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SHORT COMMUNICATION

Antibacterial activity of cyclodextrins against *Bacillus* strains

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Abstract Growth of alkaliphilic *Bacillus halodurans* C-125 both on agar plates and in liquid culture was inhibited by methyl-β-cyclodextrin (CD). Furthermore, resting cells of the strain were lysed by contact with methyl-β-CD higher than 10 mM. α-CD also showed lysis activity against *Bacillus* and related strains. The activity was not observed with Gram-negative and Gram-positive bacteria except for *Bacillus* strains. Fluorescence staining and scanning electron microscopy of cells revealed that methyl-β-CD disrupted cell membranes, and consequently, the cells were lysed. This is a novel physiological property of CDs.

Keywords Methyl-β-cyclodextrin · α-cyclodextrin · Antibacterial agent · *Bacillus* · Lysis

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Cyclodextrins (CDs) include α-, β-, γ-CDs, and methyl-β-, hydroxypropyl-β- and branched-CDs (Duchêne1991; Dodziuk 2006; Nakakuki 2005). They are synthesized from starch by cyclodextrin glucanotransferases, and some of them are modified by subsequent chemical reactions. CDs can form inclusion complexes that enclose various organic compounds in their unique cyclical structure. Therefore, they are widely used as additives to food, detergents, cosmetics, and pharmaceutical preparation to improve the physical and chemical properties of incorporated materials. However, despite a long-term research and development, very few information about antimicrobial activity of CDs against microorganism has been published to date. Jadoun and Bar (1993) reported that dimethylated β-CD (Dimeb) showed growth inhibition against *Rhodococcus erythropolis*. Around 50% growth inhibition of the strain was observed in a culture supplemented with 50 mM Dimeb. Hydroxypropyl-β-CD as well as Dimeb showed toxic effect towards *Mycoplasma capricolum* and *Acholeplasma laidlawii* (Greenberg-Ofrath et al. 1993). Recently, Donova et al. (2007) demonstrated that methyl-β-CD altered growth and cell envelope features of *Mycobacterium* sp. The strain did not grow at 180 mM methyl-β-CD.

While investigating the mechanism of adaptation to alkaline conditions of the alkaliphilic *Bacillus halodurans* C-125, we noticed that the gene BH3500 was strongly expressed under alkaline culture conditions by using microarray analysis (Zhang et al. 2005). The gene product showed similarity to flotillin-1, which has been reported to be a membrane protein and a molecular marker of lipid rafts in the plasma membrane of eukaryotic cells (Simons and Ikonen 1997). Lipid rafts are sphingolipid- and cholesterol-rich membrane microdomains (Brown and London 2000; Salzer and Prohaska 2001). The molecular function of flotillins is still unknown, and that of flotillin-like proteins in prokaryotes is also

unclear. To clarify the function of a flotillin-like protein in *B. halodurans* C-125, methyl-β-CD, which is known to be cholesterol-trapping agent (Scheiffele et al. 1997), we added it to the culture medium. Methyl-β-CD inhibited growth of this strain despite having none of the synthetic pathways for cholesterol of prokaryotes. In this report, we describe a new function of methyl-β-CD, cell lysis as well as growth inhibition of *Bacillus* and related strains.

B. halodurans strain C-125 JCM9153 (Takami and Horikoshi 1999) was usually propagated on Horikoshi II medium (Horikoshi 2006) composed of 1% (w/v) soluble starch (Wako Pure Chemical), 0.5% Bactopeptone (Difco), 0.5% yeast extract (Difco), 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.4% Na_2CO_3 (sterilized separately) (pH 10) or 0.4% NaCl (pH 7). To solidify it, 1.5% agar was added to the medium.

First, the antimicrobial activity against *B. halodurans* C-125 was examined using a diffusion method with filter paper disks (i.d. 6 mm) impregnated with 5, 10, or 20 mg methyl-β-CD (Sigma: mean degree of substitution 10.5–14.7). The filter paper disks were placed on agar plates which were spread with 0.1 ml of cell suspension (5×10^4 or 5×10^8 cells ml^{-1}), and then incubated at 37°C for 24 h. Growth inhibition zones were clearly detected around all disks at both high and low cell inoculation densities (Fig. 1). A growth test in a liquid culture (50 ml of Horikoshi medium II, pH 10) containing 0, 2.5, 5, or 7.5 mM methyl-β-CD was also done. The initial turbidity of each culture was measured at absorbance at 600 nm (A_{600}) as 0.03. After incubation at 37°C for 16 h with shaking, A_{600} of the control culture without methyl-β-CD reached 3.0, equal to that of the culture in the presence of 2.5 mM methyl-β-CD, and A_{600} of the culture with 5 mM methyl-β-CD reached 0.5. The cells did not grow at all in the culture medium containing 7.5 mM methyl-β-CD. This is a new finding of a physiological activity of methyl-β-CD.

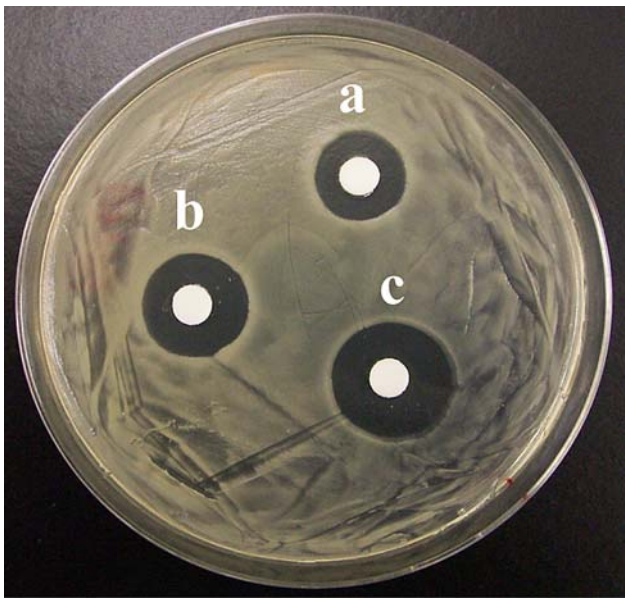

Fig. 1 Agar diffusion method. *B. halodurans* C-125 (5×10^7 cells) was spread on Horikoshi II medium, and methyl-β-CD-impregnated filter paper disks (*a* 5 mg, *b*10 mg, *c* 20 mg) were put on top. The agar was incubated for 24 h at 37°C

In addition, we found that *B. halodurans* C-125 cells were gradually lysed by adding methyl-β-CD to the resting cell suspensions. The strain was propagated aerobically in Horikoshi II medium (pH 7 or 10) at 37°C for 16 h, the cells were collected and washed by centrifugation (5,000×*g* at 4°C for 15 min) with 10 mM Na^+, K^+-phosphate buffer (pH 7.4), and then resuspended in the same buffer until A_{600} reached approximately 0.5. The cell suspensions were incubated with 10 or 20 mM methyl-β-CD or without it as a control at 37°C for 30 min under gentle shaking. After 30-min incubation, the lysis rate was calculated from A_{600} of control cell suspension minus that of cell suspension incubated with methyl-β-CD divided by that of the control cell suspension, and expressed as percentage. All experiments were done at least three times. The lysis rates of the strain grown at pH 7 were $16.5 \pm 6.1\%$ in 10 mM and $47.1 \pm 3.15\%$ in 20 mM methyl-β-CD,

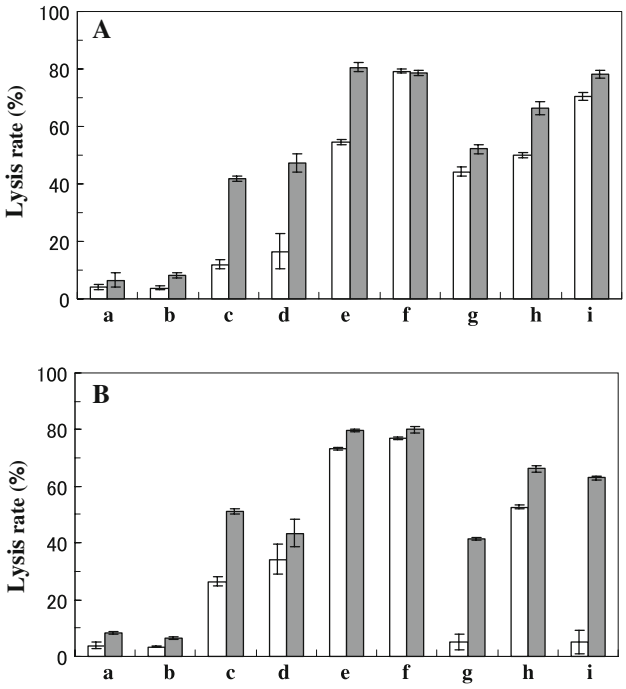

Fig. 2 Lysis of bacterial strains by CDs. Each strain was incubated with 10 mM (*white bar*) or 20 mM (*filled bar*) of methyl-β-CD (**a**) or α-CD (**b**) for 30 min at 37°C with gentle shaking. The lysis rate was calculated as described in the text. Bacterial strains used: *a Escherichia coli* JCM1649, *b Staphylococcus aureus* JCM2151, *c Bacillus subtilis* JCM1465, *d B. halodurans* C-125 grown at pH 7, *e B. halodurans* C-125 grown at pH 10, *f Paenibacillus campinasensis* JCM11200, *g Oceanobacillus iheyensis* JCM11309, *h Geobacillus kaustophilus* JCM12893, *i B. cereus* ATCC14579

whereas those of the strain grown at pH 10 were 54.6 ± 0.94% in 10 mM and 80.6 ± 1.47% in 20 mM methyl-β-CD (Fig. 2a). The effects of other CDs on lysis of *B. halodurans* C-125 cells were examined. α-CD also showed lysis activity on the strain grown at pH 7 with rates of 34.3 ± 5.29% in 10 mM and 43.5 ± 4.89% in 20 mM α-CD (Fig. 2b). As in the case of methyl-β-CD, the alkaline grown strains were more susceptible to α-CD with rates of 73.4 ± 0.42% in 10 mM and 79.7 ± 0.49% in 20 mM α-CD, (Fig. 2b), whereas even in 20 mM β- and γ-CDs, they had less activity (lysis rates of <15%). It is also the first finding of this physiological ability of α-CD. The lysis activity towards other strains, namely, *Escherichia coli* JCM1649, *Staphylococcus aureus* JCM2151, and other *Bacillus* strains was investigated. Both methyl-β-CD and α-CD showed lysis activity towards *Bacillus* and related stains, such as *B. subtilis* JCM1465, *B. cereus* ATCC14579, *Paenibacillus campinasensis* JCM11200, *Oceanobacillus iheyensis* JCM11309, and *Geobacillus kaustophilus* JCM12893 (Fig. 2a, b). Although the experimental conditions were different, *B. clausii* KSM-K16 (Kageyama et al. 2007) cells were also lysed with the rate of 36.9 ± 0.22% after 1 h incubation in the presence of 10 mM methyl-β-CD at room temperature. However, there was little activity towards *E. coli* and *S. aureus* cells. It is surprising and interesting that both methyl-β-CD and α-CD were clearly effective against only *Bacillus* and related strains. Thus, methyl-β-CD and α-CD did not distinguish between Gram-negative and Gram-positive bacteria except *Bacillus* and related strains.

After incubation of *B. halodurans* C-125 suspension with or without 10 mM methyl-β-CD, cells were stained with both SYTO 9 and propidium iodide (PI) and observed by fluorescence microscopy. The control cells (without methyl-β-CD) were stained green only by SYTO9 throughout the experiment (Fig. 3a), whereas the cells in the presence of methyl-β-CD were stained green with SYTO 9 and red with PI after 10 min incubation (Fig. 3b). Almost all cells were stained red after 30 min incubation with methyl-β-CD, and the total cell density was greatly decreased due to cell lysis (Fig. 3c). These results suggest that the cell membranes of *B. halodurans* C-125 were gradually disrupted during incubation with methyl-β-CD. Actually, many holes on the methyl-β-CD-treated cell membrane of *B. halodurans* C-125 were seen by scanning electron microscopy, as shown in Fig. 4. Furthermore, after cell suspensions of *B. halodurans* C-125 in 10 ml of 10 mM phosphate buffer (pH 7.4, $A_{600} = 1.0$) were incubated with 10 mM methyl-β-CD at 37°C for 30 min, protein concentrations in the centrifugal supernatant were measured using a DC protein assay kit (Bio-Rad) with bovine serum albumin as the standard. The protein concentration was 0.5 mg ml^{-1}, whereas the no protein was detected in the centrifuged control supernatant without methyl-β-CD. The

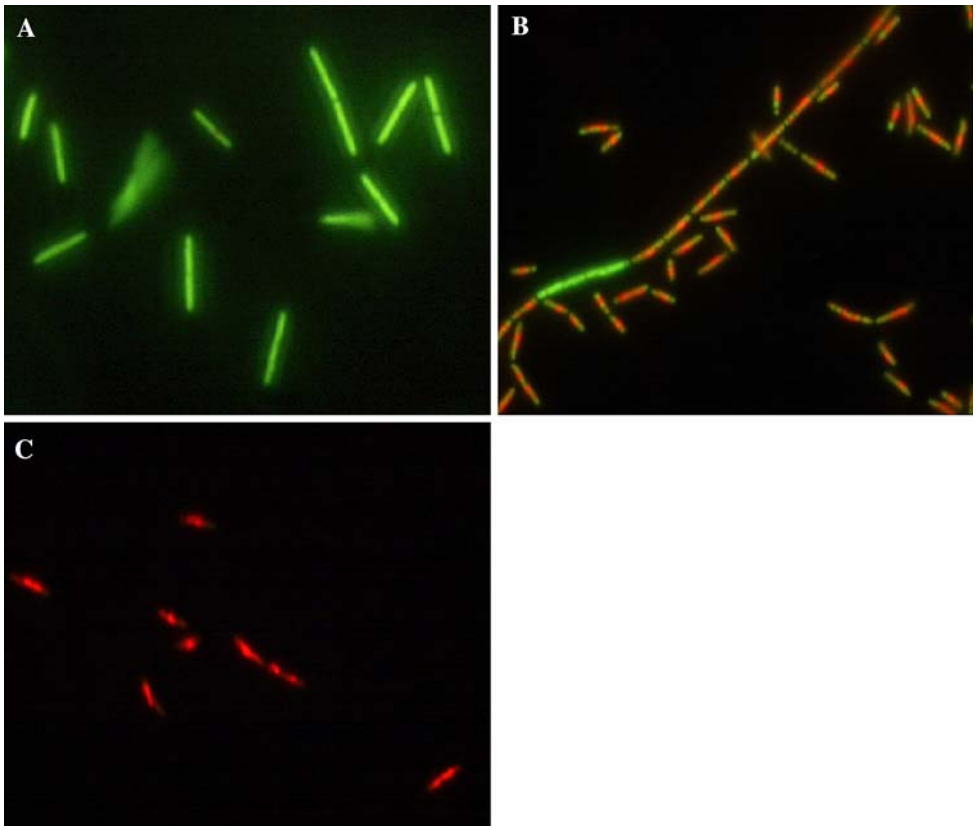

Fig. 3 Methyl-β-CD-treated *B. halodurans* C-125 cells (×1,250). The cells were stained with SYTO9 and PI before treatment with methyl-β-CD (**a**), after 10-min treatment of 10 mM methyl-β-CD (**b**), and after 30-min treatment with10 mM methyl-β-CD (**c**). The stained cells were observed by epifluorescence microscopy using a Nikon ECLIPSE 800 equipped with VFM

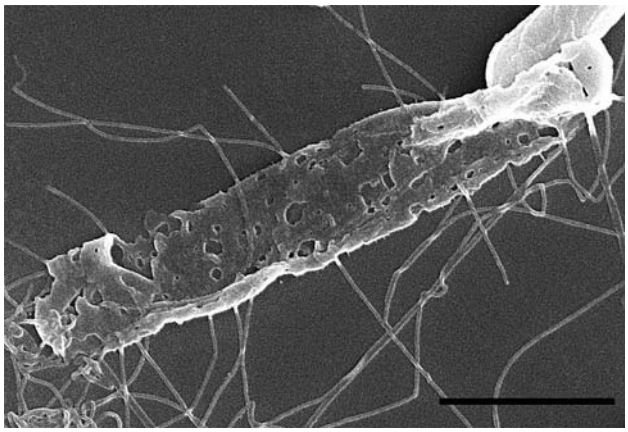

Fig. 4 Methyl-β-CD treated *B. halodurans* C-125 cell. (×25,000) Cell suspensions were incubated with 10 mM methyl-β-CD for 10 min at 37°C. The precipitated cells were prefixed with 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) for 1 h at 37°C. After washing with phosphate-buffered saline, cell suspensions were spread on a glass plate coated by poly-L-lysine (Sigma). The glass plates were postfixed with 2% osmium tetraoxide in phosphate-buffered saline for 1 h at 4°C. After rinsing with distilled water, conductive staining was performed by incubating with 1% tannic acid (pH 6.8) for 1 h. The preparations were then washed with distilled water and treated with 1% osmium tetraoxide for 1 h. The preparations were dehydrated through a graded ethanol series and freeze dried (JFD-300; JEOL) followed by coating with osmium by an osmium plasma coater (POC-3; Meiwa Shoji Co., Osaka, Japan), then observed by a FE-SEM (JSM-6700F; JEOL) at an acceleration voltage of 5 kV. *Bar* represents 1 μm

nucleic acids were measured at 260 nm and calculated to be 183 μg ml^{-1}. There were no peaks around 260 nm in the control centrifugal supernatant. These results also suggest that *B. halodurans* C-125 cells were lysed by a novel mechanism of methyl-β-CD. This previously unknown phenomenon means that both methyl-β-CD and α-CD can be used as anti-bacillus reagents and/or lysis agents for bacilliae.

One of the physiological properties of methyl-β-CD that is known is the capacity for high-affinity binding and forming an inclusion complex with cholesterol molecules. However, there are no cholesterols or cholesterol-like molecules in the cell membrane of prokaryotes including *Bacillus* and related strains. We analyzed total lipids in extracts from methyl-β-CD-treated or untreated cell membranes of *B. halodurans* C-125. The concentrations of unknown lipid-like components with molecular masses of 650–690 Da, which were determined by electrospray ionization mass spectrometry, decreased with increasing concentration of methyl-β-CD, and finally disappeared when treated with 40 mM methyl-β-CD (data not shown). On the other hand, a flotillin homologue encoded by BH3500 of *B. halodurans* C-125 showed high similarity to those of *Bacillus* sp. SG-1 (ZP_01859093), *B. clausii* KSM-K16 (YP_177441), *Geobacillus thermodenitrificans* NG80-2 (YP_001124425), and *B. pumilus* SAFR-032 (YP_001487947) with 40% to 68% identities. Furthermore, many genes for flotillin-like proteins have been found by genome sequencing of *Bacillus* strains such as *B. subtilis* (Kunst et al. 1997), *B. cereus* and *B. anthracis* (Ivanova et al. 2003), and *Oceanobacillus iheyensis* (Takami et al. 2002). This suggests that strains possessing the flotillin-like proteins are affected by methyl-β-CD or α-CD. This hypothesis is supported by the findings that the gene encoding foltilin-like protein in *B. halodurans* C-125 was strongly expressed in alkaline cultures, and the strains grown at pH 10 were more sensitive to methyl-β-CD or α-CD than cells grown at neutral pH. It is supposed that the flotillin-like proteins form a complex with unknown lipid molecules present in the cell membranes of the strains possessing flotillin-like protein, and that the complex must be very important in the construction and stabilization of their structure. Unfortunately, it is not evident why methyl-β-CD and α-CD show a strong lysis activity against *Bacillus* strains at lower concentration among tested CDs. Identification of the unknown lipid molecules trapped by methyl-β-CD or α-CD would elucidate the mechanism of higher lysis activity of both CDs, and it is our ongoing focus.

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