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The Role of Hydrogen Peroxide Produced by Polychlorinated Biphenyls in *PMR1*-Deficient Yeast Cells

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Polychlorinated biphenyls (PCBs) are well-known recalcitrant environmental pollutants. Although the metabolism of the PCBs has been intensively studied, very little is known about their mechanism of toxicity in living organisms or how they are degraded. We have examined the effects of PCBs on two different yeast strains to determine their mechanism of action. One yeast strain (K601, wild type) is resistant to the growth-inhibitory effect of PCBs, whereas the other strain (AA542, *PMR1* mutant) is susceptible. PCBs increased the level of intracellular hydrogen peroxide in AA542 cells but not in K601 cells. In the presence of α-tocopherol or ursolic acid the growth of AA542 cells was not inhibited by treatment with PCBs. These results suggest that PCBs block cell growth through production of hydrogen peroxide in the *PMR1* mutant strain, AA542. We compared superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase activities in both strains. The catalase activity in K601 cells was 10 times higher than that in AA542 cells. In contrast, there was no difference in activities of SOD and GPx between the two strains. Collectively, these observations indicate that oxidative stress causes the inhibition of cell growth observed in catalase-deficient yeast cells exposed to PCBs.

Key words: catalase, *PMR1* mutant, polychlorinated biphenyls, reactive oxygen species, *Saccharomyces cerevisiae*.

Abbreviations: PCBs, polychlorinated biphenyls; DCBs, dichlorinated biphenyls; ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione S-transferase; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate buffered saline.

Polychlorinated biphenyls (PCBs) belong to a large group of chemicals known as halogenated aromatic hydrocarbons. PCBs are biologically hazardous substances and are one of the most serious environmental pollutants because of their chemical stability, lipophilic nature, highly insulating properties, and resistance to burning (*1*–*3*). The degradation and mechanism of toxicity of PCBs have been intensively studied in an attempt to overcome the environmental and toxic problems they cause (*1*, *4*). PCBs disrupt steroid hormone function (*5*), enhance the growth of cancer cells (*6*), and cause accumulation of reactive oxygen species (ROS) in a variety of cells (*7*–*9*). PCBs can be broken down biologically, and since the first report of this biodegradation (*10*), many PCB-degrading bacteria have been screened and characterized biochemically for use in bioremediation (*4*, *11*). Various microorganisms have been shown to contain hydrolases and dioxygenases that can degrade PCBs (*1*, *12*–*13*).

A relatively low concentration of PCB (10 ng/ml) is sufficient to disrupt bovine oocyte maturation, fertilization, and developmental competence (*14*). PCBs can affect parts of the endocrine system by inhibiting secretion of estradiol in MCF-7 cancer cells (*15*–*16*) and affect thyroid hormone and vitamin A action in the rat (*17*).

In addition, PCBs reduce dopamine levels, induce cytochrome P450, increase intracellular calcium concentration, and interfere with intracellular signaling systems in various cells (*18*–*21*). Although many studies have reported that PCBs regulate cellular responses in a variety of systems, very little is known about how they achieve this.

ROS are continually generated as byproducts of respiration in aerobic organisms, and their products can modify intracellular molecules including proteins, DNA, and lipids (*22*–*24*). Nearly all aerobic organisms including bacteria and yeasts possess a range of non-enzymatic and enzymatic antioxidant defense systems such as glutathione, thioredoxin, superoxide dismutase (SOD), several peroxidases, and catalase, to counter oxidative stress. In animals, one of the major defense enzymes, SOD, scavenges the intermediates of oxygen reduction (*25*), which deprotonates the superoxide anion (O_2^-) to H_2O_2. H_2O_2 is enzymatically detoxified by catalase (*26*) and peroxidase (*27*–*28*). In *Saccharomyces cerevisiae*, ROS generated by respiration or oxidation of nutrients are enzymatically catabolized by catalase, peroxidase, and SOD (*26*–*28*). Recently, it was demonstrated that PCBs can cause production of ROS in a variety of cells (*7*–*9*). It is therefore of interest to determine if PCBs are capable of causing ROS production in *S. cerevisiae* and to elucidate the mechanism through which this occurs.

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In the present paper we examined two different strains of *S. cerevisiae* to determine the mechanism of toxicity of PCBs. Treatment of the *PMR1* mutant (AA542) with PCBs resulted in inhibition of cell growth, whereas treatment of the wild-type form (K601) had no effect. We found that PCBs suppressed cell growth in AA542 through production of hydrogen peroxide. To elucidate the mechanism of toxicity, we compared the activities of SOD, glutathione peroxidase (GPx), and catalase in K601 and AA542 strains. Activities of SOD and GPx but not catalase were detected in AA542. It is likely that this lack of catalase results in accumulation of hydrogen peroxide that leads to suppression of cell growth.

MATERIALS AND METHODS

Chemicals—2,3-Dichlorobiphenyl, 2,4-dichlorobiphenyl, and 2,5-dichlorobiphenyl were purchased from Accu-Standard (New Haven, CT, USA). 2,7-Dichlorodihydrofluorescein diacetate, α-tocopherol, ursolic acid, pyrogallol, nitroblue tetrazolium, riboflavin, tetramethyl ethylene diamine, ferric chloride, and potassium ferric cyanide were from Sigma Chemical (St. Louis, MO, USA). Yeast extract, peptone, and dextrose were from Becton Dickinson (Sparks, MD, USA). Rabbit anti-catalase was from Fitzerald Industries International (Concord, MA, USA). 2,7-Dichlorodihydrofluorescein diacetate and DCBs were dissolved in DMSO. All were stored as stock solutions at –20°C.

Yeast Strains and Cell Culture—The yeast strains used in this study were K601 (YR98:MATα *ade2 his3-Δ200 leu2-3, 112/lys2-Δ201 ura3-52*, wild type) and AA542 (YR122:MATα *ade2 his3-Δ200 leu2-3, 112/lys2-Δ201 pmr1-Δ1::LEU2 ura3-52*, *PMR1*-deficient mutant). Yeast cells were cultured in YPD medium (1% yeast extract, 2% dextrose, and 2% peptone) at 30°C with reciprocal shaking (250 rpm) and harvested during the stationary phase (*29*).

Measurement of Cell Growth—Yeast cells at stationary phase were inoculated into fresh culture medium at an optical density of 0.1 at 600 nm. The yeast cells were cultured with or without each test compound. The cell number and density was measured at intervals using a haemacytometer (Superior, Germany) or by absorbance at 600 nm (SmartSpec3000, Bio-Rad), respectively.

Measurement of Intracellular Reactive Oxygen Species (ROS)—Production of cellular ROS was analyzed by measuring the intracellular deacylation and oxidation of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2,7-dichloroflurescein (DCF). DCFH-DA is highly reactive with hydrogen peroxide (*30*). Yeast cells at stationary phase were washed with phosphate-buffered saline (PBS). After preincubation in PBS with 50 μM DCFH-DA at 25°C for 60 min, the cells were incubated with each test compound (*31*). Cells were then washed three times with PBS to remove unloaded dye. Fluorescence at 520 nm (emission) was measured with an excitation wavelength of 488 nm using a fluorescence spectrophotometer (LS 50B, Perkin Elmer). The bandwidths for excitation at 488 nm and emission at 520 nm were 10 nm and 5 nm, respectively.

Enzyme Assays by Spectrophotometry—Yeast cells from both strains were disrupted by vortexing with glass beads in 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM DTT, 0.5 mM PMSF, and 0.5 μg/ml of each of the protease inhibitors, aprotinin, leupeptin, and pepstatin A. The extracts were then clarified by centrifugation at 10,000 ×*g* (30 min, 4°C). The supernatants were aliquotted into clean tubes and stored at –70°C. Protein concentrations were estimated by the method of Bradford (*32*).

SOD activity was determined as previously described (*33*). The activity was measured by inhibition of the autoxidation of pyrogallol by anionic peroxide radicals in an alkaline solution at 420 nm. One unit of SOD activity is defined as the amount of enzyme that inhibits the reaction by 50%.

GPx activity was assayed using the cellular glutathione peroxidase assay kit (Calbiochem, Germany). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm with an extinction coefficient of 0.00622 μM^{-1} cm^{-1}. This provides a spectrophotometric means of monitoring GPx activity. One unit of GPx activity is defined as the amount of enzyme oxidizing of 1 μmol of NADPH per min at 25°C.

Catalase activity was assayed as described by Beers and Sizer (*34*) in 50 mM sodium phosphate buffer, pH 7.4, by measuring the decomposition of H_2O_2 at 240 nm using an extinction coefficient of 43.6 M^{-1} cm^{-1} and an initial H_2O_2 concentration of 54 mM. One unit of catalase activity is defined as the amount of enzyme catalyzing the breakdown of 1 μmol of H_2O_2 per min at 25°C.

Enzyme Assays Using in Gel Staining—SOD activity staining was performed as previously described (*25*). The enzyme extracts were applied to a 12% native polyacrylamide gel. After electrophoresis, the gel was stained by incubation in a solution containing 2.5 mM nitroblue tetrazolium (NBT) for 25 min, followed by incubation in 50 mM potassium phosphate buffer, pH 7.8, containing 28 μM riboflavin and 28 mM tetramethyl ethylene diamine for 20 min in the dark. The gel was placed in distilled water and exposed on a light box for 10 to 15 min at room temperature.

The method for catalase-activity staining was essentially that described by Wayne and Diaz (*35*). Protein extract was applied to an 8% native polyacrylamide gel. After electrophoresis, the gel was washed in distilled water to remove residual buffer salts and then placed in 5 mM H_2O_2 for 10 min. The rinsed gel was transferred to a freshly prepared solution of 2% ferric chloride and 2% potassium ferric cyanide. As soon as a green color began to appear in the gel, the reaction was stopped by washing the gel in distilled water.

Immunoblot Analysis—Yeast cell lysate was prepared in lysis buffer (8 M urea, Triton X-100, 1 mM DTT, 1 mM PMSF, and 40 mM Tris, pH 7.4). Total proteins (1 mg) obtained by centrifugation (14,000 ×*g*, 20 min, 4°C) were charge- and size-separated by electrophoresis on IPG strip and 12% SDS-polyacrylamide gel, respectively (*36*), then transferred electrophoretically to a nitrocellulose membrane. Nonspecific binding was blocked with TBST (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) containing 5% non-fat milk for 1 h at 25°C. The membrane was immunoblotted with primary antibody (1: 1,000 dilution of rabbit anti-catalase) for 16 h at 4°C, then exposed to a horseradish peroxidase-conjugated sec-

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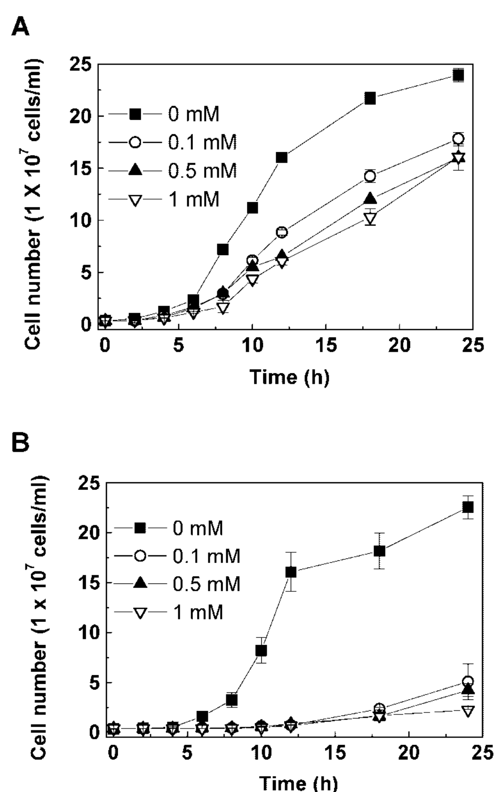

Fig. 1. **Growth curves for the yeast strains K601 (A) and AA542 (B) in response to different concentrations of 2,5-DCB.** The yeast cultures grown on YPD medium were treated with 0 mM (solid squares), 0.1 mM (open circles), 0.5 mM (solid triangles), and 1 mM (inverted open triangles) 2,5-DCB. Each value is the mean ± standard deviation of four experiments.

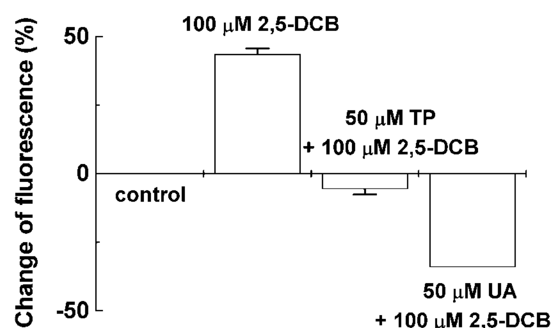

Fig. 2. **Production of hydrogen peroxide following treatment of yeast cells with 2,5-DCB.** Levels of hydrogen peroxide were measured using the fluorescent dye DCFH-DA in AA542 cells. 50 μM α-tocopherol (TP) or 50 μM ursolic acid (UA) were added to cultures 30 min before addition of 2,5-DCB. Control indicates fluorescent intensity of untreated yeast cells. Results are expressed as percentages of the control value and are the means ± standard deviations of three experiments.

ondary antibody (1:5000 dilution in TBST) for 2 h at 25°C. Immunoreactive spots were visualized using an ECL detection system.

RESULTS AND DISCUSSION

The modulating activity of PCBs as endocrine disruptors on cell growth was estimated using *S. cerevisiae* as a simple model system of eukaryotic cells. The focus of the current study was to identify how PCBs inhibit cell growth in the *PMR1*-deficient mutant strain AA542. The *PMR1* gene encodes a 104-kDa polypeptide homologous to mammalian Ca^{2+}-ATPases present in the endoplasmic reticulum (*41*). The *PMR1* product localizes to the golgi complex in *S. cerevisiae* (*24*) and has been shown to accumulate cytosolic calcium and manganese (*42*) and to exhibit in calcium dependent alterations in protein secretion (*41*, *42*). We compared the effects of PCB on cell growth in K601 and AA542 strains and then identified how PCBs regulate cell growth in both yeast strains.

PCBs Block Cell Growth in AA542 but Not in K601 Cells—We investigated the effect of various concentrations of the PCB congener 2,5-dichlorobiphenyl (DCB) on cell growth in K601 and AA542 cultures. 2,5-DCB had little effect on the growth rate of K601 cells. In contrast, 2,5-DCB (100 μM) strongly inhibited growth of AA542 cells (Fig. 1). Other DCBs such as 2,3-DCB and 2,4-DCB had similar effects to those of 2,5-DCB on growth of K601 and AA542 cells (data not shown). PCBs inhibit cell growth in a wide variety of eukaryotic cells, such as serotonergic cells and MCF-7 cells (*15–16*, *37–38*). However, the mechanism responsible for the inhibitory effect of PCB on *PMR1*-deficient cell growth has remained unresolved. Clarification of the difference observed in the growth patterns induced by PCBs in the two yeast strains may thus provide a clue to the action mechanism of PCBs on eukaryotes including mammalian cells.

PCBs Inhibit Growth of AA542 Cells through Production of Hydrogen Peroxide—It has recently been reported that PCBs can cause production of reactive oxygen species (ROS) in several systems (*7*, *39*, *40*). It therefore is important to determine if this also occurs in *S. cerevisiae* and whether it is part of the mechanism of toxicity of PCBs. To evaluate the correlation between treatment with PCBs and production of ROS in yeast, we measured intracellular levels of hydrogen peroxide using the fluorescent probe DCFH-DA following exposure of AA542 cells to 2,5-DCB (100 μM). Exposure to 2,5-DCB resulted in production of hydrogen peroxide (Fig. 2) in a concentration dependent manner (data not shown). When antioxidant, α-tocopherol (vitamin E, 50 μM) or ursolic acid (50 μM), was added 30 min before addition of 2,5-DCB to AA542 cells, hydrogen peroxide level was significantly reduced (Fig. 2). In contrast, no hydrogen peroxide accumulated in K601 yeast cells during exposure to 2,5-DCB (data not shown). Other water-soluble antioxidants such as ascorbic acid (2 mM) and DTT (1 mM) had no effects on hydrogen peroxide level produced by addition of 2,5-DCB in both K601 and AA542 cells (data not shown). This might be due to their inability to cross the cell membrane.

α-Tocopherol and ursolic acid prevented the inhibition of growth of AA542 cells by 2,5-DCB in a concentration-dependent manner (Fig. 3). A concentration of 50 μM ursolic acid completely prevented the inhibition of growth of AA542 cells caused by 100 μM 2,5-DCB, whereas a concentration of 100 μM α-tocopherol only partly reversed the inhibition (Fig. 3). Therefore, it appears that ursolic acid is more effective in protecting

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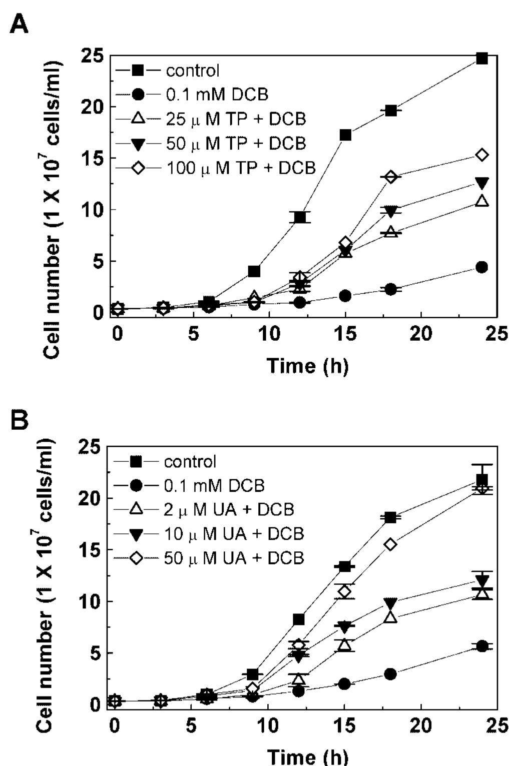

Fig. 3. **Effect of 2,5-DCB on growth of AA542 cells pre-treated with α-tocopherol (A) or ursolic acid (B).** AA542 cells were pre-treated with various concentrations of α-tocopherol (TP) or ursolic acid (UA) followed by addition of 0.1 mM 2,5-DCB. Each value is the mean ± standard deviation of three experiments.

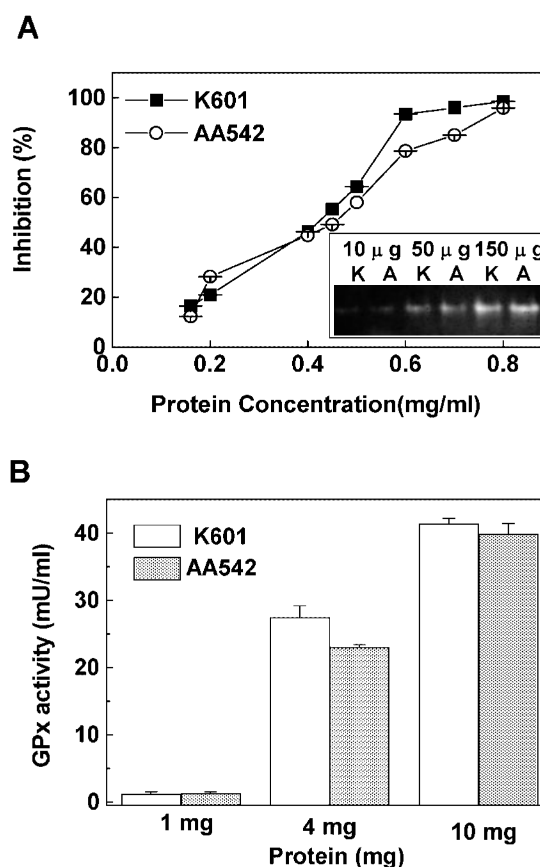

Fig. 4. **Activities of SOD and GPx in K601 and AA542 cells.** (A) SOD activity is calculated by measurement of inhibition of pyrogallol autoxidation by protein extracts from K601 or AA542 cells. The rate of autoxidation was taken from the increase in A_{420} per min. Values of the IC_{50} are 0.42 for K601 and 0.46 for AA542, respectively. Results are expressed as percentages of inhibition of autoxidation and are the means ± standard deviations of three experiments. The insert shows a native gel stained with nitroblue tetrazolium to detect activity of SOD in K601 (denoted K) and AA542 cells (denoted A). The amount of protein extract loaded in each lane is indicated. The result shown is representative of five separate experiments. (B) GPx activity calculated by measuring oxidation of NADPH to $NADP^+$ in 1 mg, 4 mg, and 10 mg protein extracts from K601 and AA542 cells. Each value is the mean ± standard deviation of three experiments.

cells against the growth inhibitory effects of 2,5-DCB than α-tocopherol (Fig. 3) and in removing hydrogen peroxide (Fig. 2). These results suggest that PCBs block cell growth through production of ROS in AA542 cells. Recent studies have demonstrated that PCBs cause accumulation of ROS in rat synaptosome (*7*) and other systems (*39*, *40*) and induce activities of cytochrome P450, glutathione S-transferase (GST), and peroxidase, which play important roles in protection against oxidative stress in mice (*39*). Our observations that ROS mediate the inhibitory effects of PCBs on *PMR1*-deficient yeast growth are consistent with previously reported results in mammalian cells.

Deficiency of Catalase in AA542 Cells Causes Accumulation of Hydrogen Peroxide—Cells are protected from oxidative stress by the enzyme defense system composed of SOD, peroxidase, and catalase (*25–28*). To identify whether the accumulation of hydrogen peroxide is due to a defect in this defense system, we measured the activities of these enzymes in K601 and AA542 cells (Table 1 and Figs. 4 and 5). Activities of SOD and GPx in both yeast strains are shown in Fig. 4 and Table 1. The activity of SOD in K601 cells (2.38 ± 0.02 units) was very similar to that in AA542 cells (2.17 ± 0.08 units). The activities of GPx were 41.42 and 39.87 units in K601 and AA542 cells, respectively. These results show that SOD and GPx are unlikely to be associated with the accumulation of hydrogen peroxide in AA542 cells.

When we measured catalase activity, we found a large difference between K601 and AA542 cells (Fig. 5 and Table 1). Catalase activity was detected in K601 cells (4.23 ± 0.3 units), very little activity was present in AA542 cells (0.39 ± 0.03 unit). To determine whether AA542 is deficient catalase at protein level, we employed immunoblot analysis. As shown in Fig. 6B, catalase was

Table 1. **Relative specific activities of enzymes in yeast strains, K601 and AA542.** Protein extracts were obtained to measure enzyme activities as described in "MATERIALS AND METHODS." Values are the means ± standard deviations of three or five independent experiments.

Enzyme activity (units/mg of protein)	K601	AA542
Superoxide dismutase	2.38 ± 0.02	2.17 ± 0.08
Catalase	4.23 ± 0.30	0.39 ± 0.03
Glutathione peroxidase	1.08 ± 0.37	1.21 ± 0.35

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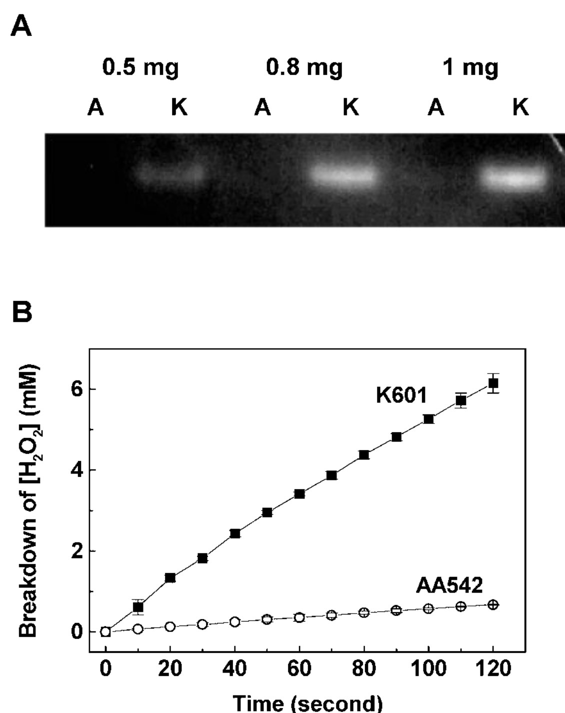

Fig. 5. **Catalase activities in K601 and AA542 cells. (A) The amount of protein extract loaded in each gel lane is indicated.** The native gel was stained for catalase activity with 2% ferric chloride and 2% potassium ferric cyanide. K and A indicate protein extracts from K601 and AA542 cells, respectively. This result is representative of four independent experiments. (B) Catalase activity (0.8 mg protein/ml) was measured by the breakdown of 54 mM hydrogen peroxide in 50 mM sodium phosphate buffer, pH 7.4. Each value is the mean ± standard deviation of three experiments.

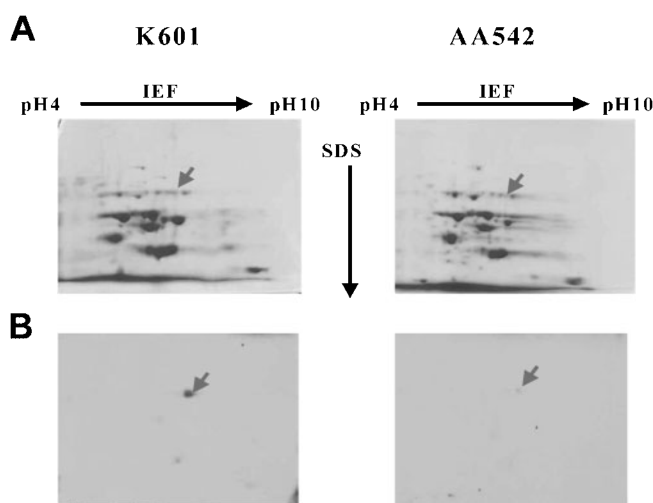

Fig. 6. **Comparison of catalase levels in K601 and AA542 strains.** (A) 2D-PAGE pattern of the K601 and AA542 proteins. Equal amounts (1 mg) of total proteins were subjected to 2D-PAGE and the proteins were detected by Coomassie Brilliant Blue staining. (B) Catalase was detected by immunoblotting. Arrows indicate the catalase.

largely present in K601 but not in AA542. Equal protein loading was confirmed on a coomassie brilliant blue-stained 2D gel (Fig. 6A). These results suggest that the accumulation of hydrogen peroxide during the exposure of catalase-deficient AA542 cells to PCBs inhibits cell growth. How does the *pmr1* mutant (AA542) result in loss of both catalase activity and protein level? It is possible that loss of the *PMR1* product leads to a block in the secretion pathway (*41*) or to cytoplasmic accumulation of Ca^{2+} and Mn^{2+}, resulting in absence of catalase activity in AA542 cells. However, to date, no biochemical evidence has been presented that directly supports the catalase-deficiency in AA542. As mentioned above, we found that SOD and GPx were present in K601 and AA542 cells, whereas catalase was present in K601 cells but virtually absent in AA542 cells (Figs. 5 and 6). Collectively, these observations indicate that oxidative stress caused by exposure to PCBs in *PMR1*-deficient yeast cells that lack catalase results in the suppression of cell growth.

Our data, however, indicate that accumulation of hydrogen peroxide caused by exposure to PCBs is not the sole factor in the blockage of cell growth in AA542. Addition of 10 μM H_2O_2 to AA542 cells produced an intracellular level of hydrogen peroxide similar to that produced by treatment with 100 μM 2,5-DCB, and the concentration of H_2O_2 in AA542 cells did not inhibit cell growth (data not shown). If accumulation of hydrogen peroxide is not the only mechanism, what are the others? Do these mechanisms involve modification of proteins, or several unknown proteins, or accumulation of other toxic compounds? Further studies to explore and identify the proteins or compounds should enable us to answer some of these questions.

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