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Toxic Effects of Catechol and 4-Chlorobenzoate Stresses on Bacterial Cells

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Catechol and 4-chlorobenzoate (4CBA) which are produced from the biodegradation of a variety of aromatic and chloroaromatics have been recognized as toxic to living organisms. In this study, the toxic effects of catechol and 4-chlorobenzoate on gram-positive and -negative bacteria were examined in terms of survival, morphology, change in fatty acids and membrane protein composition. The survival rate of the organisms during treatment for 6 h was decreased, as the concentration of each aromatic was increased. *Escherichia coli* and *Pseudomonas* cells treated with catechol and 4CBA at concentrations causing a significant decrease in their viability, showed destructive openings in their cell envelopes. *Bacillus subtilis* treated with the aromatics were reduced in cell size and *Staphylococcus aureus* cells displayed irregular rod shapes with wrinkled surfaces. The bacterial cells treated with 20 mM catechol showed increases in unsaturated fatty acids, but several saturated fatty acids were decreased. In the *E. coli* cells treated with 20 mM catechol, inner membrane proteins of 150 kDa and 105 kDa were decreased. But several kinds of the inner and outer membrane proteins were increased. In *B. subtilis* treated with 20 mM catechol, several kinds of proteins were increased or decreased in membrane proteins.

Key words: catechol, 4-chlorobenzoate, gram-positive and -negative bacteria, toxic effects

Organic solvents, such as aromatic compounds, alkanes, alkenes, and alcohols, are one of the largest groups of environmental pollutants, and generally regarded as extremely toxic to living organisms (17). Because of their persistence, toxicity, bioaccumulation, and transformation into hazardous metabolites, public concern has been attracted in terms of human health problems such as carcinogenicity, mutagenicity, and disturbance in endocrine systems. The endocrine disrupters including chlorinated and polycyclic aromatics, such as polychlorinated biphenyl and dioxin compounds, have been broadly studied for toxic effects on eucaryotic organisms including humans (3, 13).

These aromatic pollutants have been extensively studied on biodegradation by various kinds of microorganisms (4). Few reports have been made on their effects on procaryotic microbial cells. Some of the studies indicated that these aromatic compounds are partitioned into lipid bilayer membranes causing significant changes in the structure and functioning of membrane components, such as disruption of membrane potential, removal of lipids and proteins, and loss of magnesium and calcium ions (20). These adverse effects on the membrane are known to cause the loss of membrane functions, leading to cell death (7).

Lambert *et al.* (9) reported that 20 mM benzoate depressed the growth rate of *E. coli* by repressing many proteins including ribosomal-subunit proteins and transcription factors because of the reduced intracellular pH. Warth (22) also showed that 10 mM benzoic acid was toxic to *Zygosaccharomyces bailii* by dissipating the pH gradient by the reduced intracellular pH, glycolytic enzymes inhibition, and ATP depletion.

Catechol and 4-chlorobenzoate have been widely used as reagents in photography, dyeing fur and rubber and plastic production, causing considerable environmental pollution (18). These compounds are common intermediate metabolites produced via the degradation pathway of the aromatics by dechlorination and hydroxylation as shown in Fig. 1. They have been recognized to act as inhibitors to cell growth, such as DNA damage, protein inactivation, and destruction of the membrane by inhibition of lipid peroxidation (18). However, there has been no comparative study of the toxic effects between gram-positive and -negative bacteria.

We have previously reported the degradation of the aromatics and cellular responses such as production of stress-

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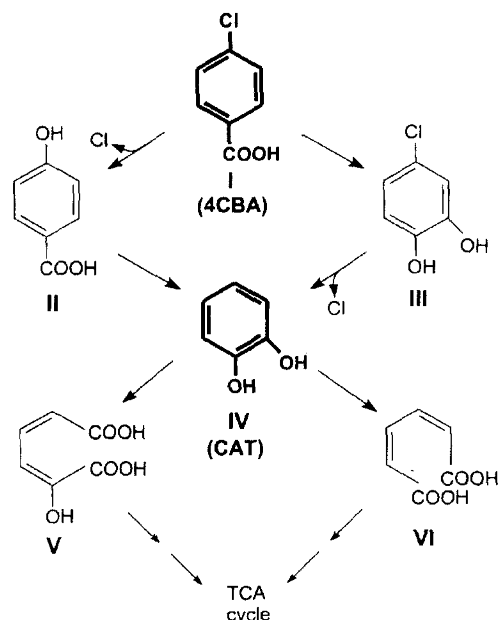

Fig. 1. The chemical structures and degradative pathway of 4-chlrobenzoate (I) and catechol (IV) used in this study.

shock proteins by *Pseudomonas* sp. DJ-12 and *E. coli* (8, 14, 15). In this study, the toxic effects of catechol and 4-chlorobenzoate on several bacteria were examined in terms of survival, morphology, changes in fatty acids and membrane protein composition. Furthermore, these effects were compared between gram-positive and negative bacteria.

Materials and Methods

Bacterial strains and cultivation

The bacterial strains used in this study are listed in Table 1. *Pseudomonas* sp. DJ-12 (6) is an isolate capable of utilizing biphenyl and 4-chlorobiphenyl as carbon and energy sources. *E. coli* LE392 is the host strain used for transformation of the degradative genes from the DJ-12 strain. Both gram-positive bacteria of *B. subtilis* and *S. aureus* were obtained from American Type Culture Collection. Those bacteria were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C (*E. coli* LE392 and *S. aureus* ATCC25923) or 30°C (*Pseudomonas* sp. DJ-12 and *B. subtilis* KCTC1028). Degradative activity of each organism was examined by resting cell assay by incubation for 16 h in MM2 medium supplemented with 1 mM concentration, as reported by Arensdorf and Focht (1) and Park *et al.* (14).

Treatment with aromatic stress and survival test

The bacteria grown in LB broth were harvested by centrifugation at 2,000 × g for 10 min. Those cells were washed three times with 10 mM phosphate buffer (pH 7.0) and then inoculated to be about 10^8 cells/ml in 20 ml MM2 medium in 100 ml Erlenmeyer flasks. For stress treatment with each aromatic, the medium was added with 4-chlorobenzoate (4CBA; Sigma Co., St. Louis, MO, USA), or catechol (Sigma Co., USA) at various concentrations as described by Park *et al.* (14). After treatment for an appropriate period of time, the viable cells were enumerated by plating on LB agar at 35°C.

Scanning electron microscopy

The colonies of each bacterial cell grown on LB agar plates for 12 h were extracted as blocks of 0.5 cm^3. These agar blocks with a colony were then treated with catechol at 10 mM (*E. coli* LE392) and 20 mM (other strains), or 4CBA at 30 mM (*B. subtilis* KCTC1028 and *Pseudomonas* sp. DJ-12) and 40 mM (*E. coli* LE392 and *S. aureus* ATCC25923) for 2 h. The colonies treated with the aromatics were pre-fixed with 2.5% glutaraldehyde in a 100 mM potassium phosphate buffer (pH 7.2) for 2 h, and then post-fixed with 1% osmium tetraoxide in the same buffer as described by Ng *et al.* (12). The fixed cells were dehydrated with a serial concentration (30 to 95%) of ethanol every 15 min and then 100% ethanol for 20 min. The cells were substituted with absolute isoamyl acetate for 15 min and then air-dried. The cells were coated with gold using a sputter coater (IB-3, Giko Engineering Co., Japan) and then examined with a scanning electron microscope (S-2500C, Hitachi Co., Japan)

Analysis of fatty acids

The bacterial cells treated with 20 mM catechol for 2 h

Table 1. Bacterial strains used in this study and the biodegradability of several aromatic compounds

Gram reaction	Strain	Degradation[a]				Source
		4CBA	CAT	4CB	BP[b]	
Negative	*E. coli* LE392	-	-	-	-	Stratagene Ltd.
	Pseudomonas sp. DJ-12	+	+	+	+	Kim *et al.* (6)
Positive	*Staphylococcus aureus* ATCC25923	-	-	-	-	Martineau *et al.* (10)
	Bacillus subtilis KCTC 1028	-	-	-	-	Wallerstein Co. Inc

[a]Degadation of each substrate was measured after incubation for 16 h at 1 mM concentration by resting cell assay.
[b]4CBA, 4-chlorobenzoate; CAT, catechol; 4CB, 4-chlorobiphenyl; BP, biphenyl.

were collected as described above, and the total fatty acids from each strain were determined by the Microbial Identification System (MIDI; Microbial ID, Inc., Newark, USA). The cell pellets were heated in 1 ml of reagent 1 (45 g sodium hydroxide, 150 ml methanol, 150 ml H_2O), at 100°C for 35 min, and cooled. The 2 ml of reagent 2 (325 ml, 6N hydrochloric acid, 275 ml methanol) was added to the cell pellets. Subsequently, the cell pellets in reaction were heated at 80°C for 1 min. The cooled cells were mixed with 1.25 ml reagent 3 (200 ml hexane, 200 ml MPDE) for 10 min. The supernatant of reaction solution was washed with reagent (10.8 g sodium hydroxide, 900 ml H_2O). The fatty acids were analyzed with a gas-liquid chromatograph (HP6890, Hewlett-Packard Co., Palo Alto, CA, USA)

Extraction of membrane proteins and SDS-PAGE

Membrane protein was extracted from the aromatic-treated cells by the Triton X-100 extraction method as described by Schnaitman (17) and Tsuboi (21). In order to purify the membrane proteins from *E. coli* or *B. Subtilis* treated with 20 mM catechol for 4 h, the cells were suspended in 50 mM Tris-Cl buffer (pH 7.6) containing 10^{-4} M $MgCl_2$ and then disrupted on ice with a sonicator (Labsonic 2000; Laboratory Supply Co. Freidberg, Germany) for 15 seconds 15 to 20 times. After centrifugation at 5,000 × g for 30 min, the supernatant was treated with 0.1% Triton X-100, and precipitated by ultracentrifugation at 40,000× g for 60 min. The pellet treated with 0.1% Triton x-100 buffer was incubated at 30°C for 30 min. After ultracentrifugation at 40,000× g for 60 min, the soluble membranes were obtained from the supernatant. The insoluble membrane proteins were obtained from pellets treated with 0.1% Triton x-100 buffer. Prior to SDS-PAGE analysis, the proteins were quantified with a protein assay kit (Sigma Co., USA) according to the manufacturer's instruction. The SDS-PAGE of the proteins was performed according to the method described by Bollag *et al.* (2) using 10% and 4% acrylamide for separation and stacking gels, respectively. The gels were electrophoresed with a running buffer (0.025 M Tris, 0.192 M

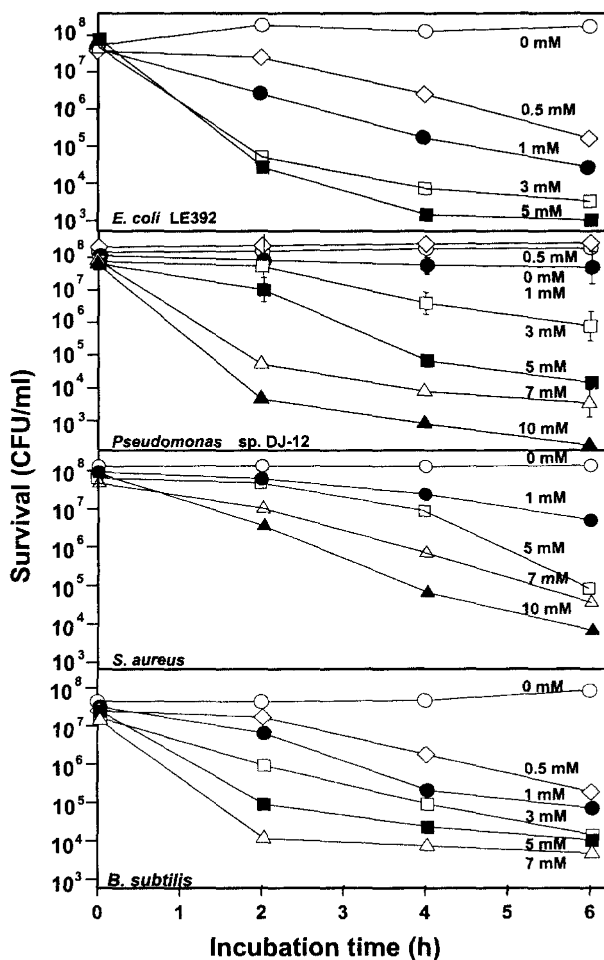

Fig. 2. Effect of catechol at different concentrations on survival of gram-negative and -positive bacteria in MM2 broth.

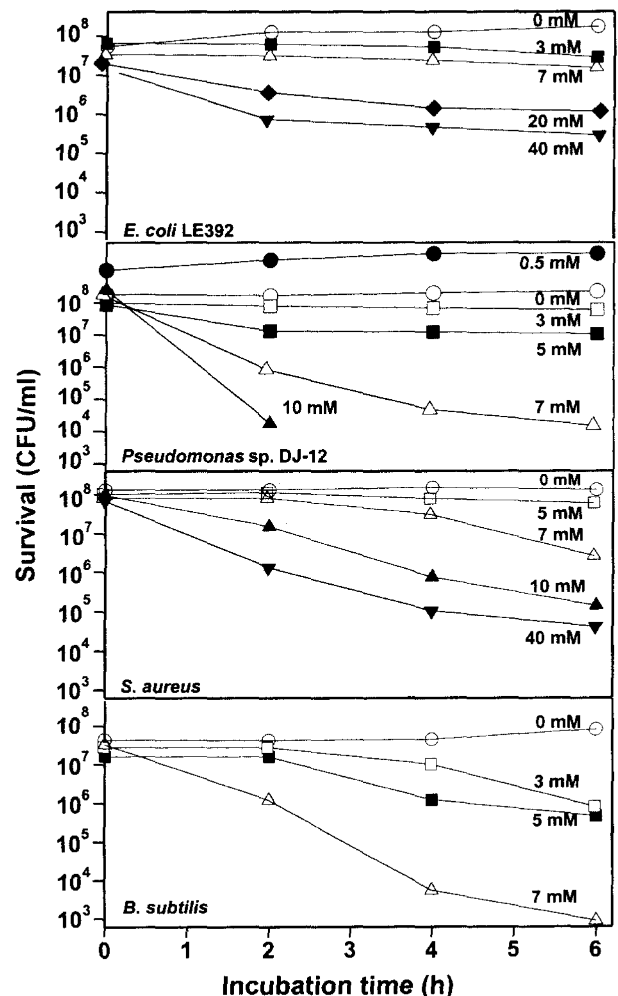

Fig. 3. Effect of 4-chlorobenzoate at different concentrations on survival of gram-negative and -positive bacteria in MM2 broth.

glycine, 0.1% SDS, pH 8.3) at 60~90V for 2.5 h and then stained with a staining solution (0.025% Coomassie brilliant blue R-250, 40% methanol, 7%, glacial acetic acid) for 2 h. The gels were destained with solution I (50% methanol, 10% glacial acetic acid) for 1 h and then with solution II (5% methanol, 7% glacial acetic acid) for 10 h.

Results and Discussion

Effects of catechol and 4CBA on survival

The survival of bacterial cells treated with catechol and 4CBA of different concentrations in MM2 medium are shown in Figs. 2 and 3, respectively. In general, the survival rate of the organisms during treatment for 6 h decreased, as the concentration of each aromatic increased. When *E. coli* LE392 was treated with 0.5 mM catechol or 40 mM 4CBA for 6 h, the survival of the strain decreased by about 2 logs. The toxic effect of catechol on survival of *E. coli* LE392 was much more drastic than that of 4CBA. The toxic effects of catechol and 4CBA to the survival of *Pseduomonas* DJ-12 was not detected at 1.0 mM or lower concentrations. In contrast, *E. coli* LE392 and *S. aures* were more severely effected by catechol than 4CBA at 5 mM or lower concentration. However, there was no clear difference in toxic effect on survival between gram-negative and -positive bacteria as well as between aromatic degraders and non-degraders.

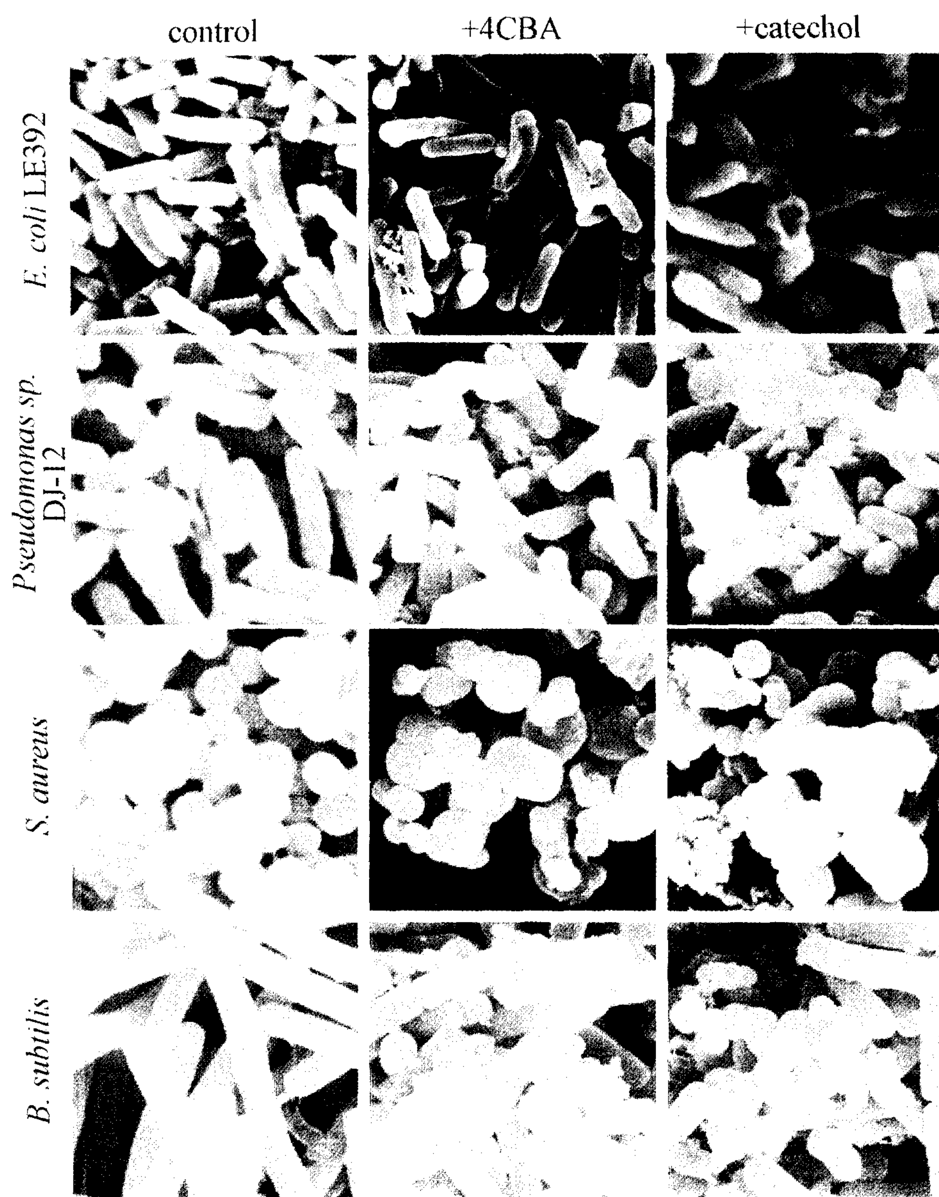

Fig. 4. Scanning electron micrographs of gram-negative and -positive bacteria treated with catechol and 4CBA for 2 h, respectively.

The survival rates of organisms have been generally reported to decrease at higher concentrations of aromatic hydrocarbons (16, 19). The toxic effects of phenolic compounds have been recognized to be due to the permeability change of membranes which was attributed by fatty acids changes (16). Schweigert *et al.* (18) reported that catechol was slightly lipophilic, but acted as both an antioxidant preventing lipid peroxidation and a pro-oxidant damaging macromolecules such as DNA and proteins. Sikkema *et al.*, (20) and Warth (22) reported that aromatic hydrocarbons such as benzoate destroyed the pH gradient and denaturated some enzyme proteins in cells. Therefore, the toxic effects of catechol and 4CBA on the survival rates of the organisms can be explained by the above mechanisms in both gram-negative and -positive bacterial cells.

Effects of catechol and 4CBA on cellular morphology

The morphological changes of the bacterial cells treated with catechol or 4CBA at the concentration were examined for any changes in cellular morphology causing a significant decrease in their viability. As seen in the micrographs (Fig. 4), *E. coli* LE392 cells treated with 10 mM catechol and 40 mM 4CBA showed several destructive openings in the cell envelopes. *Pseudomonas* sp. DJ-12 treated with 20 mM catechol and 30 mM 4CBA reduced cell size, and showed destructive openings in the cell envelopes. Also, *B. subtilis* treated with these aromatics was reduced in cell size, and *S. aureus* cells displayed irregular rod shapes with wrinkled surfaces.

Ramos *et al.* (16) and Sikkema *et al.* (20) reported that the aromatic hydrocarbons at high concentrations conferred a toxic effect on the cells due to the disruption of membrane components, and led to cell death. The bacterial cells treated with aromatic hydrocarbons, such as biphenyl, 4-chlorobiphenyl, and 4-hydroxybenzoate, have been previously reported to show destructive openings in the cell envelopes which were not observed in cells treated with ethanol and heat (15).

Effects of catechol on cellular fatty acids

E. coli LE392 and *S. aureus* treated with 20 mM catechol for 2 h was examined for any changes in total cellular fatty acids, as shown in Tables 2 and 3, respectively. *E. coli* LE392 treated with 20 mM catechol showed some increases in 17:0 fatty acid and cyclo fatty acid (19:0 cyclo w8c), but 13:0 fatty acid decreased by about 20.9%. *S. aureus* treated with catechol showed a basic increase in oleic acid (unsaturated fatty acids, 18:1 w9c), but several saturated fatty acids, such as 13:0 ISO, 14:0 ISO, 14:0, 18:0 ISO and 19:0, decreased after treatment with catechol.

Some mechanisms for changes in totals of fatty acids by aromatic pollutants have been observed in reaction to the compounds (5, 11). Matsusue *et al.* (11) reported that the proportion of arachidonic acid in PCB-treated rats was reduced by about 50%, while oleic and linoleic acids increased significantly. Heipiper *et al.* (5) reported that phenol is toxic to *P. putida,* as a disrupter of membrane permeability by increasing the unsaturated fatty acids in the cell membrane. Therefore, the increase of unsaturated fatty acids in *E. coli* and *S. aureus* treated with 20 mM catechol had a toxic effect on the cells, and led to cell death by distruption of cell envelopes as in Fig. 4, as described by Matsusue *et al.* (11) and Heip-

Table 2. Effect of catechol on the fatty acid composition of *E. coli* LE392

Fatty acids	Distribution (mol % of total fatty acid)		
	Untreated cells (control)	Catechol-treated cells (% change of the control)	
12:0	8.23	7.96	
13:0	0.81	0.64	(20.9% ↓)
14:0	8.71	7.66	
13:0 3OH/15:1 i I/H	0.83	0.94	
15:0	2.14	1.95	
16:1 ISO I/14:0 3OH	18.46	18.03	
16:1 w7c/15 iso 2OH	2.45	2.13	
16:0	27.47	28.38	
17:0 CYCLO	15.46	15.33	
17:0	1.81	2.09	(15.5% ↑)
18:1 w7c/w9t/w12t	4.49	4.77	
19:0 CYCLO w8c	3.76	4.90	(30.3% ↑)
15:1 ISO H/13:0 3OH	0.83	0.94	
16:1 w7c15 iso 2OH	2.45	2.13	
18:1 w7c/w9t/w12t	4.49	4.77	

Table 3. Effect of catechol on the fatty acid composition of *S. aureus* ATCC25923

Fatty acids	Distribution (mol % of total fatty acid)		
	Untreated cells (Control)	Catechol-treated cells (% change of the control)	
13:0 ISO	0.41	<0.1	(75.6% ↓)
14:0 ISO	2.40	2.30	(18.9% ↓)
14:0	0.53	<0.1	(81.1% ↓)
15:0 ISO	13.27	12.69	
15:0 ANTEISO	46.11	45.97	
16:0 ISO	1.16	1.23	
16:0	2.31	2.71	(17.3% ↑)
17:0 ISO	5.16	5.36	
17:0 ANTEISO	11.27	11.45	
17:0	0.72	0.82	
18:0 ISO	0.44	<0.1	(77.3% ↓)
18:1 w9c	0.23	0.75	(226.1% ↑)
18:0	7.82	8.33	
19:0 ISO	1.40	1.49	
19:0 ANTEISO	1.80	1.83	
19:0	1.26	1.46	(18.9% ↑)
20:0	3.69	3.62	

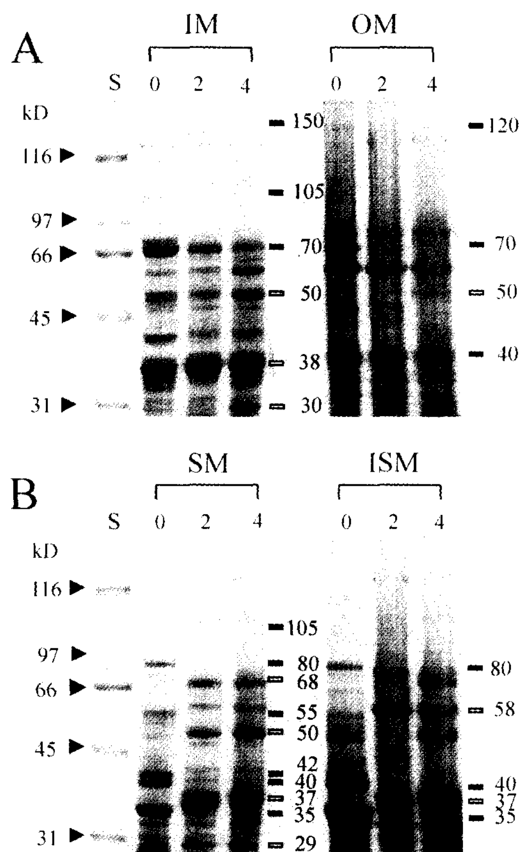

Fig. 5. SDS-PAGE of membrane proteins purified from *E. coli* (A) and *B. subtilis* (B) treated with catechol for 0, 2, and 4 h, respectively. Lanes: S, Size marker; IM, inner membrane; OM, outer membrane; SM, soluble membrane; ISM, insoluble membrane. Open bars denote increased bands, and closed bars denotes decreased band.

iper *et al.* (5).

Effects of catechol on membrane proteins

The membrane proteins in *E. coli* LE392 and *B. subtilis*, which were treated with 20 mM catechol for 2 or 4 h, were analyzed by SDS-PAGE as shown in Fig. 5. In the *E. coli* LE392 treated with 20 mM catechol, the inner membrane proteins of 150 kDa, 105 kDa, and 70 kDa decreased, but the proteins of 50, 38, and 30 kDa increased. Some outer membrane proteins (120, 70, and 40 kDa) increased in *E. coli* LE392 treated with catechol. In *B. subtilus* treated with 20 mM catechol, the soluble membrane proteins of 105, 80, 55, 42, 40, and 35 kDa decreased and the proteins of 68, 50, 37 and 29 kDa increased. On the other hand, the 2 kinds of insoluble membrane proteins (58 and 37 kDa) increased. There have been some reports that gram-negative bacteria are more resistant to deleterious lipophilic compounds (7), but it is hard to describe a general rule of difference between gram-positive and -negative bacteria on the effects of aromatics on membrane proteins.

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