the two hybrid thioredoxins can serve as a cofactor for *E. coli* methionine sulfoxide reductase *in vivo*.

E. coli BH2012 (F⁻, *trxA*) will not support the growth of the T7 bacteriophage because the host thioredoxin is an essential accessory protein for viral DNA polymerase (Mark & Richardson, 1976). This same strain containing plasmid pJS20, encoding for *E. coli* thioredoxin, allows the growth of T7, but the strain BH2012 harboring plasmid pJS50, coding for *C. nephridii* thioredoxin C3, did not support phage growth (Table 4). Strain BH2012 with plasmid pJS40 encoding the C3-E hybrid thioredoxin, gave rise to plaques after infection (Table 4). In contrast, the strain with plasmid pJS30 coding the E-C3 hybrid thioredoxin, did not support phage growth (Table 4). The mutant T7 phage (T7*tas*) with a broad specificity of thioredoxin requirement, which presumably has a mutation in the T7 gene 5 (Lim *et al.*, 1987), was also able to replicate in the presence of either hybrid thioredoxin (Table 4). As shown in Table 4, the C3-E hybrid thioredoxin is similar to *E. coli* thioredoxin, whereas the E-C3 hybrid resembles *C. nephridii* thioredoxin C3. These results indicate that the C-terminal domain of *E. coli* thioredoxin is required to specify the requirement of thioredoxin for bacteriophage T7 growth.

E. coli thioredoxin is also required for filamentous phage assembly (Russel & Model, 1985; Russel & Mo del, 1986). The two hybrid thioredoxin genes were transferred into the M13mp18 vector by digestion with *Eco*RI and *Hin*dIII, and subsequent ligation. Phage M13 mp18 RF DNAs carrying hybrid thioredoxin genes were isolated, and their inserts were identified by digestion with *Eco*RI and *Hin*dIII. These M13mp18 derivatives containing the hybrid thioredoxin genes were used to test the growth capacity in a thioredoxin-deficient strain.

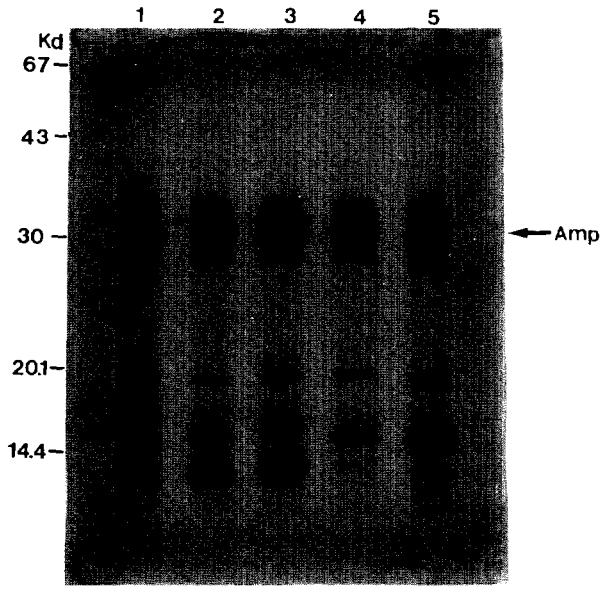

Fig. 3. Expression of hybrid thioredoxins using T7 RNA polymerase/promoter system. Lane 1 : BH5262/pGP1-2/pTZ18R; lane 2 : BH5262/pGP1-2/pJS20; lane 3 : BH5262/pGP1-2/pJS30; lane 4 : BH5262/pGP1-2/pJS40; lane 5 : BH5262/pGP1-2/pJS50.

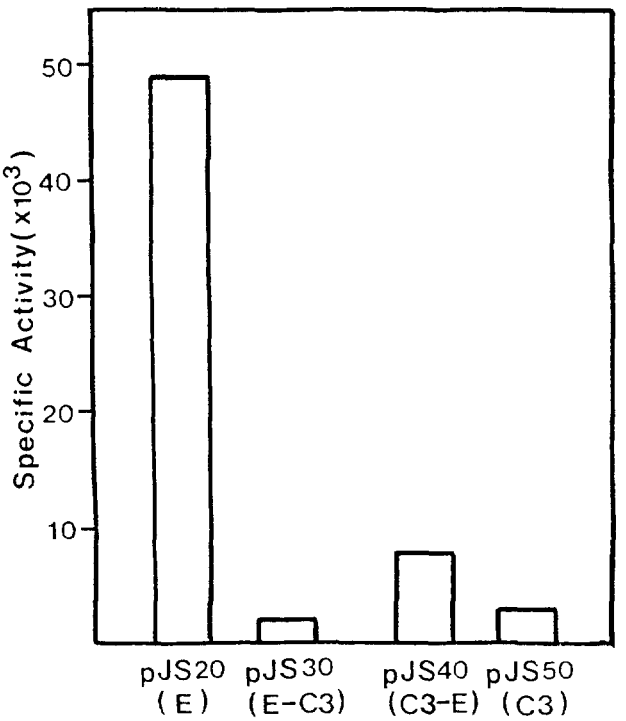

Fig. 4. Thioredoxin activity in exponential phase of *E. coli* strain JF521 carrying various thioredoxin genes. For an assay of thioredoxin activity, thioredoxin-catalyzed reduction of DTNB was monitored in the presence of *E. coli* thioredoxin reductase and NADPH. Specific activities are shown in OD_{412}/min/mg protein.

The phage M13mp18 derivatives harboring the *E. coli* thioredoxin gene, the E-C3 hybrid thioredoxin gene, and the C3-E hybrid thioredoxin gene could make plaques in the strain JF521(F', *trxA*), whereas the phage M13mp18 derivative harboring *C. nephridii* thioredoxin C3 gene could not (Table 5). These results from T7 and M13 infections suggest two aspects of the structure-function relationship of thioredoxin. One aspect is that the C-terminal domain of *E. coli* thioredoxin may be required for the growth of phages T7. The other is that phages T7 and M13 may use different surfaces of thioredoxin for their growth.

Expression of the hybrid thioredoxin genes in *E. coli*

Because strains BH5262/pGP1-2/pJS30 and BH 5262/pGP1-2/pJS40 carried a hybrid thioredoxin gene under the T7 promoter, their gene expression products could be labelled with ^{35}S-methionine by a T7 RNA polymerase/promoter system (Tabor & Richardson, 1985). An autoradiographic pattern of labelled proteins showed the production of two hybrid thioredoxins (Fig. 3). The mRNA produced from the *E. coli* thioredoxin gene contained two potential translational initiation sites, one of which could initiate the synthesis of a protein 19 amino acids longer (called extended thioredoxin, M.W., 15,000 daltons) than the *E. coli* thioredoxin of 108 amino acid residues (M.W., approximately 12,000 daltons) (Lim *et al.*, 1985; Lim *et al.*, 1991; Lim *et al.*, 1992). This study shows that the *E. coli* thioredoxin gene expresses both thioredoxin and extended thioredoxin with the expected molecular weights (Fig. 3, Lane 2). The reading frame predicted from the nucleotide sequence of the *C. nephridii* thioredoxin C3 gene contained one potential translational initiation site and *C. nephridii* thioredoxin C3 consisted of 145 amino acid residues (M.W., approximately 15,000 daltons). An autoradiographic pattern showed the production of *C. nephridii* thioredoxin C3 having a molecular weight of 15,000 daltons on SDS-PAGE (Fig. 3, Lane 5). Strain BH5262/pGP1-2/pJS30 produced two proteins with molecular weights of 15,000 and 12,000 daltons, similar to the *E. coli* thioredoxin gene products, whereas strain BH5262/pGP1-2/pJS40 produced a single protein with a molecular weight of 15,000 daltons, like *C. nephridii* thioredoxin C3 (Fig. 3, Lanes 3 and 4). This finding originated from two different regulatory regions of the *E. coli* thioredoxin gene and the *C. nephridii* thioredoxin C3 gene. The production of the E-C3 and C3-E hybrid thioredoxins is from the two hybrid thioredoxin genes.

Reaction with *E. coli* thioredoxin reductase

E. coli thioredoxin is a substrate for *E. coli* thioredoxin reductase (Holmgren, 1985). The activities of hybrid thioredoxins were measured by DTNB assay with crude extracts prepared from *E. coli* strain JF521 harboring chimeric thioredoxin genes. Assays were carried out

with heat-treated extracts since thioredoxins are generally known to be heat-stable. As shown in Fig. 4, the extract from the pJS30-containing strain showed a much reduced catalytic efficiency compared with the extract from the pJS20-containing strain. Considering that they are produced from the same *E. coli trxA* promoter, the difference in catalytic efficiency reflects either a low affinity of the E-C3 hybrid thioredoxin for *E. coli* thioredoxin reductase, or a relatively unstable character of the E-C3 hybrid thioredoxin. The C3-E hybrid thioredoxin was a better substrate for *E. coli* thioredoxin reductase than *C. nephridii* thioredoxin C3 (Fig. 4). Further study to characterize the hybrid thioredoxin constructed in this study should proceed with their purification.

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