RESEARCH ARTICLE

The role of periplasmic antioxidant enzymes (superoxide dismutase and thiol peroxidase) of the Shiga toxin-producing *Escherichia coli* O157:H7 in the formation of biofilms

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This study examined the role of the periplasmic oxidative defense proteins, copper, zinc superoxide dismutase (SodC), and thiol peroxidase (Tpx), from the Shiga toxin-producing *Escherichia coli* O157:H7 (STEC) in the formation of biofilms. Proteomic analyses have shown significantly higher expression levels of both periplasmic antioxidant systems (SodC and Tpx) in STEC cells grown under biofilm conditions than under planktonic conditions. An analysis of their growth phase-dependent gene expression indicated that a high level of the *sodC* expression occurred during the stationary phase and that the expression of the *tpx* gene was strongly induced only during the exponential growth phase. Exogenous hydrogen peroxide reduced the aerobic growth of the STEC *sodC* and *tpx* mutants by more than that of their parental strain. The two mutants also displayed significant reductions in their attachment to both biotic (HT-29 epithelial cell) and abiotic surfaces (polystyrene and polyvinyl chloride microplates) during static aerobic growth. However, the growth rates of both wild-type and mutants were similar under aerobic growth conditions. The formation of an STEC biofilm was only observed with the wild-type STEC cells in glass capillary tubes under continuous flow-culture conditions compared with the STEC *sodC* and *tpx* mutants. To the best of our knowledge, this is the first mutational study to show the contribution of *sodC* and *tpx* gene products to the formation of an *E. coli* O157:H7 biofilm. These results also suggest that these biofilms are physiologically heterogeneous and that oxidative stress defenses in both the exponential and stationary growth stages play important roles in the formation of STEC biofilms.

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Abbreviations: CuZnSOD, copper and zinc superoxide dismutase; **LB**, Luria-Bertani; **PVC**, polyvinyl chloride; **ROS**, reactive oxygen species; **SodC**, superoxide dismutase; **STEC**, Shiga toxin-producing *E. coli* O157:H7; **Tpx**, thiol peroxidase

1 Introduction

Biofilms are a complex community of microorganisms that attach to and grow on either biotic or abiotic surfaces in a wide variety of environments [1]. The biofilm plays a key role in protecting pathogenic bacteria from the host defenses [2]. The accumulation of a biofilm also causes fouling of indus-

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trial equipment, which can decrease the level of water transfer in water distribution systems, and the spoilage of foods leading to the transmission of disease [3]. Biofilm formation appears to be a highly coordinated process that is influenced by both genetic and environmental factors [4, 5]. The expression of a distinct set of genes that differentiate sessile cells from planktonic cells is essential for the successful formation of a biofilm [5–7]. The most significant changes that occur during biofilm development were observed in the proteins involved in the resistance to oxidative damage, exopolysaccharide production, and the metabolism [6, 7]. Recent studies using community proteomics have also shown that the proteins involved in oxidative stress are highly expressed in natural microbial biofilms [8]. Bacterial growth within a biofilm is believed to be heterogeneous, with maximum growth occurring at the surface–liquid interface, and minimal growth within the interior regions of the community. DeBeer *et al.* [9] measured the oxygen concentration in various regions of a biofilm, and revealed significant levels of dissolved oxygen at all levels of the water channels. It was also reported [9–11] that the water channels inside a biofilm structure appear to transport oxygen into the biofilm. However, diffusion limitations and oxygen utilization produce very low oxygen levels at the center of the cellular microcolonies. Therefore, the physiology of the biofilm cells is not anoxic. Although the inner space of the biofilm tends to have low oxygen concentrations, these low oxygen concentrations are sufficient to support the cellular metabolism through which intercellular or extracellular reactive oxygen can be generated [9–11]. It was also reported that the cells are located on the surface of a biofilm would experience a higher oxygen concentration than those inside the biofilm. The subset of the genes involved in the defense against oxidative stress under biofilm conditions is different from that in planktonic cells. Such genes may be required to protect the bacterial cells from damage by reactive oxygen species (ROS) during the cellular metabolisms within the biofilm. Recently, many proteomics or microarrays used to examine biofilm cells revealed strong expression of the proteins involved in proteins folding and the responses to oxidative stress [8, 12]. This led to the conclusion that biofilm cells experience oxidative stress even in low oxygen concentrations. It is possible that oxidative stress on biofilm cells might result from the fact that the increased cell densities deplete the locally available nutrients such as iron [13, 14]. Iron starvation leads to oxidative stress [15, 16].

ROS can be generated by the host immune responses, environmental pollutants, and the incomplete reduction of oxygen to water during the bacterial aerobic metabolism [17]. Under normal conditions, $<$1.0% of the electrons from the electron transport chain are transferred to oxygen to produce superoxide (O_2^-) [18]. In order to cope with these exogenous and endogenous oxidative burdens, bacterial cells have several enzyme-based defense systems that involve catalase and superoxide dismutase [19]. The regulated adaptive responses of microorganisms to oxidative stress have been studied extensively using *Escherichia coli* and *Salmonella enterica* as the model microorganisms [19]. The *sodC* gene encodes copper and zinc superoxide dismutase (CuZnSOD), and many Gram-negative bacteria export this protein to their periplasmic compartments where they protect the cells from oxidative stresses [20]. Because the superoxide anion cannot cross the bacterial inner membrane, periplasmic CuZnSOD is believed to be involved in protecting the cells from superoxides derived from various periplasmic or environmental sources [21]. The level of superoxide dismutase (SodC) activity according to the β-galactosidase activity is extremely low during exponential growth but increases at least 100-fold during the stationary phase. Northern blot analysis showed that the *sodC* transcript was observed only in the late-stationary-phase cultures, paralleling the increase in SodC activity [2, 20]. Many *sodC*-deficient mutants are more susceptible to death from extracellular superoxides than the wild-type variants [22, 23]. However, Gort *et al.* [20] reported that the *sodC* gene is not induced by ROS but is induced in the stationary phase by RpoS. Furthermore, mutants of *E. coli* and *Salmonella typhimurium* that lacked CuZnSOD were not found to be sensitive to exogenous superoxide, because both were killed more rapidly by exogenous hydrogen peroxide in the early stationary phase than their parental strains. The molecular basis for this phenotype is unknown [20].

Another periplasmic oxidative defense enzyme, thioredoxin-dependent thiol peroxidase (Tpx) from *E. coli*, belongs to the peroxiredoxin (Prx) family. Although the levels of *tpx* mRNA and its protein did not change after exposure to various oxidants [24], Tpx functions in oxidative stress defenses against organic hydroperoxides (ROOH) and H_2O_2 [25]. The high-level expression of the *tpx* gene during the growth phase without induction by H_2O_2 suggested that it plays an important role in the detoxification of peroxides in the periplasmic space during growth [26]. Microorganisms in a biofilm show a heterogeneous behavior with respect to their metabolic activity, growth status, and gene expression [27]. The aim of this study was to determine the proteins upregulated during the formation of a biofilm, particularly those involved in the antioxidant systems. It was found that both Tpx and SodC are essential for biofilm formation. Because of their differences in transcriptional regulation during aerobic growth, this study investigates the requirement and regulation of the different types of periplasmic antioxidant systems, SodC and Tpx, in biofilm formation.

2 Materials and methods

2.1 Bacterial strains and growth media

E. coli O157:H7 ATCC 43894 producing Shiga-like toxins 1 and 2, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *E. coli* O157:H7 ATCC 43894 (hereinafter *E. coli* O157:H7) was cultured at 30°C in Luria-Bertani (LB) media or a modified M9 medium

containing (*per* liter of H_2O) 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5% casamino acid (vitamin free), 0.1% $MgSO_4 \cdot 7H_2O$, and 0.5% glucose. Unless otherwise stated, all the other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) and BioRad (Richmond, CA, USA).

2.2 DNA and plasmid manipulation

Table 1 lists the bacterial strains, plasmids, and primers used in this study. PCR and cloning for vector construction were performed according to the method described by Ausubel *et al.* [28]. *E. coli* O157:H7-R (rifampicin-resistance *E. coli* O157:H7) were selected from spontaneous mutations on (LB) media containing rifampicin (200 μg/mL) after prolonged incubation and sequential transfers. The bacteria cultures were grown at 37°C for *E. coli* in LB or the modified M9 medium with vigorous aeration by shaking at 220 rpm. Kanamycin (100 μg/mL) was added when deemed necessary.

2.3 Construction of *sodC, tpx, rpoS,* and *oxyR* mutants

The suicide vector, pVIK112 [29], was used to construct the *sodC* and *tpx* mutants. The SodC-KO1/SodC-KO2 primer pair was used to target the internal region of the *sodC*. The amplified 257-bp fragment was subcloned into the *Eco*RI cloning site of pVIK112 to create pVIK-sodC. The 367-bp fragment of the internal region of the *tpx* gene amplified using the primer pair (Tpx-KO1/Tpx-KO2) was cloned into the *Eco*RI cloning site of the pVIK112 vector, generating pVIK-tpx. The 422-bp, 418-bp fragments of the internal region of the *rpoS*, *oxyR* genes amplified using the primer pairs (ErpoS-KO1/ErpoS-KO2, EoxyR-KO1/EoxyR-KO2), were cloned into the *Eco*RI cloning site of the pVIK112 vector, generating pVIK-ErpoS and pVIK-EoxyR, respectively. The same transformation, conjugation, and mutant confirmation procedures used for constructing the mutants are described elsewhere [30]. *E. coli* O157:H7-R was used as the recipient strain.

2.4 Conditions for planktonic and biofilm cultures

Planktonic and biofilm cultures were prepared using a slight modification of the method described by Tremoulet *et al.* [31, 32]. Autoclaved Glass Fiber Filter Thick (GFF; 25 mm diameter, Pall Life Science, USA) was used for the biofilm cultures. Sterile GFF was inoculated with 500 μL of the bacterial culture (~10^6 CFU/mL). The adhesion of the bacterial cells to the glass fibers was complete within 20 min. The inoculated GFF was washed with 10 mL of PBS for 10 s and placed on the sterile GFF in Petri dishes containing modified M9 agar. The biofilms were incubated at 30°C for 5 days. The viable cells were determined using serial dilution techniques and extra solution was recovered for a further proteomic study.

Table 1. Bacterial strains, plasmids, and primers used in this study

(a)

Bacterial strain/plasmid	Description	Reference
Strains		
E. coli S17-1λ pir	Tra+ R6K strain, used for biparental conjugation	Lab stock
E. coli Top10	*mcrA,* Δ*(mrr-hsdRMS-mcrBC), Φ80lacZΔM15*	Promega
E. coli O157:H7 43894	Shiga toxin 1, 2 producing *E. coli* O157:H7	Lab stock
E. coli O157:H7-R	Rifampicin-resistance strain of *E. coli* O157:H7	This study
E. coli O157:H7-sodC	*sodC* mutant, insertion of pVIK-sodC into *E. coli* O157:H7	This study
E. coli O157:H7-tpx	*tpx* mutant, insertion of pVIK-tpx into *E. coli* O157:H7	This study
E. coli O157:H7-oxyR	*oxyR* mutant, insertion of pVIK-oxyR into *E. coli* O157:H7	This study
E. coli O157:H7-rpoS	*rpoS* mutant, insertion of pVIK-rpoS into *E. coli* O157:H7	This study
Plasmids		
pVIK112	R6K*oriV*, suicide vector, *lacZ* fusion	
pVIK-sodC	Internal *sodC* fragment region in pVIK112	This study
pVIK-tpx	Internal *tpx* fragment region in pVIK112	This study
pVIK-ErpoS	Internal *rpoS* fragment region in pVIK112	This study
pVIK-EoxyR	Internal *oxyR* fragment region in pVIK112	This study

(b)

Primer	Sequence 5′-3′
SodC-KO1	CGC GAA TTC CCG ATA AAG GTC TGG AGT T
SodC-KO2	CGC GAA TTC GGT AGC TTT GCC GTC ATT
Tpx-KO1	CGC GAA TTC AAC ATT TTC CCG AGT ATT G
Tpx-KO2	CGC GAA TTC ACG CGC TGC CAG ACC TTT CA
EoxyR-KO1	CGC GAA TTC CCG TGC TGC GTG AGG TG
EoxyR-KO2	CGC GAA TTC TGA TCG CGC AAA CAG TGA C
ErpoS-KO1	CGC GAA TTC TCT GGC GTT GCT GGA CCT TAT C
EropS-KO2	CGC GAA TTC CTT CCG GAC CGT TCT CTT TTT CAT
MCS-R	ACC ATG GTC ATA GCT GTT TCC TG

Underlined *Eco*RI cloning site.

2.5 Preparation of crude protein extract

The bacterial cells were centrifuged (4°C, 8000 × *g*, 30 min) and then resuspended in a lysis buffer (40 mM Tris-HCl, 1% Tritox X-100, 1 mM EDTA, 1 mM $MgSO_4 \cdot 7H_2O$; pH 8.0) containing 1 mM PMSF. The biofilm-grown cells that had been attached to the GFF were immersed directly into the same lysis buffer after washing the surface in PBS. The cells were lysed on ice by sonicating (Dr. Hielscher, Teltow, Germany) five times for 30 s with an 80% pulse duration. After sonication, the cell debris and glass fibers were removed by centrifugation, and the supernatants were recovered. For protein precipitation, chilled TCA/acetone (10% TCA and 0.07% 2-mercaptoethanol in acetone) was added to the supernatants, and the mixture was kept overnight at −20°C. After centrifugation, the pellet was washed twice with chilled acetone. The pellet was air dried in a hood and resuspended in a solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.5% ampholytes pH 3–10). The protein concentration was measured using a modification of the Bradford method [33]. The protein samples were stored at −80°C until needed for proteome analysis.

2.6 2-DE

2-DE was performed using a slight modification of the method described by Görg *et al.* [34]. To perform the first dimension, the samples were mixed with a rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.2% ampholytes pH 3–10, and a trace of Bromophenol blue) resulting in a final protein level of 500 μg of protein in 320 μL. The samples were rehydrated on ReadyStrip IPG Strips (17 cm, pH 3–10, BioRad) overnight at 20°C under mineral oil. IEF was carried out using a PROTEAN™ IEF cell (BioRad) according to the manufacturer's instructions under the following conditions: 250 V (30 min), 500 V (30 min), 1000 V (30 min), 8000 V (2 h), 8000 V (25 000 V·h), and 500 V (15 min). After IEF, the strips were incubated for 10 min in equilibration buffer I (6 M urea, 2% SDS, 20% glycerol, 130 mM DTT, 0.375 M Tris-HCl, pH 8.8), and for an additional 15 min in equilibration buffer II (6 M urea, 2% SDS, 20% glycerol, 135 mM idoacetamide, 0.375 M Tris-HCl, pH 8.8). After equilibration, the strips were transferred to 12.5% SDS-PAGE gels (16 cm × 20 cm) for the second dimension. The separation was performed using a PROTEAN™ II xi system (BioRad) with 10 mA *per* gel for 1 h and thereafter with 20 mA *per* gel at 4°C. The protein spots were visualized using the blue silver staining method [34]. The stained gels were scanned using a densitometry instrument (800 × 1600 dpi, UMAX, UTA 2100XL, USA) and the spots were analyzed using PDQuest software (BioRad) according to the manufacturer's instructions. Four gels resulting from two independent experiments were obtained, and two gels of good quality were used for analysis. Only spots showing significant changes in intensity (more than 2.5-fold) were considered in this study.

2.7 Protein identification by MALDI-TOF/MS

The sample preparation for MALDI-TOF/MS was performed using a method described elsewhere [35]. Briefly, the protein spots of interest were excised and destained by washing with 25 mM ammonium bicarbonate containing 50% ACN. The gels were dehydrated by adding 100% ACN, rehydrated in ice by adding 20 μL of 25 mM ammonium bicarbonate containing 10 mg/mL of sequencing grade modified trypsin (Promega, Madison, WI, USA). After incubation at 37°C for 20 h, the peptides were extracted with 0.1% TFA in 50% ACN. The supernatants were recovered and dried in a freeze dryer. The samples were reconstituted in 0.1% TFA and concentrated with C_{18} ZipTips™ (Millipore, Bedford, MA, USA). The purified peptides were eluted with a saturated matrix solution (CHCA in 60% ACN and 0.1% TFA). The monoisotopic masses (M + 1) of the tryptic fragments were measured in a Perspective Biosystem MALDI-TOF/MS voyager DE-STR Mass Spectrometer (Framingham, MA, USA). The spectra were searched and identified using the MASCOT system (http://matrixscience.com) with an *E. coli* subset of the NCBI database.

2.8 Northern blot analysis

The total RNA was isolated from 2 to 3 mL of cells using a RNase Mini kit (Qiagen) according to the manufacturer's instructions. The RNA concentrations were estimated from the absorbance at 260 nm. Samples of RNA (10 μg) were loaded onto the denaturing agarose gels containing 0.25 M formaldehyde and electrophoresed. The gels were then stained with ethidium bromide to visualize the rRNA (23S and 16S). The fractionated RNA was transferred to nylon membranes (Scheicher & Schuell, NH, USA) using a Turboblotter (Scheicher & Schuell). The amount of *sodC* or *tpx* mRNA was determined by hybridizing the membrane with an *sodC* or *tpx*-specific, a ^{32}P-labeled probe prepared by PCR amplification with the primer pairs, SodC-KO1/SodC-KO2 and Tpx-KO1/Tpx-KO2.

2.9 Survival of *sodC* and *tpx* mutants with exogenous H_2O_2

Different concentrations of oxidative stress agents (paraquat, menadione, and H_2O_2) were used to determine the survival rates of the wild-type and mutant strains under various oxidative stress conditions. The LB-cultured *sodC* and *tpx* mutant cells were collected at the stationary and exponential phases, respectively, and washed three times with autoclaved PBS (pH 7.5). Approximately, 10^9 CFU/mL were inoculated into PBS (5 mL) containing different agents, and the cultures incubated at 37°C with constant agitation (220 rpm). At each time point (5 min, 1, 2, and 3 h), the cells were harvested and washed with PBS. The number of viable cells was determined by dilution in LB and plating them on LB plates.

2.10 Biofilm assay

The assay for biofilm formation was carried out using a slight modification of a method reported elsewhere [36]. Polystyrene and polyvinyl chloride (PVC) 96-well microtiter plates (BD Science, USA) were used as the abiotic surfaces for biofilm formation. The overnight cultures of *E. coli* O157:H7 were washed twice with PBS and inoculated at $\sim 10^5$ CFU/mL in a fresh modified M9 minimum medium supplemented with 0.1% glucose. Incubation at 25°C without agitation continued for either 24 or 48 h, the microtiter plates were rinsed thoroughly twice with sterile water followed by a 0.1% w/v solution of crystal violet (CV) to stain the attached cells. After staining at room temperature for 30 min, the CV was removed and the wells were rinsed three times with sterile water. The dye was dissolved in 95% ethanol. The absorbance of the solubilized dye at 595 nm was then determined.

2.11 Attachment assay

The HT-29 cell line was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were routinely cultured in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Prior to the attachment assay, the HT-29 monolayers were washed three times in PBS to remove the culture medium and any nonattached cells. The *E. coli* O157:H7 ($\sim 10^6$ CFU/mL), which was grown in a modified M9 medium, was then cultured overnight. Incubation continued at 37°C in a 5% CO_2 atmosphere. Before the detachment procedure, monolayers of the HT-29 cells were washed six times with PBS to remove the nonattached bacteria. The adherent cells were released from the well plates using trypsin-EDTA for 5 min at 37°C. An antibiotic-free medium was then added to each of the wells, which were then agitated to dissociate the HT-29 cells. Serial dilutions of the mixtures were then plated onto MacConkey agar (Difco, USA) and incubated for 48 h at 37°C. The attachment ability (CFU/ mL) was determined by cell counting.

2.12 Capillary biofilm system

The biofilms were grown in glass capillary tubes under continuous flow conditions using the methods described elsewhere [37]. The capillary tubes (0.3 mm × 0.3 mm inside dimensions; Vitrocom, Mountain Lakes, NJ, USA) were inoculated with 1 mL of the wild-type and mutant cultures of *E. coli* O157:H7 that had been incubated overnight, and grown in modified M9 media supplemented with 0.1% glucose. The inoculum was syringe injected to fill the inside of the glass tubes. The tubing upstream of the glass tube was clamped, and the system was allowed to stand without flow for 1 h at room temperature. After inoculation, the capillary tubes were mounted on a standard microscope slide. The system was switched to continuous culture mode by delivering modified M9 medium supplemented with 0.1% glucose and antibiotics to a homemade carboy (1 L) at a flow rate of 0.1 mL/min. The developing biofilms were visualized using transmitted light and 10× objective lenses with an Olympus CH2 microscope.

2.13 Statistical analysis

Unless indicated otherwise (in the figure legends) all experiments were replicated at least three times. The statistical significance was assessed by ANOVA, followed by a Duncan's test in the SAS software package (Ver. 9.1). A p value <0.05 was considered significant.

3 Results and discussion

3.1 Identification of proteins expressed at higher levels in biofilm cells

A proteomics approach was used to determine the proteins involved in biofilm formation. The total proteins of Shiga toxin-producing *E. coli* O157:H7 (STEC) cultured either in planktonic or biofilm conditions for 5 days at 30°C were analyzed by 2-DE. The total proteins from the biofilm cultures were extracted from the mature biofilms on day 5, in order to insure that the results were relatively close to the actual protein profiles under the biofilm conditions. Among the 450 spots detected, 26 genes were differentially up- or down-regulated 2.5- to 5.9-fold under biofilm conditions. Changes in the spot intensity of at least 2.5-fold were considered and selected for MALDI-TOF/MS analysis (Fig. 1 and Table 1). Using computer-aid analysis, the proteins that were expressed only under one growth condition (18 spots for planktonic cells and 22 spots for biofilm cells) were identified. Further analysis of those spots is currently under way. This study was interested in identifying the spots that appeared on both culture conditions because of the physiological heterogeneity of the biofilm structure. Fourteen proteins including CuZn-SOD, OmpF, a single-strand binding protein, and Tpx were up-regulated, and 12 proteins including the phosphotransferase system (PTS) enzyme (glucose-specific IIA component), cyclic AMP receptor protein (CRP), and UDP-*N*-acetylglucosamine acetyltransferase were down-regulated (Fig. 1B, Table 2). The proteins identified could be classified into six categories based on their function: (i) oxidative stress response (spots 3, 5, and 11), (ii) general metabolism (spots 2, 8, 9, 13, 14, 18, 19, and 25), (iii) DNA replication (spots 12 and 15), (iv) membrane maintenance (spots 1, 4, 21, and 26), (v) phage related proteins (spots 6, 7, and 22), and (vi) unknown proteins (spots 10, 16, 17, 20, 23, and 24).

Among the proteins involved in the general metabolism, succinate dehydrogenase induced a two-fold change under biofilm conditions. Succinate dehydrogenase is also involved in the bacterial iron homeostasis and the oxidative stress response system [15, 16]. The expression of succinate dehy-

Table 2. Summary of the *E. coli* O157:H7 proteins identified by MALDI-TOF/MS indicating different expression levels in the biofilms

Spot no.	Protein	MW	Theoretical p*I*	MOWSE score	%Coverage	NCBI accession no.	Fold difference in biofilm
1	Chain A, Structure Of The Hypothetical Protein Ygiw From *E. Coli*	11898	4.73	55	50%	gi|47168420	+5.9
2	PTS system, glucose-specific IIA component	18255	4.73	33	27	gi|15802950	−2.9
3	Putative thiosulfate sulfurtransferase	30864	4.56	123	47	gi|38704097	+2.8
4	OmpF porin deletion	36381	4.69	71	40	gi|1943074	+2.9
5	Thiol peroxidase (Tpx)	17824	4.75	78	58	gi|13361368	+4.4
6	Putative phage inhibition, colicin resistance and tellurite resistance protein	20467	4.69	78	53	gi|15801103	−2.6
7	Nucleoside channel; receptor of phage T6 and colicin K	33586	5.06	87	50	gi|15800140	+2.5
8	Glutamate-1-semialdehyde aminotransferase (aminomutase)	45326	4.74	94	37	gi|15799838	−4.9
9	Transaldolase B	35197	5.11	78	35	gi:16128002	−2.6
10	Unkown					UI[a)]	+3.2
11	Copper, zinc superiode dismutase (SODC)	17670	5.95	28	32	gi|15802060	+2.7
12	ssDNA-binding protein	18946	5.44	91	55	gi|15804651	+2.8
13	Lipoamide dehydrogenase (NADH)	50942	5.79	72	31	gi|30061682	+3.0
14	Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	65008	5.85	204	56	gi|16128698	+2.5
15	DNA polymerase III catalytic subunit	10559	6.13	26	35	gi|263398	+3.2
16	*yciD* gene product	18330	6.17	62	45	gi|902460	+3.6
17	Putative enzyme	27012	6.14	94	53	gi|15803556	−2.6
18	Anthranilate synthase component II	57158	6.08	158	53	gi|15831089	−4.9
19	Acetaldehyde dehydrogenase; Alcohol dehydrogenase; Aldehyde–alcohol dehydrogenase	96622	6.32	110	31	gi|26247570	+2.5
20	Hypothetical protein ycfP	23621	6.50	80	56	gi|26247251	−3.5
21	Molybdate-binding periplasmic protein	24903	6.38	197	72		+2.5
22	Unknown protein encoded by prophage CP-933T	14849	7.96	40	47	gi|15802321	−2.7
23	Unknown					UI	−2.6
24	Unknown					UI	−3.0
25	Cyclic AMP receptor protein	23796	8.37	82	47	gi|30065357	−2.5
26	UDP-*N*-acetylglucosamine acetyl-transferase; lipid A biosynthesis	28318	6.63	65	35	gi|15799863	−2.6

a) UI: Unidentified.

drogenase is reduced considerably during anaerobiosis using an ArcAB two-component regulatory system [38]. Higher levels of *sdhA* (encoding a succinate dehydrogenase) have also been observed in the microarray analysis of *Staphylococcus aureus* cultivated under biofilm conditions [7]. A three-fold higher expression level of the SodC and Tpx proteins together with a two-fold induction of succinate dehydrogenase suggests that the STEC cells can experience oxidative stress under biofilm conditions. This is supported by the finding that a higher level of expression of the *sodA* (Mn-containing superoxide dismutase) gene was induced during the transition to biofilm growth both in *E. coli* and *S. aureus* biofilms

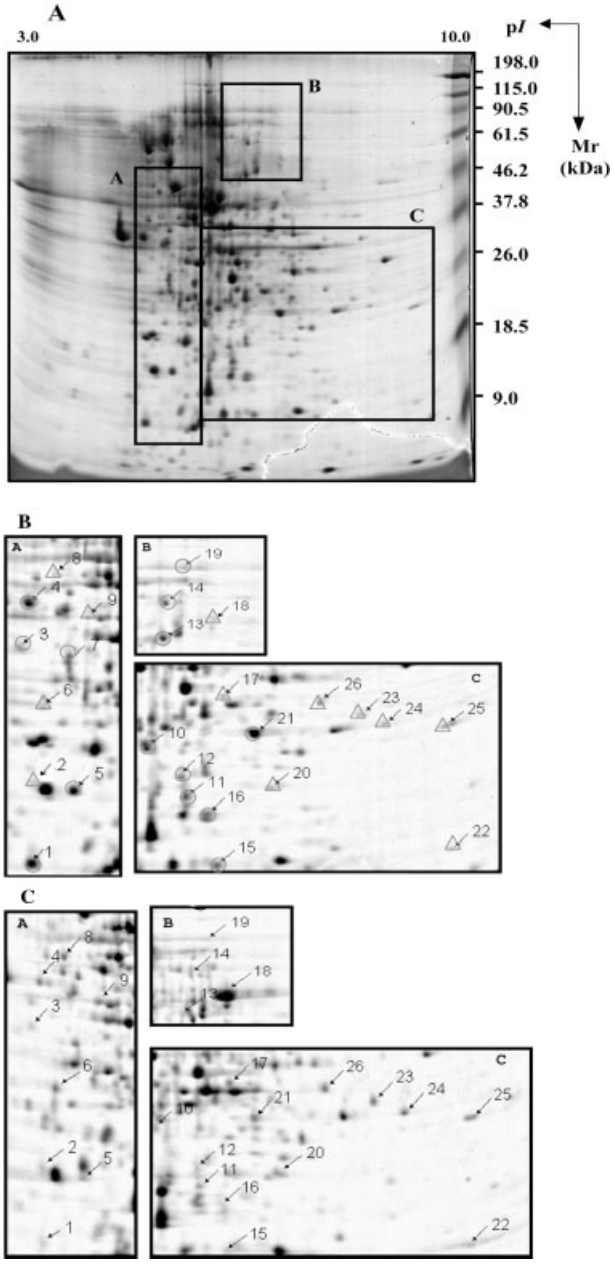

Figure 1. Proteomic analysis of *E. coli* O157:H7 cells under biofilm conditions. (A) 2-DE patterns of the *E. coli* O157:H7 whole cell lysate proteins from the cells grown under biofilm conditions for 5 days. The proteins were subjected to IEF (pH 3–10) and resolved in the second dimension by 12.5% SDS-PAGE. The proteins were separated, detected by silver staining, and the data were analyzed, as described in Section 2. Twenty-six genes differentially expressed under biofilm conditions were analyzed. Table 2 shows the results. The proteins analyzed by MALDI-TOF/MS are indicated by spot number. The spots indicated by circles are those proteins with an increased level in biofilms and the triangle spots indicate a decreased level of proteins in the biofilms. (B) Detailed view of a silver-stained 2-DE gel under biofilm conditions. (C) Detailed view of a 2-DE gel under planktonic conditions. The experiment was repeated at least three times with similar results.

[7, 12], and that the level of *sodC* mRNA was higher in enterohemorrhagic *E. coli* O157 (EHEC) cells attaching to eukaryotic plasma membranes [39]. The induction of two periplasmic oxidative defense systems, SodC and Tpx proteins under the STEC biofilm conditions indicates that the antioxidant systems in the periplasmic location play an important role in the defense against oxidative stress. Although oxygen limitations and spatial physiological heterogeneity have been observed between biofilms [40], the levels of a certain subset of the oxidative stress defense system were significantly higher in both natural and laboratory microbial biofilms [8, 12]. This shows that the subset of bacterial defenses against oxidative stress can make important contributions to the survival and persistence of biofilm-grown cells. This interpretation is consistent with recent findings showing that catalase and superoxide dismutase can protect *Pseudomonas aeruginosa* biofilms from exposure to exogenous hydrogen peroxide [41, 42].

3.2 Transcriptional regulation of the *sodC* and the *tpx* under biofilm conditions

These results suggest that the *sodC* and *tpx* genes involved in the bacterial oxidative stress responses may play a significant role in the development of a biofilm. Consistent with the proteomic approach, Northern blot analysis suggested that the *sodC* gene mRNA level was elevated under the same biofilm conditions used for the proteomic analyses (Fig. 2A). However, a lower level of *tpx* expression was observed under biofilm conditions, compared with expression under planktonic culture conditions (Fig. 2A). The *tpx* mRNA level differs from the level of the Tpx protein when its expression patterns in the biofilm and planktonic culture conditions were compared (Figs. 1 and 2A). There is no satisfactory explanation for the discrepancy between the level of the Tpx protein and the level of *tpx* mRNA under biofilm conditions. The Tpx protein under biofilm conditions might not destabilize quickly enough to result in the accumulation of large amounts of the Tpx protein for biofilm-cultured cells. Further analysis of the role of *tpx* mRNA and its protein stability in the biofilm cell physiology remains to be determined.

The growth phase-dependent transcription of the *sodC* and *tpx* was examined after collecting the total mRNA during growth in LB media (Figs. 2B and C). Northern blot analysis showed that the *sodC* promoter activity is much higher during the stationary growth phase than during the exponential growth phase (Figs. 2B and 2C). Overnight (16 h) culture-grown cells also showed a higher *sodC* mRNA expression (Figs. 2B and C, lane 1). However, the expression of the *tpx* gene increases in the early exponential phase, and a significantly low expression level of *tpx* was detected in the overnight cultured cells (Figs. 2B and D). The growth phase-dependent expression of both *sodC* and *tpx* genes correlate with previous studies, where the *sodC* gene was reported to be induced in the stationary phase by RpoS [18] and a relatively high expression level of the Tpx protein was detected during

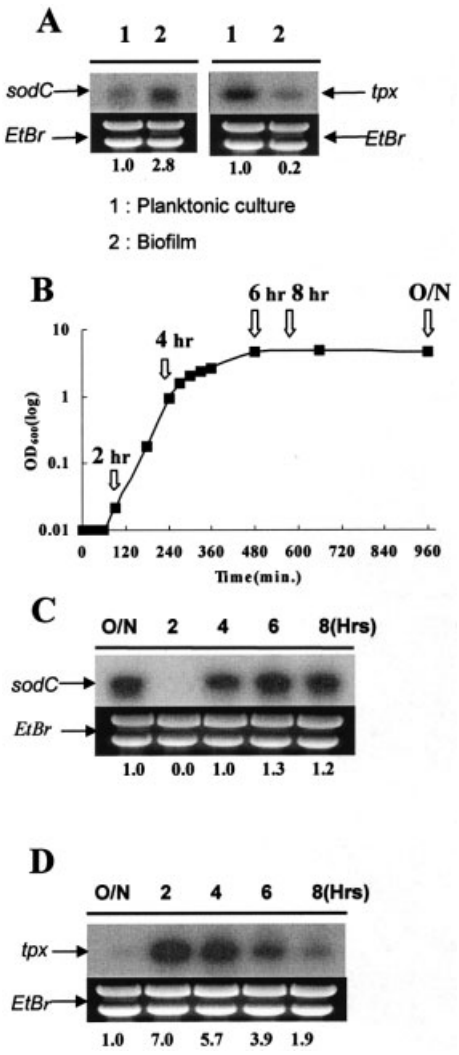

Figure 2. Northern blot analysis of the *sodC* and *tpx* genes. The ethidium bromide-stained (EtBr) gel prior to blotting is also shown to demonstrate the consistent loading in all lanes; The mRNA induction ratio is indicated below *EtBr* figure. (A) The expression of the *sodC* and *tpx* genes under biofilm and planktonic conditions. (B) The time-dependent growth phase of wild-type STEC grown in LB media and the time for collecting the total mRNA (shown by the arrows) for Northern blot analysis. (C) Growth phase-dependent expression of the *sodC*. (D) Growth phase-dependent expression of the *tpx* gene; O/N, overnight culture (16 h).

growth [26]. Tpx has functions in the oxidative stress defenses against ROOH and H_2O_2 even though it was reported that the level of *tpx* mRNA and its protein did not change upon exposure to various oxidants [24, 25]. The high level of *tpx* gene expression during the growth phase without induction by H_2O_2 is believed to be a requirement for peroxide detoxification in the periplasmic space during growth [26]. In agreement with a previous study using *E. coli* cells, a deletion of *rpoS* in STEC cells resulted in the loss of *sodC* expression in the stationary phase [20]. Gort *et al.* [20] reported that the *sodC* of *E. coli* is not induced by ROS, including paraquat or H_2O_2, either in the exponential or in stationary phases [20]. In contrast to their results, the present study found that the *sodC* mRNA of STEC appears to be strongly expressed after treating the cells with H_2O_2 for 10 min during exponential growth (Fig. 3A). This induction was not detected when using other stress agents including paraquat, menadione, and NaCl. In contrast to *sodC* expression, there was no significant change in the level of *tpx* mRNA after treating the cells with various stress agents (Fig. 3A, right panel). This suggests that Tpx is a housekeeping-type periplasmic antioxidant system. These results are consistent with previous results showing that the cellular level of Tpx was unchanged upon exposure to oxidative stress [43].

sodC is controlled by RpoS [20], and many H_2O_2-inducible genes are regulated by OxyR in *E. coli* [44]. Therefore, the involvement of OxyR and RpoS on *sodC* induction in the presence of H_2O_2 was examined. The induction of *sodC* was

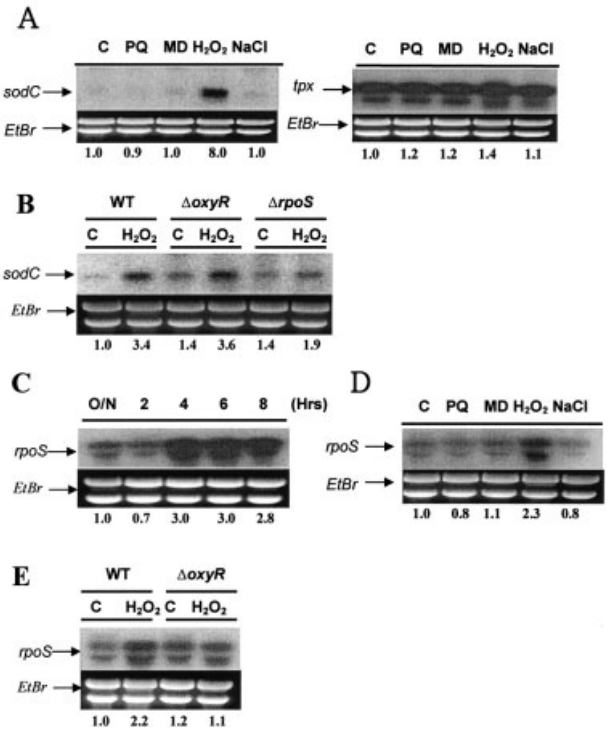

Figure 3. Induction of the *sodC* and *tpx* genes by oxidative and osmotic stresses in different genotypic backgrounds: control (C; without any additional chemical), paraquat (PQ; 1.0 mM), menadione (MD; 1.0 mM), and hydrogen peroxide (H_2O_2; 3.0 mM) and NaCl (0.85 M). Total mRNA was extracted at 10 min exposure after treatment with the referenced agents. (A) Northern blot analysis of the *sodC* and *tpx* genes under oxidative and osmotic conditions. (B) Induction of *sodC* by H_2O_2 in the wild-type STEC, *oxyR* mutant, and *rpoS* mutant. (C) Growth phase-dependent expression of the *rpoS* gene. (D) *In vivo* expression analysis of the *rpoS* gene under various stress conditions. (E) Induction of the *rpoS* gene by H_2O_2 the in wild-type STEC and *oxyR* mutant.

diminished in the absence of RpoS during H_2O_2 exposure in the exponential phase (Fig. 3B, lane 6), suggesting that the induction of *sodC* is regulated partly at the transcriptional level by RpoS. The expression of *sodC* was still induced by H_2O_2 in the absence of OxyR but the fold-difference was not the same, as observed in the wild-type cells (Fig. 3B, lane 4). However, in the absence of OxyR, the basal expression of *sodC* without the H_2O_2 treatment increased slightly during exponential growth probably because of the indirect effects of the *oxyR* mutant background. It is likely that the endogenous H_2O_2 generated in the *oxyR* mutant is slightly higher than in the wild-type cells [17]. Since the level of RpoS can increase as a result of environmental stress during the exponential phase [45], the induction of *sodC* by the H_2O_2 treatment may result from the increase in the RpoS levels under such conditions. The expression of the *rpoS* gene was not up-regulated until 4 h after the cells grown overnight had been inoculated into fresh LB media (Fig. 3C). Significant induction of the *rpoS* gene was observed during the exponential phase (after 2 h) after the H_2O_2 treatment, but not by the other stress agents (Fig. 3D). These results provide the first evidence that the induction of the *rpoS* gene by H_2O_2 increases the level of *sodC* expression. In the *oxyR*-minus background, the basal level of the *rpoS* mRNA transcript increased slightly without the H_2O_2 treatment (Fig. 3E). Surprisingly, this suggests that H_2O_2 does not induce *rpoS* in the *oxyR* mutant (Fig. 3E). It appears likely that the activated OxyR in STEC cells controls *rpoS* expression at the transcriptional level, which is in contrast to other reports [46]. Overall, OxyR might also contribute to the induction of the *sodC* indirectly through the transcriptional up-regulation of the *rpoS*. However, there may be unknown regulators other than the RpoS that control *sodC* transcription because the induction of the *sodC* was not completely abolished in the *rpoS*-minus background (Fig. 3B). The precise mechanisms for the transcriptional activation of the *rpoS* gene in STEC cells remain to be determined.

3.3 Growth and sensitivity of the STEC *sodC* and *tpx* mutants to salicylate and exogenous oxidative stress

The aerobic growth of the STEC *sodC* and *tpx* mutants and the wild-type cells were determined in LB medium using exogenous H_2O_2 (1.0 mM). The growth of these mutants was slower than the wild-type strain after supplementation with exogenous H_2O_2 (Fig. 4A). After the wild-type and mutants cells were grown overnight in LB media, the culture was diluted 100-fold in 10 mL of fresh LB media and grown until the OD_{600} was approximately 0.2. Aliquots of each strain (exponentially grown ($\sim 10^8$ cells)) was diluted serially and spotted onto LB agar plates in the presence and absence of salicylate (10 mM). The mutant cells could not grow when a 10^{-3} dilution was spotted on the agar plate, and the size of their colonies was also smaller than that of wild-type cells (Fig. 4B). Salicylate induces phenotypic multiple antibiotic

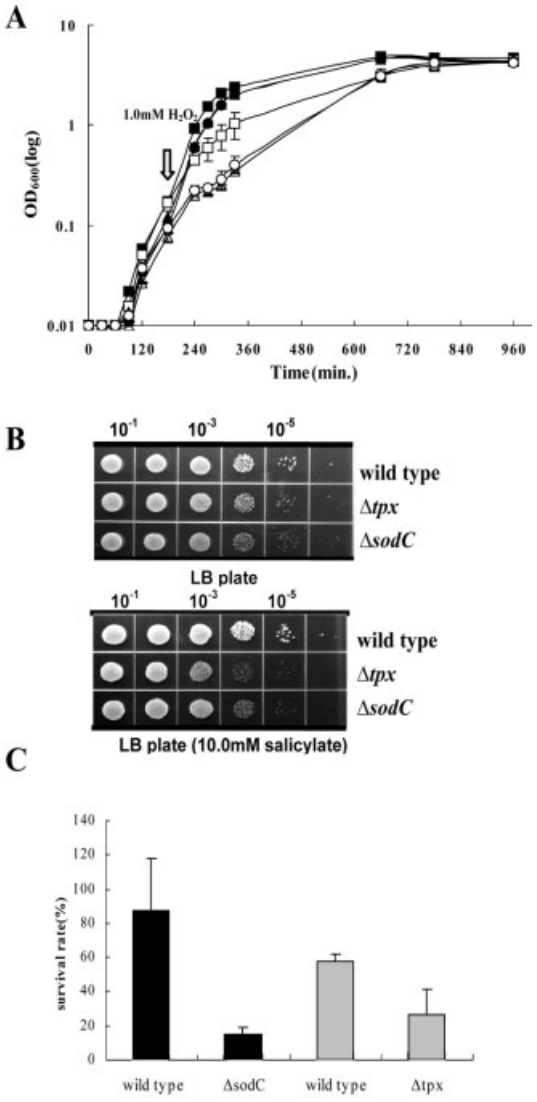

Figure 4. Growth and sensitivity of the wild-type STEC, *sodC*, and *tpx* mutants under various oxidative stresses. (A) Growth curves of wild-type (filled diamond), the *sodC* (filled triangle), and the *tpx* (open circle) mutants in the LB medium. The growth of each strain was monitored by measuring the OD_{600} of the cultures. The arrow indicates the time for H_2O_2 addition (1.0 mM; the measured OD_{600} was near 0.3). (B) Phenotypic assays of the wild-type and mutant strains of STEC. The cells ($\sim 10^8$) in exponential growth were diluted serially and spotted on LB agar supplemented with and without salicylate (10.0 mM). (C) The survival of the *sodC* and *tpx* mutants after exposure to exogenous H_2O_2 and *t*-BOOH. The LB-cultured *sodC*, and *tpx* mutant cells were collected at the stationary and exponential phases, respectively, and then washed with PBS (pH 7.5). Approximately, $\sim 10^9$ CFU/mL was inoculated into PBS (5 mL) containing H_2O_2 (90.0 mM; black bar) and *t*-BOOH (0.3 mM; gray bar), and the cultures were incubated at room temperature with vigorous aeration (220 rpm). After 3 h incubation, the cells were harvested and washed with PBS. The number of viable cells was determined by dilution with LB and plating on top of agar on LB plates. The mean values for three independent experiments are shown along with their SDs.

resistance in various bacterial species and can stimulate oxygen consumption [47, 48]. It has also been shown that the growth of *P. aeruginosa* and *S. epidermidis* in the presence of salicylate reduces the level of extracellular polysaccharide production needed for biofilm formation [49]. Growth in the presence of salicylate also increases the mutation frequency whereby *S. aureus* becomes resistant to fluoroquinolones and fusidic acid *in vitro* [50]. Many genes, such as oxidative stress genes (*sodA* and *fumC*), are modulated and by both paraquat and salicylate with the same trend [51]. These results suggest that the *sodC* and *tpx* gene products of STEC cells can counteract the inhibitory effects of salicylate stress, most likely the oxidative stress. This indicates that SodC and Tpx are essential for efficient growth under salicylate-amended conditions.

The viability of the *sodC* mutant was severely reduced when the stationary phase-cells of the wild-type and the *sodC* mutant were treated with 90 mM H_2O_2 (Fig. 4C; see Section 2). After 3 h incubation in PBS (5 mL) under exogenous oxidative stress conditions, the viability of the *sodC* mutant was reduced to 10% of the level of the wild-type cells. The survival rates of the stationary phase-cells were compared because the maximum *sodC* induction was observed at the stationary phase in an RpoS-dependent manner. A deletion of the *tpx* gene did not result in a loss of viability at the exponential phase after a treatment with various concentrations of exogenous H_2O_2 (3–90 mM), compared with the wild-type cells (data not shown). However, a significant loss of viability of the *tpx* mutant was observed with *t*-butyl hydroperoxide (*t*-BOOH) (Fig. 4C). The effect of *t*-BOOH over H_2O_2, at the same concentration (0.25 mM), on the viability of nonpathogenic *E. coli* has been reported [52]. An explanation for a higher sensitivity of the *tpx* mutant to *t*-BOOH is not simple. This result is believed to be due to *E. coli* Tpx having substrate specificity toward alkyl hydroperoxide over H_2O_2 [52].

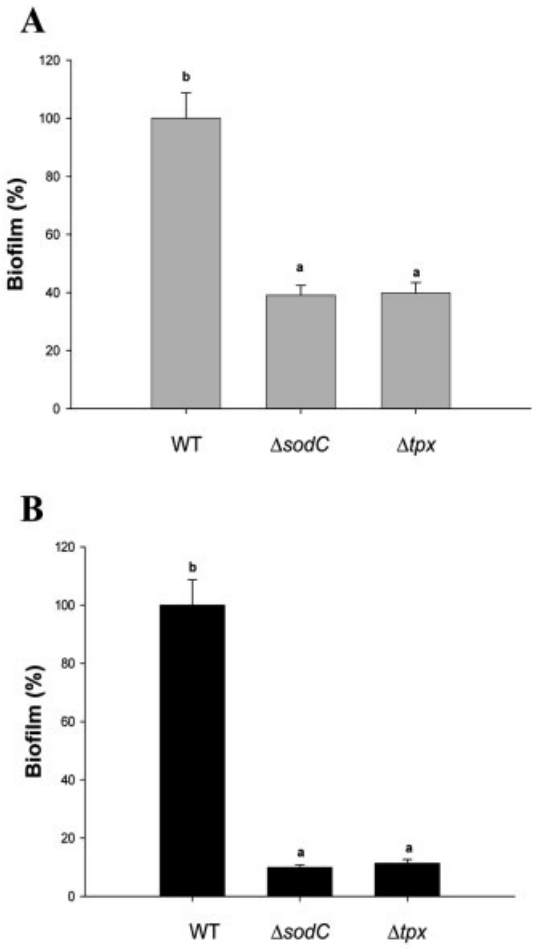

Figure 5. Quantification of biofilm formation by the wild-type and *sodC, tpx* mutants on the abiotic surfaces under static aerobic growth conditions in modified M9 medium supplemented with 0.1% glucose for 24 h. The formation of abiofilm on polystyrene (A) and PVC (B). The mean values for three independent experiments are shown along with SD.

3.4 Requirement of SodC and Tpx for biofilm formation on abiotic and biotic surfaces

The formation of a biofilm of the wild-type and mutants cells was examined in a modified M9 medium under aerobic conditions. The mutant strains showed significant reductions in attachment to both the polystyrene and PVC microtiter plate surfaces during static aerobic growth (Fig. 5), even though the growth rates of both the wild-type and mutants were similar under aerobic growth conditions. A more severe effect on biofilm formation was observed on the surface of the PVC plates (Fig. 5B). The human colon carcinoma line (HT-29) was used as a model biotic epithelial cell surface. HT-29 shows the markers of mature colon enterocytes, including the ability to express secretary components [53], and has been used to investigate bacterial adhesion to epithelial cells [54, 55]. An overnight culture of wild-type and mutant cells (~10^6 CFU/mL), grown in a modified M9 medium, was used for the attachment assay. The adherent cells were released from well plates and counted using serial dilutions. The attachment ability of the wild-type and mutant cells was similar, as demonstrated by the CFU counts after 2 h incubation (Fig. 6). However, more wild-type cells than mutant cells were recovered after 4 h incubation (~5.6 *vs.* ~4.4 CFU/mL, (Fig. 6). The mutants still attached to this cell line with no difference at earlier time points. This suggests that SodC and Tpx play a role in the growh of STEC cells after adherence to an epithelial cell surface, which is presumably due to SodC and Tpx playing important roles against membrane stresses during an infection. The surface of the bacterial pathogens is the first point of contact with the host and is the major target for the host defense system. While it is well known that the production of ROS by NADPH oxidase on the phagocytic cell memebrane plays an important role in the defense against a pathogenic infection [56], there is no

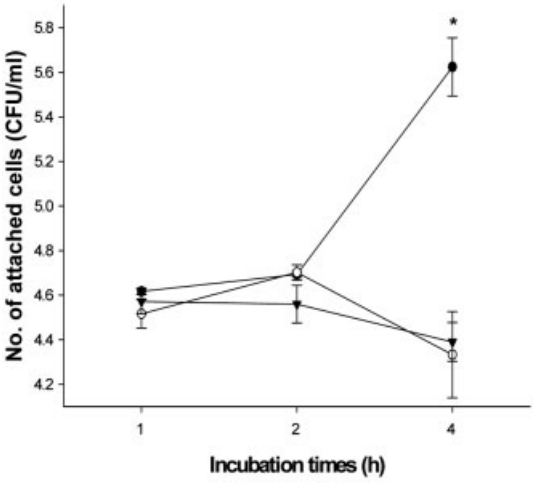

Figure 6. Attachment assay on the HT-29 cell line by the wild-type (filled circles), the *sodC* (open circles), and the *tpx* (filled triangles) mutant cells after 1, 2, and 4 h. After incubating each cell with the HT-29 cell line, the detachment was carried out according to the experimental procedure and serial dilutions were performed on MacConkey agar plate to count the number of cells.

information available on the presence of specific oxidative attack in epithelial cells. However, several reports have suggested that ROS damage may be involved in the killing of bacteria by epithelial cells [57]. This hypothesis is consistent with the recent finding that the level of *sodC* mRNA is higher in attached enterohemorrhagic *E. coli* [39], and many *in vivo* experiments that suggest a role for SodC in the virulence and pathogenicity of infecting microorganisms [58, 59]. The involvement of Tpx in the attachment and pathogenicity of *E. coli* strains is not completely understood. This is the first mutational study showing that SodC and Tpx of pathogenic *E. coli* can contribute to the growth of STEC cells attaching to a biotic surface. STEC biofilm formation was only observed with the wild-type STEC cells in glass capillary tubes under a continuous flow-culture system condition, compared with the STEC *sodC* and *tpx* mutants (Fig. 7). This provides evidence that SodC and Tpx are also essential for the establishment of a biofilm under continuous flow conditions. From the observation of the attachment to the three different types of surfaces (polystyrene, PVC, and HT-29 epithelial cell), it is concluded that SodC and Tpx play key roles in the establishment of a biofilm.

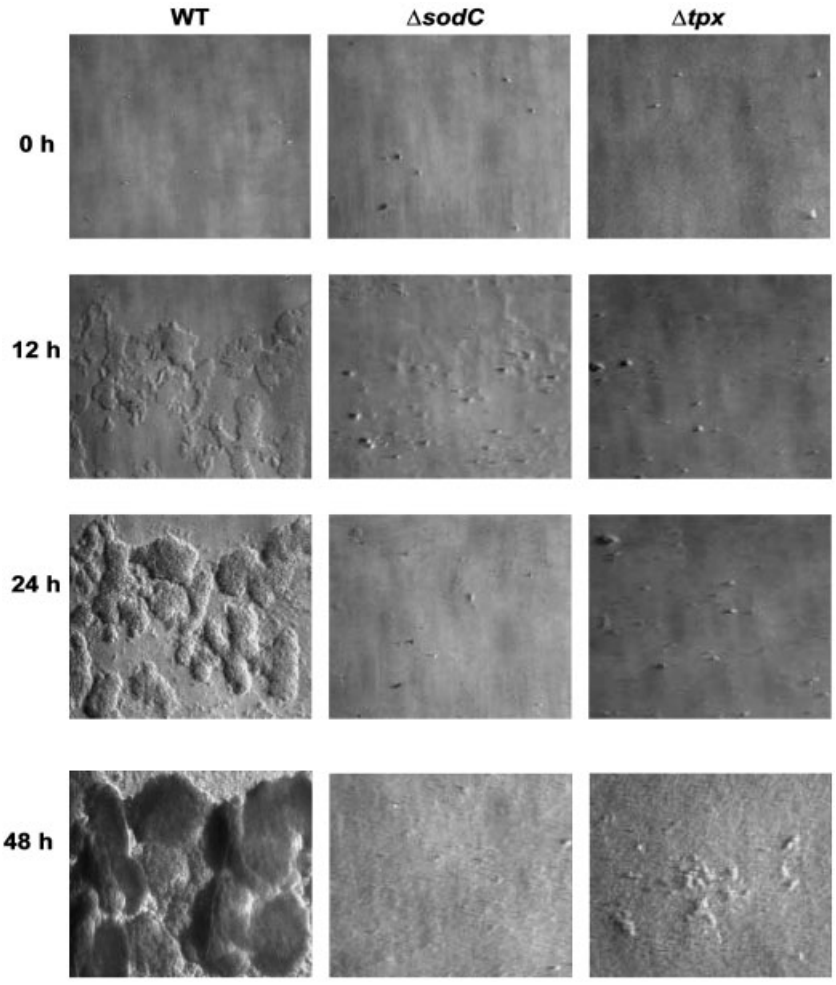

Figure 7. Quantification of biofilm formation using glass capillary tubes and a continuous flow system. The biofilms developed by wild-type, *sodC*, and *tpx* mutants were observed at different time points, as indicated.

4 Concluding remarks

Bacterial cells in a biofilm are believed to resemble stationary phase cells and grow slowly within a biofilm [60]. The stationary sigma factor (RpoS)-dependent expression of *sodC* has been identified in many analyses of biofilm gene expression [7, 12, 31]. These results support the idea that SodC is strongly expressed in a biofilm and plays a key role in its establishment. However, DNA microarray data using an *E. coli* biofilm revealed that compared with the pattern of gene expression in the stationary phase, many genes are also differentially regulated under biofilm conditions [12]. Compared with planktonic cells, only a small portion (1.84%, 79 genes) of the *E. coli* genes show significantly altered expression during biofilm growth, including the exponential and stationary phases [12]. The functions of a majority of these "biofilm-specific" genes identified from DNA microarray studies are unknown. Although it is not known if the Tpx proteins are abundant in biofilm cells because of discrepancies between the proteomic and *tpx* mRNA data, this study demonstrated that the *tpx* gene is essential for the development of a stable biofilm. Since cells in a biofilm have a heterogeneous physiology and variable growth rates [27, 37], the coordination of a subset of exponentially expressed genes (including *tpx)* appears to be essential for the stable formation of a biofilm. Tremoulet *et al*. [31] carried out a similar proteomic analysis examining the differences between the protein patterns of *E. coli* O157:H7 biofilm and planktonic cells [31]. The proteins identified in this study are not consistent with the observations reported by Tremoulet *et al*. [31], possibly because of the different culture conditions used in the two biofilm studies. The bacterial culture conditions can influence the formation of a biofilm [61]. Therefore, the subsets of genes that are highly expressed in biofilm under different culture conditions might also be altered. Several global analyses of genes that are differentially expressed in a biofilm have been examined using different microorganisms [7, 12, 31]. Only a few genes such as those for adhesion and defense against oxidative stress are commonly found in those experiments. This study focused on the expression of the genes associated with the oxidative stress response because proteomic analysis of a natural biofilm indicates that the proteins involved in refolding and oxidative stress response are expressed at high levels [8]. To the best of our knowledge, this is the first mutational report showing the contribution of *sodC* and *tpx* genes products to the formation of an *E. coli* O157:H7 biofilm. A higher *sodC* expression level has been reported in a biofilms made from other bacteria [12, 39], and a higher *tpx* expression level has been recently demonstrated in *Campylobacter* biofilms [62]. However, they did not perform any further experiments to demonstrate the involvement of those two genes. This study also provides the first line of evidence showing that the induction of the *rpoS* gene by H_2O_2 increases the level of *sodC* expression in *E. coli* O157:H7. These results suggest that biofilms are physiologically heterogeneous. They do not resemble stationary phase-cells, and the defenses against oxidative stress in both the exponential and stationary growth stages are likely to play important roles in the formation of an STEC biofilm.

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