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- Note -

Toluene Tolerance in Solvent Tolerant *Pseudomonas* sp. Strains By Antioxidant Defense Systems

Woo Hong Joo[1*], Hye Jung Choi[1], Da Som Kim[1], Yong-Kweon Cho[2] and Dong Wan Kim[2]

[1]*Department of Biology and Chemistry, Changwon National University, Changwon 51140, Korea*
[2]*Department of Biohealth Science, Changwon National University, Changwon 51140, Korea*

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To elucidate whether or not solvent-tolerant bacteria use anti-oxidative defense systems to defend themselves against toxic solvents, oxidative enzyme activity and total anti-oxidative capacity (T-AOC) were investigated in two tolerant strains of *Pseudomonas* sp. under toluene stress. The superoxide dismutase (SOD) activities of solvent tolerant BCNU 106 exhibited relatively increased levels at a toluene concentration of 100 mg/l, where those of solvent tolerant BCNU 171 increased at 200 mg/l. A greater than three-fold increase in catalase (CAT) levels was observed at concentrations of 200 and 300 mg/l in BCNU 106, and a two-fold increase was monitored at the same concentrations in BCNU 171. High glutathione S-transferase (GST) levels were also observed in the solvent tolerant bacteria. Higher levels of T-AOC was expressed in the solvent tolerant strains than in the ordinary non-tolerant KACC 10266. The highest plateau of SOD in BCNU 171 was observed at 1 hr of toluene exposure. CAT levels plateaued at 1 hr and 14 hr in BCNU 106 and reached the highest plateau at 3 hr in BCNU 171. The highest peak of T-AOC occurred at 9 hr in BCNU 106, and two high peaks occurred in BCNU 171, at 1 hr and at 9 hr of toluene exposure. The solvent-tolerant bacteria showed active antioxidant responses and could survive under harsh environments, including the presence of solvents, through means of antioxidant defense systems.

Key words : Antioxidant defense, *Pseudomonas*, solvent tolerance, solvent-tolerant bacterium, toluene

Introduction

Tolerance towards various solvents in bacteria has been reported [9], and since then many solvent tolerant bacterial strains have been isolated from natural environments such as garden soil, forest soil, wastewater, and deep sea [13, 15]. It was determined that solvent tolerant bacteria could survive under harsh and extreme environments, including exposure to various solvents, by incorporating a combination of diverse solvent tolerance mechanisms common to solvent tolerant bacteria [13, 15].

Organic solvents including benzene, toluene, ethylbenzene and xylene are toxic for cells, even including organic solvent tolerant bacterial cells. The toxicity of solvents is measured by the disruption of membrane functions, impairment in respiratory chains and following generation of reactive oxygen species (ROS) in electron transfer chains [6]. Therefore, detoxification of cellular ROS is an essential prerequisite for survival in the presence of organic solvents. Most bacteria possess several antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), Alkyl hydroperoxide reductase (Ahp), and glutathione S-transferase (GST), that function primarily in ROS detoxification.

The involvement of anti-oxidant defense systems in organic solvent tolerance was demonstrated in *Pseudomonas* sp. BCNU 106 [4]. However, whether this phenomena is common to other organic solvent tolerant bacteria has not been elucidated. Therefore, total antioxidant defense status including antioxidative enzymes and T-AOC was investigated in two organic solvent tolerant bacteria, *Pseudomonas* sp. BCNU 106 and *Pseudomonas* sp. BCNU 171, in comparison with an organic solvent non-tolerant *P. putida* strain.

***Corresponding author**
Tel : +82-55-213-3453, Fax : +82-55-213-3459
E-mail : whjoo@changwon.ac.kr

Materials and Methods

Strains and culture conditions

Two organic solvent tolerant bacteria, *Pseudomonas* sp. BCNU 106 and BCNU 171 [6, 7], were used in this study. Solvent non-tolerant strain *P. putida* KACC 10266 (ATCC

12633; the putida type strain), obtained from the Genebank Information Center in the Rural Development Administration, Korea, was used as a comparative control strain.

Cell growth under various amounts of toluene

Two organic solvent tolerant *Pseudomonas* sp. BCNU 106 and BCNU 171, and one solvent non-tolerant *P. putida* KACC 10266 were pre-cultured in Luria-Bertani (LB) medium at 37℃ or 26℃, respectively, for 18 hr, and then inoculated into 20 ml of the same medium (final concentration of 1.0×10^7 CFU/ml containing 0, 100, 200, 300, or 400 mg/l toluene. Bacterial cells were cultivated at 37℃ or 26℃, respectively, with shaking at 130 rpm for 24 hr. All bacterial cultures were grown in LB medium supplemented with and without toluene (200 mg/l) and were monitored at 600 nm at regular intervals of 3 hr for 24 hr.

Preparation of crude bacterial extracts

After cell cultivation for 6 hr (mid-log phase), or at 0, 0.5, 1, 1.5, 3, 5, 9, 14 and 24 hr, bacterial cells were collected by centrifugation at 10,000 *g* for 10 min, washed twice with ice-cold 0.9% sodium chloride solution, and then re-suspended in fresh 0.9% sodium chloride solution. Cell suspensions were lysed with a probe sonicator (Vibra-Cell VCX130, Sonics, USA) in an ice-water bath. The supernatant was precipitated with centrifugation at 10,000 *g* for 10 min, transferred to a new centrifuge tube for enzyme assays and stored at -20℃ until use.

Enzyme assay

Levels of SOD, CAT and GST and total anti-oxidative capacities (T-AOC) were measured with a spectrophotometer (Shimazu UV 1601, Kyoto, Japan) at 550, 405, 412 and 520 nm, respectively, using commercial kits A001-1, A007-1, A004 and A015 (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Protein concentration was also assayed photometrically at 595 nm using a commercially available assay kit A045 (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of SOD activity was defined as the amount of the crude enzyme that corresponded to 50% inhibition of the oxidation rate of 0.1 mM pyrogallol in 1 ml of solution at 25℃ [8]. One unit of CAT activity was defined as the amount of lysate that degraded 1 μM of H_2O_2 at pH 7.0 and 25℃ in 1 min [12]. One unit of GST activity was defined as the amount of lysate that degraded 1.0 μM of GSH at 37℃ in 1 min. One unit of T-AOC was defined as an increase in absorbance of 0.01 at 37℃ in 1 min. Total protein concentration in cell lysates was determined by a modified Lowry procedure using bovine serum albumin as the standard. The specific SOD, CAT and GST activities were expressed as U/mg protein.

Results

Bacterial cell growth

Organic solvent tolerant *Pseudomonas* sp. BCNU 106 showed better growth than solvent non-tolerant *P. putida* KACC 10266 in the presence of toluene (v/v, 200 mg/l) at concentrations not detrimental to *P. putida* KACC 10266 (Fig. 1). Another solvent tolerant *Pseudomonas* sp. BCNU 171 showed better growth to the first 6 hr than solvent tolerant *Pseudomonas* sp. BCNU 106 in the presence of toluene (v/v, 200 mg/l). After this time period, BCNU 171 showed worse growth than BCNU 106 in the presence of toluene (Fig. 1).

Effects of toluene concentrations on SOD, CAT, GST, and T-AOC response

Organic solvent tolerant BCNU 106 and BCNU 171 pos-

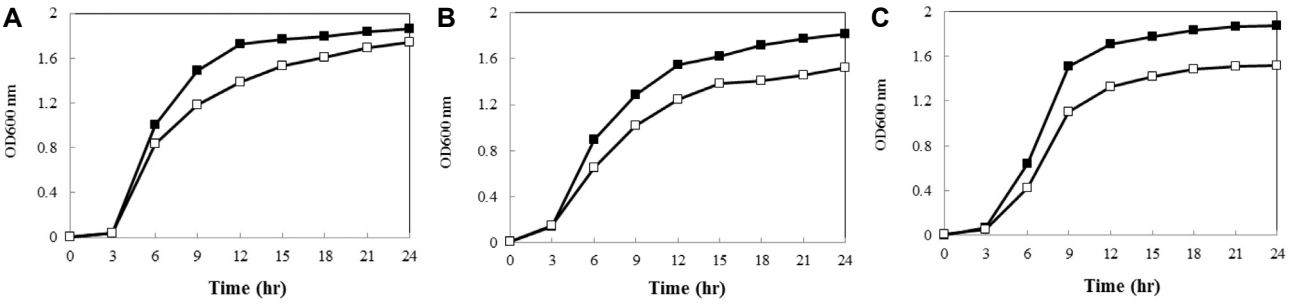

Fig. 1. Growth of *Pseudomonas* sp. strains. Growth of solvent tolerant *Pseudomonas* sp. BCNU 106 (A), solvent tolerant *Pseudomonas* sp. BCNU 171 (B), and solvent non-tolerant *P. putida* KACC 10266 (C) in the presence (white squares) or absence of 200 mg/l toluene (black squares). Each value is the mean ± S.E. (n=3).

sessed higher SOD levels than those of solvent non-tolerant KACC 10266 at all tested toluene concentrations (Fig. 2A). The SOD levels of solvent tolerant BCNU 106 increased 1.62-fold at 100 mg/l toluene, those of solvent tolerant BCNU 171 increased 1.98-fold at 200 mg/l, but SOD levels of solvent non-tolerant KACC remained almost stationary within 0 mg/l to 200 mg/l toluene (Fig. 2A, Table 1). CAT activities of the two solvent tolerant bacteria were also higher than KACC 10266 (Fig. 2B). More than a three-fold increase in CAT levels was observed at 200 and 300 mg/l in solvent tolerant BCNU 106 and more than a two-fold increase in CAT levels was also monitored at 200 and 300 mg/l in BCNU 171, while CAT levels decreased rapidly with increases in toluene concentrations in solvent non-tolerant KACC 10266 (Fig. 2B, Table 1).

High GST levels were observed in solvent tolerant bacteria (Fig. 2C). T-AOC was expressed at higher levels in solvent tolerant BCNU 106 than in solvent tolerant BCNU 171 and solvent non-tolerant KACC 10266 (Fig. 2D). T-AOC levels of solvent non-tolerant KACC 10266 was slightly higher than that of solvent tolerant BCNU 171 at 100 mg/l of toluene, but T-AOC levels of solvent non-tolerant KACC 10266 were lower than that of solvent tolerant BCNU 171 from 200 mg/l exposure (Fig. 2D). The highest activity fold change of T-AOC levels was seen at 100 mg/l toluene in solvent tolerant BCNU 106, and at 200 mg/l toluene in solvent tolerant BCNU 171 (Fig. 2D, Table 1).

Effects of exposure times on SOD, CAT, GST, and T-AOC response

SOD levels of BCNU 106 increased 1.96-fold at 1 hr and 1.79-fold at 14 hr after exposure to toluene, respectively, compared with control levels (at 0 hr). The highest plateau of SOD (2.68-fold increase) in BCNU 171 was observed at 1 hr and peaks were observed at 30 min and 14 hr in solvent non-tolerant KACC 10266 (Fig. 3A, Table 2). The CAT levels reached a plateau at 11.965-fold and 9.94-fold change, at 1 hr and 14 hr, respectively, in BCNU 106 (Fig. 3B, Table

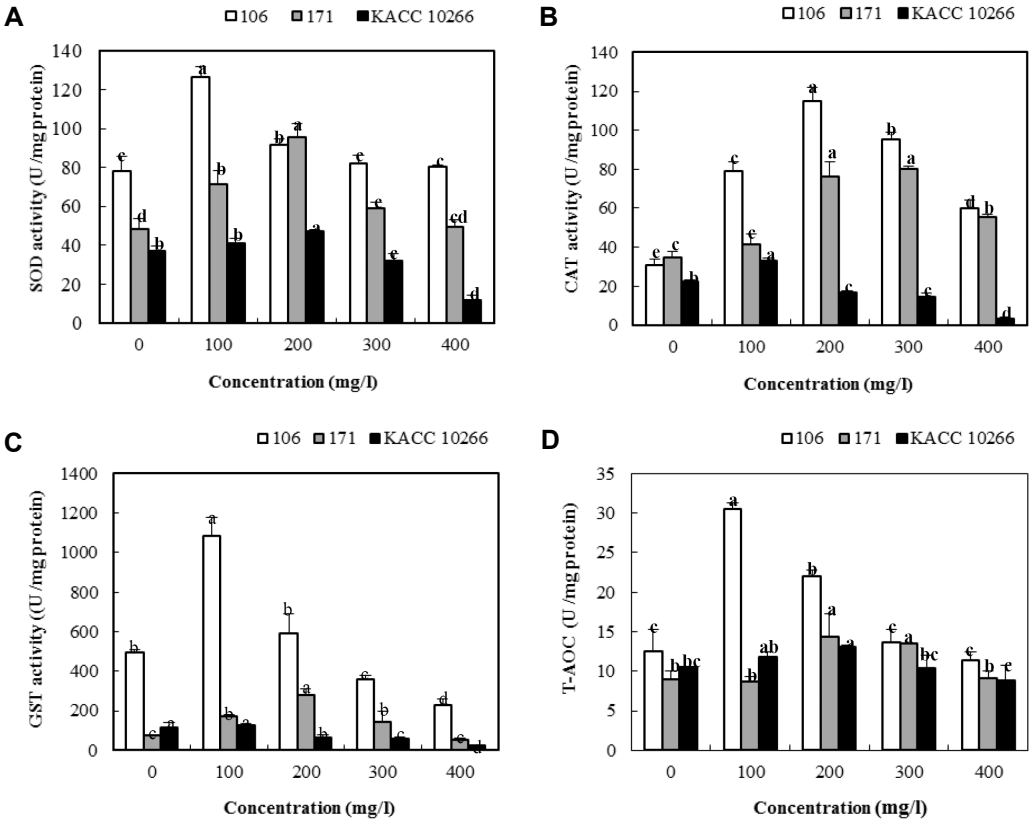

Fig. 2. Effect of exposure concentration on activities of SOD, CAT, and GST and T-AOC in bacteria. (A) SOD activities, (B) CAT activities, (C) GST activities, and (D) T-AOC in three bacteria (solvent tolerant *Pseudomonas* sp. BCNU 106, solvent tolerant *Pseudomonas* sp. BCNU 171, and solvent non-tolerant *P. putida* KACC 10266) at mid-log phase (6 hr). Each value presents mean ± S.E. (n=3). Different letters in the same row indicate a significant difference at $p<0.05$ by Duncan's multiple range test.

Table 1. The relative activities (fold changes) of SOD, CAT, and GST and relative T-AOC in response to toluene exposure at mid-log phase (6 hr)

	BCNU 106	BCNU 171	KACC 10266	BCNU 106	BCNU 171	KACC 10266
mg/l	Fold changes of SOD activity			Fold changes of CAT activity		
0	1	1	1	1	1	1
100	1.62±0.07a	1.48±0.15b	1.10±0.07b	2.56±0.15c	1.20±0.14c	1.44±0.05a
200	1.18±0.04b	1.98±0.15a	1.27±0.02a	3.72±0.22a	2.19±0.22a	0.76±0.05b
300	1.06±0.05c	1.22±0.07c	0.86±0.11c	3.08±0.12b	2.30±0.04a	0.57±0.11c
400	1.04±0.01c	1.02±0.08c	0.32±0.06d	1.95±0.12d	1.59±0.04b	0.21±0.10d
mg/l	Fold changes of GST activity			Fold changes of T-AOC		
0	1	1	1	1	1	1
100	2.20±0.19a	2.31±0.13b	1.06±0.11a	2.44±0.06a	0.98±0.07b	1.13±0.04ab
200	1.19±0.22b	3.75±0.38a	0.57±0.09b	1.76±0.06b	1.60±0.34a	1.24±0.08a
300	0.73±0.04c	1.94±0.71b	0.49±0.08c	1.09±0.13c	1.51±0.02a	0.99±0.11bc
400	0.47±0.06c	0.69±0.16c	0.20±0.03d	0.91±0.09d	1.02±0.11b	0.84±0.14c

Each value is the mean ± S.E. (n=3). Different letters in the same row indicate a significant difference at $p<0.05$ by Duncan's multiple range test.

2). CAT levels in BCNU 171 reached the highest plateau (2.50-fold increase) at 3 hr, and those in KACC 10266 reached the highest peak values (5.83-fold increase) at 9 hr (Fig. 3B, Table 2). The GST levels of solvent tolerant bacteria decreased immediately after exposure to toluene and increased thereafter to the first peak (Fig. 3C), whereas GST levels of solvent non-tolerant KACC 10266 increased *ab initio* up to the first peak after exposure to toluene (Fig. 3C). The

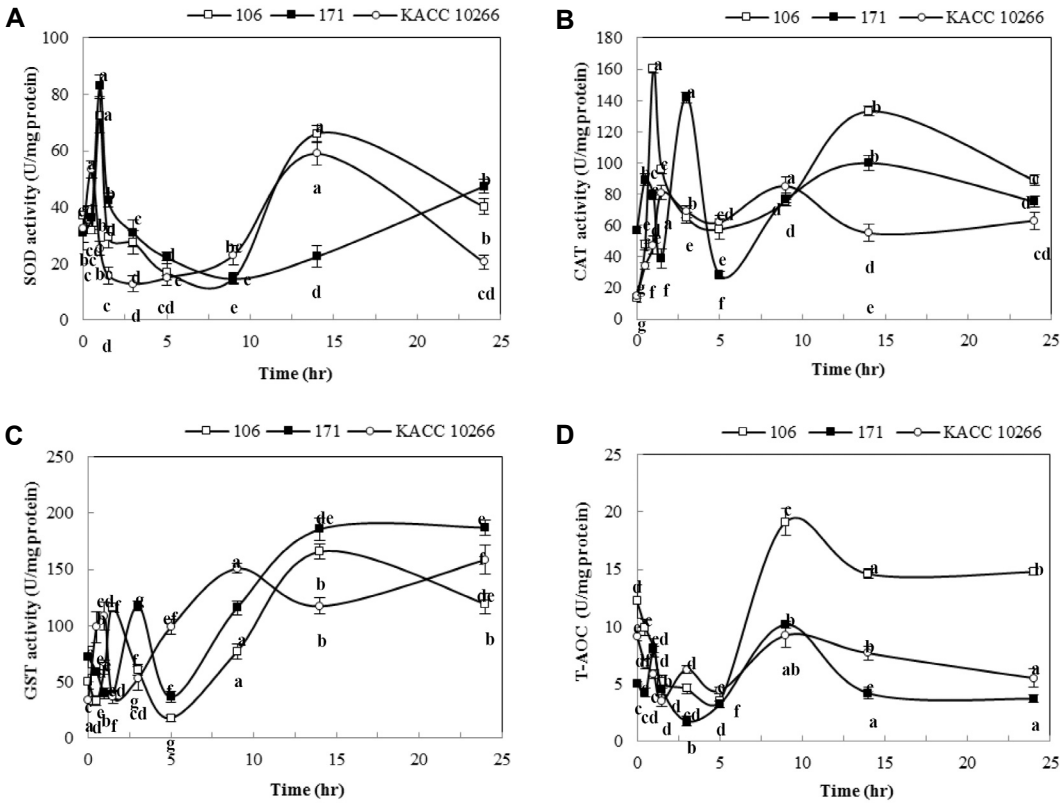

Fig. 3. Exposure time dependent variation in activities of SOD, CAT, and GST and T-AOC in bacteria. (A) SOD activities, (B) CAT activities, and (C) GST activities and (D) T-AOC in three bacteria in the presence of 200 mg/l toluene. Each value presents mean ± S.E. (n=3). Different letters in the same row indicate a significant difference at $p<0.05$ by Duncan's multiple range test.

Table 2. Relative SOD, CAT, and GST activities and relative T-AOC (fold changes of peak values versus initial value) in the presence of 200 mg/l toluene

	Time 0	Peak 1	Peak 2	Time 0	Peak 1	Peak 2	Peak 3
	Fold changes of SOD activity			Fold changes of CAT activity			
BCNU 106	1	1.96±0.16b	1.79±0.08a	1	11.97±0.22a	9.94±0.24a	
BCNU 171	1	2.68±0.12a		1	1.57±0.06c	2.50±0.06c	1.76±0.08a
KACC 10266	1	1.59±0.10c	1.77±0.12a	1	5.53±0.31b	5.83±0.39b	
	Fold changes of GST activity			Fold changes of T-AOC			
BCNU 106	1	2.31±0.07b	3.30±0.13b	1	1.57±0.09a		
BCNU 171	1	1.63±0.06c	2.60±0.09c	1	1.59±0.06a	2.03±0.06a	
KACC 10266	1	3.28±0.27a	4.55±0.09a	1	0.69±0.03b	1.01±0.11b	

Each value is the mean ± S.E. (n=3). Different letters in the same row indicate a significant difference at $p<0.05$ by Duncan's multiple range test

fold changes of GST activities were 2.31-fold and 3.30-fold, at 1 hr 30 min and 14 hr, respectively, in BCNU 106, 1.63-fold and 2.60-fold, at 3 hr and 24 hr, respectively, in BCNU 171 (Fig. 3C, Table 2). The highest peak (1.57-fold increase) of T-AOC levels occurred at 9 hr exposure to toluene in BCNU 106. Two high peaks (1.59-fold increase at 1 hr and 2.03-fold increase at 9 hr) occurred in BCNU 171 (Fig. 3D, Table 2). The T-AOC levels, however, showed about the same level or less than the control in KACC 10266 (Fig. 3D, Table 2).

Discussion

Several chaotropes including aromatic compounds cause oxidative stress [5, 7]. The anti-oxidative defense response toward oxidative stress was exhibited both by enzymatic and non-enzymatic modules, with governing regulatory networks [10]. The enzymatic antioxidative defense response to organic solvents, especially toluene, have been demonstrated in solvent tolerant *Pseudomonas* sp. BCNU 106 [4]. In this study, anti-oxidative systems including both enzymatic anti-oxidative and non-enzymatic anti-oxidative systems of solvent tolerant bacteria to toluene were investigated to determine whether this involvement occurred in other organic solvent tolerant bacteria. In general, SOD and CAT activities of solvent tolerant bacteria were higher than those of solvent non-tolerant bacterium within concentrations that do not limit survival outcomes. Moreover, CAT activity, more than SOD activity, was enhanced in solvent tolerant bacteria, suggesting that CAT may play a key role in struggling with toluene-mediated oxidative stress by enzymatic anti-oxidative systems. On the other hand, GST plays an important role in ROS detoxification and the regulation of cellular redox balance [14], and T-AOC represents the capacity of the non-enzymatic antioxidant defense system [11] and is useful for assessing an organism's antioxidant status [16]. By increasing amounts of toluene, changes in the relative activities of GST decreased in all bacteria beyond 200 mg/l or more concentration of toluene suggesting that toluene inhibited GST activities. Changes in GST activities could enhance glutathione availability for redox reactions and mitigate damage caused by toluene as observed in cadmium-exposed *Bradyrhizobium* sp. [1].

Solvent tolerant *Pseudomonas* sp. BCNU 106 and BCNU 171 showed almost the same or higher relative activity (fold change) of T-AOC than that of solvent non-tolerant bacterium in the presence of toluene. T-AOC increased in solvent tolerant *Pseudomonas* sp. BCNU 106 and BCNU 171 during exposure to 200 mg/l toluene, whereas solvent non-tolerant bacterium remained at the same level or less than the control to toluene under 200 mg/l concentration, demonstrating the involvement of non-enzymatic oxidative defense response in solvent tolerance.

In conclusion, our study demonstrated that SOD and CAT activities were elevated in solvent tolerant bacteria, supporting that the enzymatic anti-oxidative system is a solvent tolerance mechanism to mitigate the destructive toxicity of organic solvents to cells. The relative activity of GST was depressed in all bacteria in the presence of more than 200 mg/l toluene concentration. This finding suggests that toluene decreased GST activity and that GSH could possibly restore cell redox balances and reduce toluene stress damage. T-AOC in solvent tolerant bacteria was higher than that in solvent non-tolerant bacterium, suggesting that non-

enzymatic oxidative defense response is also involved in solvent tolerance.

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초록 : 항산화 방어 시스템에 의한 유기용매 내성세균 *Pseudomonas* sp. 균주에서의 톨루엔 내성

주우홍[1]* · 최혜정[1] · 김다솜[1] · 조용권[2] · 김동완[2]
([1]창원대학교 생물학화학융합학부, [2]창원대학교 생명보건학부)

유기용매 내성세균들이 항산화 방어시스템에 의하여 독성 유기용매로부터 자신을 보호하는지를 밝히기 위해 두 균주의 유기용매 내성 세균에서 항산화 효소 활성과 총 항산화능(T-AOC)을 조사하였다. 톨루엔 농도 100 mg/l에서 유기용매 내성세균 BCNU 106의 슈퍼옥사이드 디스무타아제(SOD) 레벨은 상대적으로 증가하였으며, 유기용매 내성세균 BCNU 171의 경우에는 톨루엔 농도 200 mg/l에서 상대적으로 증가하였다. 톨루엔 농도 200와 300 mg/l에서 유기용매 내성세균 BCNU 106에서 카타라제(CAT) 레벨이 3배 이상 증가함이 확인되었고, BCNU 171에서는 CAT 레벨이 2배 이상 증가함이 확인되었다. 또한 유기용매 내성세균에서 글루타치온 S- 전이효소(GST) 레벨이 높은 것으로 조사되었다. 총 항산화능도 유기용매에 대하여 내성을 보이지 않는 일반 세균인 KACC 10266보다 유기용매 내성세균들에서 높은 것으로 조사되었다. 한편 톨루엔 농도 200 mg/l 존재 하에서 BCNU 171 균주의 SOD 레벨은 1시간 후 정점에 도달하였다. BCNU 106에서 1시간과 14시간 후 CAT 레벨이 정점에 도달하였고, BCNU 171균주에서는 3시간 후 정점에 도달하였다. T-AOC 레벨의 정점은 BCNU 106에서는 톨루엔에 노출 9시간 후에 나타났으며, BCNU 171에서는 1시간과 9시간 후 두 번 T-AOC 레벨의 정점이 나타났다. 모든 유기용매 내성 세균은 왕성한 항산화 방어 반응을 보였으며, 이러한 항산화 방어 시스템을 통하여 유기용매 내성 세균이 유기용매를 비롯한 가혹한 환경하에서 생존할 수 있는 것으로 판단된다.