Support Information for Tetrabromobisphenol A (TBBPA) Exhibits Specific Antimicrobial Activity against Gram-positive Bacteria without Detectable Resistance

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Methods:

Materials: Bacterial strains used in this study are listed in Table 1. The strains were obtained from State Key Laboratory of Agricultural Microbiology, Huazhong Agriculture University and the State Key Laboratory of Crop Biology, Shandong Agricultural University. The strains were cultured under normal conditions described previously \cite{1}. The chemicals used in this study are as follows: TBBPA, 2,2-bis-(4-hydroxyphenyl) propane (BPA) and triclosan were obtained from Alfa Aesar Company; 2,2-bis-(3,5-dichloro-4-hydroxyphenyl)propane (TCBPA) from Tokyo Chemical Industry; 2,2',6,6'-tetrabromobisphenol A diallyl ether (TBBPADE) and 4,4'-isopropylidenebis [2-(2,6-dibromophenoxy) ethanol] (TBBPABE) from Sigma-Aldrich; 2,2-bis-(3,5-dibromo-4-hydroxyphenyl)sulfone (TBBPS) from Beijing Apis Biotechnology; Stock solutions of 10 mg/ml of these chemicals were prepared in dimethyl sulfoxide (DMSO). The antibiotics used in this paper are ampicillin and tetracycline that were purchased from Biosharp Company.

Susceptibility test. The minimum inhibitory concentrations (MICs) were determined with the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines. The organisms in the exponential growth phase were diluted to a final inoculum of $1 \times 10^5$ to $5 \times 10^5$ CFU/ml. The MIC was defined as the lowest concentration of a compound that completely inhibited bacterial growth \cite{2}. Compounds were taken from a stock solution of 20 mg/ml in 100% dimethyl sulfoxide (DMSO).

Preparation of bacterial samples for SEM and TEM. When the OD$_{600}$ of the culture of B. subtilis reached approximately 0.6-0.8, TBBPA was added to a final concentration of 1×MIC to the case, or equal amount of DMSO was added to the control. One hour later the cultures were centrifuged and prepared for scanning electron microscopy (SEM) and transmission electron
microscopy (TEM). SEM and TEM was performed following the method described by Wang et al\textsuperscript{[1]} and Zhu et al\textsuperscript{[3]}, respectively.

**Microarray and data analysis.** When the OD\textsubscript{600} of *B. subtilis* culture reached approximately 0.6-0.8, the cultures were challenged with 16 μg/ml of TBBPA or equal amount of DMSO (control). The cells were harvested 1 hour later and cell pellets were directly snap-frozen in dry ice. We then submitted the samples to KangChen Biotech (Shanghai, China) for an mRNA microarray assay. Sample processing including labelling, hybridization, scanning, and normalization, and data analysis was performed by KangChen Biotech (Supplementary file 2).

**RNA isolation and reverse transcription.** *B. subtilis* cells were grown till the OD\textsubscript{600} reached approximately 0.6-0.8. Each compound (TBBPA, TCBPA, BPA, TBBPADE, TBBPABE and TBBPS) at different concentrations or DMSO (control) were added respectively. The cells were harvested 1 hour later and the RNA was isolated according to the Bacterial RNA Kit (Omega Bio-tek) then was reverse transcribed with M-MLV reverse transcriptase (High Capacity cDNA Reverse Transcription kit; Promega, UK Ltd.)

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR).** The reaction was performed on cDNA generated from RNA samples (each experiment was performed at least three times). Three genes of *dlt* operon identified by microarray analysis as up-regulated by TBBPA, *dltA* was chosen to be tested by qRT-PCR with *rpsJ* gene as the internal control. The used primers were listed in Table S1. qRT-PCR was run using the SYBR Green Realtime PCR Master Mix (TOYOBO CO., LTD.) according to the manufacturer’s procedure. The amplification reaction was carried out using the following program: 95°C for 10 min, 55 cycles of (95°C for 30 s, 57°C for 30 s), and 72°C for 30 s. Expression of the *dlt* and autolysin genes was calculated as fold
changes using the following formula: fold change = $2^{-\Delta\Delta Ct}$

$$-\Delta\Delta Ct = (Ct_{\text{gene}} - Ct_{\text{rpsJ}})_{\text{condition I}} - (Ct_{\text{gene}} - Ct_{\text{rpsJ}})_{\text{condition II}}$$

81 **Background information on cell wall synthesis and degradation in Gram-positive bacteria.**

82 A significant difference between Gram-positive and Gram-negative bacteria is the presence of teichoic acids (TAs) specifically in Gram-positive bacteria cell wall. The TAs are closely related to the synthesis and degradation of cell wall, which is regulated by the *dlt* operon and autolysins.

85 The *dlt* operon is responsible for adding positively charged D-alanine via esterification to teichoic acids (TAs), as DltA ligates D-alanines in the cytoplasm of the cell and the ligated form is then transported by the carrier protein DltC\[^5\]. The TAs, including wall teichoic acid (WTA) and lipoteichoic acid (LTA), are a major structural component specifically present in the gram-positive cell wall. WTA is covalently linked to the peptidoglycan, while LTA is amphiphilic with its glycolipid being anchored in the membrane and its poly-glycerophosphate (Gro-P) chain extending into the wall. Together with peptidoglycan, WTA and LTA construct the network or matrix that provides functions relating to the elasticity, porosity, tensile strength, and electrostatic steering of the cell wall envelope\[^6\]. Protonated D-alanyl ester residues, one of the principal substituents of TAs in many low-G+C gram-positive bacteria, are covalently linked to these chains and provide counter ions for determining the net negative charge of the TA. The bacterial cell wall is a dynamic structure that undergoes coordinated synthesis and degradation during cell growth and division. The degradative phase, referred to as “autolysis”, is mediated by autolysins \[^7\]. Negatively charged D-alanyl-TAs provide binding sites for cationic autolysins by electrostatic interaction, therefore, D-alanyl-TAs play an important role in modulating the activities of autolysins by means of changing the amount of binding sites\[^8\].
Table S1. Oligonucleotide primers used for qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Primer target</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dltA</em> F</td>
<td>GGGACTTTACATTGCGTGAC</td>
</tr>
<tr>
<td>R</td>
<td>CACAAAGGAAGGTTTGATG</td>
</tr>
<tr>
<td><em>rpsJ</em> F</td>
<td>CGGTTACGAAGCAATCCAG</td>
</tr>
<tr>
<td>R</td>
<td>ATACGCATCCATTCCACT</td>
</tr>
<tr>
<td><em>yocH</em> F</td>
<td>AAATAAGAACCCGAACGGA</td>
</tr>
<tr>
<td>R</td>
<td>GCCCAATTAGAAGCATCAC</td>
</tr>
<tr>
<td><em>cwlO</em> F</td>
<td>CAATCGAGGTGCTTCCCCA</td>
</tr>
<tr>
<td>R</td>
<td>TCGCAGAGGATAAAGTCA</td>
</tr>
</tbody>
</table>

*a* Based on the annotation of *B. subtilis* 168 genome.

*b* Forward (F) and reverse (R) primers.

dltA: D-alanine--poly(phosphoribitol) ligase subunit 1  
rpsJ: 30S ribosomal protein S10  
yocH: cell wall-binding protein  
cwlO: peptidoglycan D,L-endopeptidase
Figure S1. Effects of TBBPA on *yocH* and *cwlO* mRNA. Bs168 was grown to an OD$_{600}$ of 0.6-0.8 and incubated for 90 min with the addition of different concentrations of TBBPA. Mean values from three different experiments are shown. The error bars indicate ±standard deviations. The results shown are representative of experiments performed at least three times.
Figure S2: The schematic diagram of the proposed processes involved in cell wall damage caused by TBBPA. ATO:Alditol; LTA: lipid teichoic acid; WTA: wall teichoic acid.
References


