Antibacterial mechanisms of *Orostachys cartilaginous* cell cultures: effect on cell permeability and respiratory metabolism of *Bacillus subtilis*

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Abstract

*Orostachys cartilaginous* of Crassulaceae family is a plant native to the Changbai Mountain area, China. Although *O. cartilaginous* has various medicinal values, its product development and production are restricted by the insufficient resource available. *O. cartilaginous* cell cultures possess an efficient antibacterial effect against *Bacillus subtilis*, but the underlying mechanism is not clear yet. Therefore, this study investigated the effects of extract from bioreactor cultured *O. cartilaginous* cells (OE) on *B. subtilis* cell permeability and respiratory metabolism to provide a reference for the further utilization of *O. cartilaginous* cell cultures. Results showed alkaline phosphatase activity, electrical conductivity, nucleic acid and protein contents in *B. subtilis* suspensions were significantly increased (**p** < 0.01) by OE treatment, indicating the occurrence of cell damage or increase in cell permeability. OE inhibited *B. subtilis* respiration, and the combination groups of OE + iodoacetic acid (IA) and OE + sodium phosphate (SP) showed low superposition rates (approximately 35%), revealing that OE likely affected IA- and SP-represented metabolic pathways. The activities of *B. subtilis* enzymes, specifically, hexokinase and pyruvate kinase in the embden-meyerhof-parnas (EMP) pathway and glucose-6-phosphate dehydrogenase in the hexose monophosphate (HMP) pathway, decreased after OE treatment. This result proved that OE inhibited *B. subtilis* respiration by regulating the EMP and HMP pathways.

Key message

- Extract from *Orostachys cartilaginous* cell cultures inhibited *Bacillus subtilis* growth.
- Bacterial cell contents leaked out after extract treatment.
- Extract inhibited bacterial respiration by regulating embden-meyerhof and hexose monophosphate pathways.

Keywords  Cell cultures · *Bacillus subtilis* · Leakage of bacterial cell contents · Inhibition of respiration

Introduction

*Orostachys cartilaginous* A. Bor is a species of the *Orostachys* genus, which belongs to the Crassulaceae family (Tian et al. 2019). It is mainly distributed in Changbai Mountain area, China, and the whole plants of this species have various pharmaceutical effects, such as anti-inflammation, antifebrile, hemostatic, antidote, and anticancer effects because they contain polysaccharides, phenolics, flavonoids, steroids, and triterpenoids (Park et al. 1991). However, the raw materials of *O. cartilaginous* plants are insufficient due to the excessive collection of their wild resources and the
incomplete system of artificial cultivation; this situation critically affects production and development of their products (Piao et al. 2017). Cell culture technology is an alternative method for producing plant materials for species that cannot provide materials via the traditional route (Effertth 2019). Therefore, researchers have attempted to establish cell culture systems, and cells of various species, including O. cartilaginous, have been successfully produced in large-scale bioreactors (Zhang et al. 2017; Tian et al. 2019). Nevertheless, to date, cultured cells of O. cartilaginous have been rarely applied because of various reasons, including the lack of information on the various bioactive effects.

Plant bacteriostatic substances have strong antibacterial effects with a wide spectrum, high safety, and low toxicity and side effects (Naz et al. 2015). Studies have indicated that bacteriostatic substances affect bacterial cell walls and membranes (Cui et al. 2018; Dong et al. 2020; Hu et al. 2019). The cell wall, as the outermost layer of microbial cells, can block macromolecular substances from entering the cell and resist osmotic pressure. The cell membrane is the basis of the maintenance of cell integrity and the normal metabolism of substances and energy (Gao et al. 2020). The action of a variety of plant bacteriostatic agents on the cell wall and membrane system results in the destruction of the integrity of the membrane structure, thereby affecting cell metabolism and changing cell permeability (Song et al. 2020). The main pathways for respiratory metabolism include the tricarboxylic acid (TCA) cycle; embden-meyerhof-parnas (EMP) pathway; and the hexose monophosphate (HMP) pathway, which is essentially the oxidative metabolic pathway of intracellular sugars (Bajpai et al. 2013; Li et al. 2016; Oliveira et al. 2015). Once respiratory metabolism is inhibited, cells cannot obtain the energy required for basic life needs; this effect leads to cell death indirectly (Liu et al. 2020). The effects of plant bacteriostatic substances from various species on bacterial permeability and respiratory metabolism have been investigated to clarify their antibacterial mechanism (Hu et al. 2019).

Bacillus subtilis is a kind of conditional pathogenic bacteria (Masengu et al. 2014). When the resistance of an organism is low, the pathogenic B. subtilis can cause several diseases, such as endophthalmitis, adult sepsis and pharyngeal abscess (Luo et al. 2016; Richard et al. 1988; Oggioni et al. 1998). At present, the abuse of chemical antibiotics, the increase of bacterial resistance, and the emergence of multi-drug resistant bacteria have become the common problems in the practical treatment (Li et al. 2020). Therefore, finding safe and efficient natural substances has become an urgent need. Our previous study found that O. cartilaginous cell cultures can efficiently inhibit B. subtilis growth, but its underlying mechanism has yet unclear. Therefore, this study investigated effects of extract from bioreactor cultured O. cartilaginous cells (OE) on cell permeability and respiratory metabolism of B. subtilis to clarify the antibacterial mechanism, and provided a reference for the disease treatment through regulating the key factors or enzymes.

Materials and methods

Plant materials and extract preparation

Orostachys cartilaginous cells were produced by using the method of Piao et al. (2017). Approximately 15 g/L fresh cells were inoculated into a 5 L airlift balloon-type bioreactor containing 4 L of Murashige and Skoog (1962) medium with 3.5 mg/L benzylaminopurine, 0.1 mg/L α-naphthalene acetic acid and 30 g/L sucrose. The pH of the medium was adjusted to 5.8 prior to autoclaving. The bioreactor was aerated at the flow rate of 400 mL/min air and maintained at 25 °C under 30 µmol/m²/s light intensity with a 16 h photoperiod. Cells were harvested after 25 days of bioreactor culture. The cells were washed three times with tap water and placed in a drying oven (50 °C) for 48 h.

The dried cells were soaked in 70 % (v/v) ethanol (solid:liquid ratio = 1:15) with condensation reflux for 1 h at 60 °C. Then, the filtrate was collected after vacuum filtration. The residue was repeatedly extracted twice in accordance with the above conditions. All filtrates were combined together and concentrated by using a rotary evaporator (R201DII, Shanghai Shenshun Biological Technology Co., Ltd., Shanghai, China). Afterward, the concentrated extract was freeze–dried (Beijing Boyi Kang Experimental Instrument Co., Ltd., Beijing, China). The dried extract was dissolved and diluted in deionized water, and used in the experiments.

Bacterial culture

The bacterial strain of pathogenic B. subtilis (CGMCC 1.3358) (China General Microbiological Culture Collection Center, Beijing, China) was uniformly streaked on beef peptone (BP) solid medium (3 g/L beef extract + 10 g/L peptone + 5 g/L sodium chloride + 20 g/L agar, pH 7.0–7.2). After 12 h of incubation at 37 °C, the bacterium was transferred to liquid BP medium, and incubated for 12 h on a shaker (Changzhou JT LiangYou Instrument Co., Ltd, Changzhou, China) with continuous shaking (100 rpm) at 37 °C, and used in the experiments.
**Determination of electrical conductivity value, alkaline phosphatase activity, and nucleic acid and protein contents**

Electronic conductivity (EC) value, and alkaline phosphatase (AKP) activity, nucleic acid and protein contents were determined by using the method of Jin et al. (2020). *B. subtilis* suspension (approximately 1×10^8 CFU/mL) was centrifuged for 4 min at 5000 rpm (CR22G, Hitachi Koki Co. Ltd., Tokyo, Japan), and the precipitated bacterial cells were collected. After adequately rinsing with phosphate-buffered saline (PBS, pH 7.4), *B. subtilis* cells in the OE group were added with 10 mM PBS and MIC of OE (8 mg/mL) that was previously determined by Tian et al. (2019), and those in the control group were added with same volume of BP liquid medium. After 2 h of incubation (130 rpm) at 37 °C, the EC value was measured by using an EC measurement instrument (DDSJ-308 A, INESA Scientific Instrument Co., Ltd., Shanghai, China).

A total of 2 mL of *B. subtilis* suspension (approximately 1×10^8 CFU/mL) was inoculated into 100 mL of liquid BP medium, and OE was added with its MIC (8 mg/mL). The same volume of the liquid BP medium was added to the control group. After 2 h of incubation (130 rpm) at 37 °C, the mixture was centrifuged for 5 min at 5,000 rpm, and the supernatant was collected to determine AKP activity and nucleic acid and protein contents. Nucleic acid content was determined at an optical density (OD) of 260 nm (UV-2600, Shimadzu Corporation, Kyoto, Japan) according to Jin et al. (2020). The AKP activity and protein content were determined by using an AKP assay kits (Nanjing JianCheng Bioengineering Institute, Nanjing, China) and Coomassie Brilliant Blue (CBB) (Nanjing JianCheng Bioengineering Institute, Nanjing, China) and Coomassie Blue (CBB) (Nanjing JianCheng Bioengineering Institute), respectively, in accordance with the manufacturer’s instructions. The values of AKP and the protein were measured at 520 (iMark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 595 nm (UV-2600, Shimadzu Corporation), respectively.

The intracellular protein content of *B. subtilis* was measured by applying the methods for bicinchoninic acid (BCA) assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis described by Jin et al. (2020). In brief, 1 mL of *B. subtilis* suspension (approximately 1×10^8 CFU/mL) was inoculated into 100 mL of liquid BP medium and treated with OE at 1/2MIC (4 mg/mL) or 0.5 mg/mL typical respiratory inhibitors of iodoacetate acid (IA), malonate acid (MA), and sodium phosphate (SP), or a combination of both (i.e. OE + IA, OE + MA, and OE + SP). Each mixture was exposed to air for 5 min, and the DO value was measured at 1 min intervals within 10 min by using a DO meter (Shanghai ZhenMai Instruments Co., Ltd., Shanghai, China). The difference in DO values per min was calculated as the respiratory rate.

The inhibitory rate of respiration (IR) against *B. subtilis* and superposition rate (SR) was determined by measuring dissolved oxygen (DO) (Jin et al. 2020). In brief, 2 mL of *B. subtilis* suspension (1×10^8 CFU/mL) was mixed with 10 mL of PBS (pH 7.4) and then, OE with 1/2MIC (4 mg/mL) or 0.5 mg/mL typical respiratory inhibitors of iodoacetate acid (IA), malonate acid (MA), and sodium phosphate (SP), or a combination of both (i.e. OE + IA, OE + MA, and OE + SP). Each mixture was exposed to air for 5 min, and the DO value was measured at 1 min intervals within 10 min by using a DO meter (Shanghai ZhenMai Instruments Co., Ltd., Shanghai, China). The difference in DO values per min was calculated as the respiratory rate.

IR and SR were calculated by using the following equations (Cui et al. 2018).

\[
IR(\%) = \frac{R_0 - R_1}{R_0} \times 100
\]

\[
SR(\%) = \frac{R_1 - R_2}{R_1} \times 100
\]

\( R_0 \) is the respiratory rate of the control, \( R_1 \) is the respiratory rate of OE or each respiration inhibitor, and \( R_2 \) is the respiratory rate of the combination of OE with the respiration inhibitors, and the \( SR \) of OE with different typical respiratory inhibitors (IA, MA, and SP).

**Determination of enzyme activities**

The activities of key enzymes in the EMP pathway (i.e. hexokinase [HK], phosphofructokinase [PFK], pyruvate kinase [PK]), and that in HMP pathway (i.e. glucose-6-phosphate dehydrogenase [G6PD]) were determined by using the
relevant assay kits in accordance with the manufacturer’s instructions. HK, PFK, and PK assay kits were purchased from Nanning Jiancheng Bioengineering Institute (Nanjing, China), and G6PD assay kit was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). A total of 1 mL of *B. subtilis* suspension (1×10^8 CFU/mL) was mixed with 1 mL of OE with 1/2MIC (4 mg/mL) in the OE groups. The control group was added with the same volume of liquid BP medium. The mixture was incubated (130 rpm) at 37 °C for 2 h and centrifuged for 4 min at 5,000 rpm. The supernatant was used to determine enzyme activities at OD of 340 nm (UV-2600, Shimadzu Corporation).

### Statistical analysis

The results were expressed as the mean ± standard error of six replicates. Statistical analysis was performed via ANOVA followed by Student *t*-test by using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). *p* < 0.05 was considered significant.

### Results

#### Effect of OE on cell permeability of *B. subtilis*

AKP generally locates between bacterial cell membranes and cell walls, and it cannot be identified in a bacterial suspension. Figure 1 shows that AKP activity in *B. subtilis* suspension significantly increased (*p* < 0.01) after 2 h of OE treatment, but did not change in the OE-untreated control group, indicating that the cell walls of *B. subtilis* were probably destroyed by OE treatment, leading the release of the AKP.

The leakage of cell contents was also found in the present study. Figure 2A shows that the EC value of *B. subtilis* suspension was remarkably (*p* < 0.01) elevated in the OE treatment group, but that in the control group did not increase from 0 to 2 h of incubation. The values of

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*Fig. 1:* Effect of extract from *Orostachys cartilaginous* cell cultures (OE) on alkaline phosphatase activity of *B. subtilis* suspension. Data are the mean ± standard error (n = 6). **indicates that the values were considered significant at *p* < 0.01 versus the control group.

*Fig. 2:* Effect of extract from *Orostachys cartilaginous* cell cultures (OE) on electronic conductivity A and nucleic acid content B in *B. subtilis* suspension. Data are the mean ± standard error (n = 6). **indicates that the values were considered significant at *p* < 0.01 versus the control group.

*Fig. 3:* Effect of extract from *Orostachys cartilaginous* cell cultures (OE) on protein leakage. A, protein content in *B. subtilis* suspension. B, protein content in *B. subtilis* cells. C, gel electrophoresis image of proteins in *B. subtilis* cells. Data are the mean ± standard error (n = 6). **indicates that the values were considered significant at *p* < 0.01 versus the control group.

*Fig. 4:* Effect of extract from *Orostachys cartilaginous* cell cultures (OE) on respiratory metabolism of *B. subtilis*. A, respiration inhibitory rate. B, superposition rate. Data are the mean ± standard error (n = 6). iodoacetate acid (IA), malonate acid (MA), and sodium phosphate (SP) are typical inhibitors of the respiratory metabolic pathways.
OD$_{260}$ nm in *B. subtilis* suspension significantly ($p < 0.01$) increased after OE treatment (Fig. 2B), indicating a leakage of the nucleic acid from *B. subtilis* cells. Furthermore, extracellular and intracellular protein contents were detected to investigate the protein leakage. The high protein contents of *B. subtilis* suspension increased after 2 h of OE treatment, and was obviously higher ($p < 0.01$) than that of the control (Fig. 3A), indicating protein leakage. Intracellular protein content was also identified through BCA assay and SDS-PAGE analysis. The BCA assay showed that OE treatment significantly decreased ($p < 0.01$) the intracellular protein content (Fig. 3B). SDS-PAGE analysis revealed similar result (Fig. 3C) and showed that the protein bands of the OE group were lighter than those of the control group. These results indicated that residual cellular protein contents decreased in correspondence with the increase in extracellular protein content.

**Effect of OE on respiratory metabolism of *B. subtilis***

MA, IA, and SP are the typical respiratory inhibitors in TCA cycle, EMP, and HMP pathways, respectively. To clarify whether the OE also has the inhibitory effect on bacterial respiration, the *I*$_R$ values of OE, MA, IA, and SP were measured. Figure 4A shows that the inhibitory respiratory degree of OE against *B. subtilis* was not entirely consistent with the three typical inhibitors but *I*$_R$ of OE reached over 40%, indicating an inhibitory effect of OE. Therefore, the $S_R$ value (namely, $I_R$ value of the combination of a typical respiratory inhibitor with OE) was further determined to identify the respiratory metabolic pathways that was regulated by OE. Figure 4B shows that the $S_R$ of OE + IA and OE + SP were lower than those of OE + MA, revealing that OE likely regulated the IA- or SP-represented respiratory metabolic pathways. Consequently, the key enzymes in the EMP (i.e. HK, PFK, and PK) and HMP (i.e. G6PD) were identified to prove the above result. After 2 h of OE treatment, HK and PK activities significantly decreased ($p < 0.01$) but PFK activity did not show an obvious change (Fig. 5). In addition, a significant reduction in G6PD activity was also observed under OE treatment for 2 h (Fig. 6).

**Discussion**

The rapid development of plant cell culture technology offers the possibility of acquiring sufficient materials from rare and precious plant species. For nearly 20 years, cell culture protocols have been extensively studied to improve culture systems. However, little is known regarding the bioactivity of cultured cells, thus delaying cell application.
Previous studies have indicated that *O. cartilaginous* cell cultures contain many bioactive compounds such as phenolics and polysaccharides (Piao et al. 2017; Zhang et al. 2017) that are known as antibacterial substances. The antibacterial effects of the field-grown plants of *Orostachys* species have been investigated by Ding and Wang (2013), and Ren et al. (2011). Furthermore, the inhibitory effect of *O. cartilaginous* cultured cells against *B. subtilis* has also been reported in our previous study (Tian et al. 2019). However, the antibacterial mechanisms of *Orostachys* species including field-grown plants and cultured cells have not been investigated to date.

In recent years, drug-resistant bacterial strains are increasing and the pathogenic bacteria are constantly changing because of the abuse of antibiotics. Therefore, many bacterial strains, previously considered to be non-pathogenic or conditionally pathogenic, may also cause disease (Yassin et al. 2012). *B. subtilis* is gram positive bacteria and is generally thought to be beneficial for human (Seo et al. 2011). However, clinical studies have indicated that *B. subtilis* can cause endophthalmitis, sepsis and postoperative infection disease when body’s immunity is low. In particular, the eye disease caused by *B. subtilis* or other *Bacillus* species accounts for about 40% of endophthalmitis (Vahey and Flynn 1991). Therefore, the pathogenicity of *B. subtilis* should be paid attention to avoid missed diagnosis and delayed treatment (Barth et al. 1973).

Extracts from several plant species exhibit the antibacterial effect on *B. subtilis* (Lalitha et al. 2021; Umamaheswaril and Murthy 2012; Yan et al. 2014). The present study also found that OE inhibited *B. subtilis* growth, indicating a potential application of *O. cartilaginous* cell cultures. Furthermore, the present study found that OE increased AKP activity in *B. subtilis* suspension (Fig. 1), and the levels of EC, nucleic acid, and protein in *B. subtilis* suspensions increased after OE treatment (Figs. 2, 3), demonstrating that bacterial cells were destroyed or cell permeability was increased by OE treatment. At present, many researchers have conducted the studies of antibacterial mechanisms in terms of cell permeability change. For example, Liu et al. (2017) elucidated that baicalin exerts its antibacterial effect by enhancing the cell permeability of *Escherichia coli*, resulting in the extensive leakage of cell contents. Li et al. (2019) indicated that a chitosan-gallic acid derivative disrupts the cell membranes of *E. coli* and *Staphylococcus aureus*, resulting in the cytoplasm leakage and EC increase. However, antibacterial mechanisms of *Orostachys* species have not been reported to date. The result of the present study implied that *B. subtilis* cell walls were destroyed and cell membrane permeability was enhanced by OE treatment, leading the cell contents (including electrolyte, nucleic acid, and protein) leaked out, which subsequently resulted in inhibition of *B. subtilis* growth or death.

In addition to the cell permeability change, antibacterial mechanisms based on respiration inhibition have also been...
widely studied. MA, IA, and SP are the typical inhibitors in TCA cycle, EMP, and HMP pathways, respectively. Studies often combine a typical inhibitor with the tested substance and determine the $R_I$ value of the combination (i.e. $S_R$) to identify the respiratory metabolism pathway that is regulated by the substance (Jin et al. 2020; Zheng et al. 2015). A low $S_R$ indicates that substance has a high probability of affecting a typical inhibitor-represented pathway (Tong et al. 2005). Studies on antibacterial mechanisms in respiratory metabolism have showed that different antibacterial substances are involved in various pathways (Cui et al. 2018, 2019; Hu et al. 2019). The present study found that OE inhibited $B. subtilis$ respiration (Fig. 4A), and the groups of OE + MA and OE + SP showed low $S_R$ (Fig. 4B). These results implied that OE was involved in the EMP and HMP pathways. HK, PFK, and PK are the key enzymes in the EMP pathway (Fig. 5). OE treatment decreased the activities of HK and PK in the present study but did not significantly affect PFK activity, proving that OE participated in the EMP pathway by regulating HK and PK activities. Furthermore, G6PD is the key enzyme of the HMP pathway (Fig. 6), and its activity was decreased by OE treatment. This result therefore indicated that OE also participated in the HMP pathway by regulating G6PD activity. This involvement indicated that $B. subtilis$ respiration was inhibited through an inhibition of key enzymes related to respiratory metabolic pathways.

Conclusions

OE treatment increased AKP activity, EC value, and nucleic acid and protein contents in $B. subtilis$ suspension, indicating the cell damage or increment in cell permeability. $B. subtilis$ respiration was inhibited by OE treatment, and low $S_R$ was found in the combination groups of OE + IA and OE + SP. The HK, PK, and G6PD activities of $B. subtilis$ decreased after OE treatment, revealing that OE inhibited $B. subtilis$ respiration by regulating the EMP and HMP pathways.

Author contributions YXL conducted experiment of cell permeability. XLI conducted experiment of respiratory metabolite. YNX cultured bacterial strain. XCP designed the experiments. MLL wrote the paper.

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Data availability Data are available by e-mail on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication The authors declare that they consent for publication.

Consent to participate The authors declare that they consent to participate.

Ethical approval Not applicable.

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