Identification of methyl 4-hydroxybenzoate as a natural anti-\textit{Helicobacter pylori} agent from the culture broth of \textit{Bacillus subtilis} A81

\textbf{Accepted 13th February, 2018}

\textbf{ABSTRACT}

Novel bacilli producing anti-\textit{Helicobacter pylori} compounds were screened from natural sources, and a new strain, \textit{Bacillus subtilis} A81, was found to have strong anti-\textit{Helicobacter pylori} activity. To determine the molecular structure of the anti-\textit{Helicobacter pylori} compound(s) in \textit{Bacillus subtilis} A81 cultures, we fractionated the culture supernatant containing activities with solvent extraction and silica gel chromatography. By analyzing with $^1$H and $^{13}$C nuclear magnetic resonance analyses, one compound was identified to be methyl 4-hydroxybenzoate (methylparaben). \textit{Helicobacter pylori} was found to be relatively more susceptible (10-fold or more) to methylparaben than other bacteria, \textit{Staphylococcus aureus} and \textit{Escherichia coli}. To our knowledge, this might be the first report describing methylparaben as a natural anti-\textit{Helicobacter pylori} product derived from \textit{B. subtilis}.

\textbf{Keywords:} anti-\textit{Helicobacter pylori} activity, \textit{Bacillus subtilis}, methyl 4-hydroxybenzoate, methylparaben, nuclear magnetic resonance, silica gel column chromatography.

\textbf{INTRODUCTION}

\textit{Helicobacter pylori} is a pathogenic bacterium infecting the stomach, an organism that causes chronic stomatitis and recently is thought to be the cause of almost all stomach cancer cases (Peek and Blaser, 2002). Therefore, it is crucial to eradicate \textit{H. pylori} to keep the stomach healthy. Standard therapy for \textit{H. pylori} infection called triple therapy includes two antibiotics and one proton pump inhibitor (PPI) (Malferttheiner et al., 2007). Although this triple therapy is promising, the development of resistance to antibiotics has become problematic, reducing successful eradication of \textit{H. pylori}. To address such a resistance problem, novel types of antibiotics and/or PPIs have been proposed to replace the traditional ones (Nishizawa et al., 2015; Suzuki et al., 2016). However, other novel types of anti-\textit{H. pylori} agents are also now in demand.

In Japan, several \textit{B. subtilis} strains (\textit{Bacillus subtilis} var. natto) have been historically used for fermentation of the traditional soy food “Natto”, and Sumi (2006, 2008) reported that ethanol extracts of some strains of \textit{B. subtilis} var. natto have anti-\textit{H. pylori} activity. Moreover, other strains of \textit{Bacillus} sp. were also reported to produce anti-\textit{H. pylori} compounds, for example, amicoumarin A (Pinchuk et al., 2001) and pyloricidin (Nagano et al., 2001; Nakao et al., 2001). Therefore, \textit{Bacillus} sp. can be thought as a possible source of novel anti-\textit{H. pylori} agents. In the present study, we searched for novel \textit{Bacillus} sp. strains producing anti-\textit{H. pylori} compounds.

\textbf{MATERIALS AND METHODS}

\textbf{Isolation of bacilli from natural sources}

To isolate bacilli from natural sources, we washed rice straw, wheat straw and withered grasses with phosphate-buffered saline (PBS) and then heat-treated the washing solutions at 65°C for 10 min. Thereafter, the heat-treated solutions were spread on nutrient agar plates and incubated at 37°C for 48 h. A total of 16 off-white colonies with rough surfaces were isolated.
Evaluation of anti-\textit{H. pylori} activity \textit{in vitro}

Anti-\textit{H. pylori} activities of isolated probale bacilli earlier mentioned were screened by mixing these bacilli separately with \textit{H. pylori} No.130 (1\times10^6 cfu/ml) in Brain Heart Infusion (BHI) broth containing 5\% horse serum and then incubating the mixtures at 37°C for 24 h under micro-aerobic conditions according to the method previously described (Aiba et al., 2015), where viable number of \textit{H. pylori} was counted after culturing for 4 days at 37°C on BHI agar containing 10\% horse serum and antibiotics, 25 µg/ml tetrazolium violet, 5 µg/ml trimethoprim, 2.5 units/ml polymyxin B, 10 µg/ml vancomycin, and 5 µg/ml bacitracin under micro-aerobic condition. Determination of minimum inhibitory concentrations (MICs) was performed by culturing \textit{H. pylori} on the agar plate as earlier mentioned containing various concentration of test compounds.

Anti-\textit{H. pylori} activity of each fraction of purification steps was also determined according to the method earlier mentioned. In this study, we defined “one unit” as the amount of activity in 1 ml necessary to reduce the viable number of \textit{H. pylori} to 1/100 of that in the control condition.

Evaluation of anti-\textit{H. pylori} activity \textit{in vivo}

Anti-\textit{H. pylori} activity of \textit{B. subtilis} A81 culture broth was examined \textit{in vivo} according to the method previously described (Aiba et al., 2017). In brief, \textit{B. subtilis} A81 was cultured in 0.5\% glucose, 1.0\% peptone, 1.0\% yeast extract and 0.5\% carboxymethylcellulose for 48h at 37°C and used for this study. 25 male germ-free mice (BALB/c, 4-weeks old) were orally administered with \textit{H. pylori} No. 130 (10^9 cfu/mice) once daily for three consecutive days. After 4 weeks, 5 mice were sacrificed and the viable numbers of \textit{H. pylori} in the stomach were determined to confirm successful infection of \textit{H. pylori}. Thereafter, 0.5 ml of \textit{B. subtilis} A81 culture broth (~5 \times10^6 cfu/ml; n=10) or the same volume of phosphate buffered saline (PBS; n=10) was orally administered to each mouse once daily for two weeks and finally the viable number of \textit{H. pylori} in the stomach was determined. In another experiment, \textit{B. subtilis} A81 culture broth was administered after autoclaved to know the heat-stability of the active components. This animal study was approved and carried out in accordance with the guidelines of Institutional Animal Care and Use Committee at Tokai University.

Purification and identification of anti-\textit{H. pylori} compounds from culture broth

To determine the molecular structure of the anti-\textit{H. pylori} compounds in \textit{B. subtilis} A81 cultures, we fractionated the activities with silica gel column chromatography. The molecular structures of the purified fractions were determined using Nuclear Magnetic Resonance (NMR) analysis performed in CDCl3 solution. Details of fractionation and NMR are further described in the results and discussion.

RESULTS AND DISCUSSION

Isolation of bacilli having anti-\textit{H. pylori} activity

Among sixteen (16) isolates of probable bacilli from natural sources earlier described, thirteen (13) of them had no effect on the viable number of \textit{H. pylori} in the mixing culture. However, the remaining three (3) isolates significantly reduced the number of \textit{H. pylori}, two of which reduced the microbial count to <1\times10^4 cfu/ml and another, to <1\times10^3 cfu/ml. The isolate having the strongest anti-\textit{H. pylori} activity was identified as \textit{B. subtilis} as determined according to Standard Methods of Analysis in Food Safety Regulation 2004 – Microbiology (Japan Food Hygiene Association), and confirmed by comparing the DNA sequence of its 16S ribosomal RNA gene with that of a type strain (\textit{B. subtilis} DSM10) and that of another reference strain (\textit{B. subtilis} CYBS-6); that is, the homologies were 99.9 and 100\%, respectively. We designated this bacillus strain as \textit{B. subtilis} A81.

Evaluation of anti-\textit{H. pylori} activities of \textit{B. subtilis} A81 both \textit{in vitro} and \textit{in vivo}

Although the aforementioned screening for anti-\textit{H. pylori} activity of bacilli was performed by mixing cultures of live bacilli and \textit{H. pylori}, we further examined the properties in detail and found the following: (1) The anti-\textit{H. pylori} activity survived heat-treatment by autoclaving (115°C, 20 min); (2) the culture supernatant of \textit{B. subtilis} A81 had anti-\textit{H. pylori} activity; (3) the lysate of \textit{B. subtilis} A81 made by sonication also had anti-\textit{H. pylori} activity; (4) anti-\textit{H. pylori} activity was efficiently extracted into ethyl acetate at either pH7 or 2 but not so efficiently at pH10.

Thereafter, anti-\textit{H. pylori} activity of \textit{B. subtilis} A81 culture broth was examined \textit{in vivo} according to the method as earlier described. Figure 1A shows that the culture broth of \textit{B. subtilis} A81 strongly reduced the number of viable number of \textit{H. pylori} in the stomach as compared to PBS group. Since the anti-\textit{H. pylori} activity of \textit{B. subtilis} A81 culture both survived autoclaving even in this \textit{in vivo} study (Figure 1B), some heat-stable soluble factor(s) might have been responsible for the anti-\textit{H. pylori} activity of \textit{B. subtilis} A81 not only \textit{in vitro} but also \textit{in vivo}.

Determination of the molecular structures of anti-\textit{H. pylori} compounds in \textit{B. subtilis} A81 culture

To determine the molecular structure of the anti-\textit{H. pylori} compounds in \textit{B. subtilis} A81 cultures, we fractionated the
activities (Figure 2). In summary, the culture supernatant of B. subtilis A81 was obtained after its cultivation in the bouillon broth (0.5% bonito extract, 0.5% glucose, 0.5% polypeptide, 0.5% carboxymethylcellulose, and 5% NaCl) for 48h at 37°C, and freeze-dried to afford 144 g of powder. (fraction-A). The resulting powder was extracted with ethyl acetate at pH2. The solvent of the extract was evaporated to afford about 790 mg of oily material (fraction-B). This oily material was dissolved in ethyl acetate, applied to a silica gel column and eluted with ethyl acetate: hexane = 1:10 to 1:5. The eluate was evaporated, dissolved in chloroform, applied to another silica gel column and eluted with chloroform. The eluate was evaporated to give rise to 2.7 mg of a thin film (fraction-C). The remaining material retained in the first silica gel column was eluted sequentially with acetone and methanol. These fractions were combined and evaporated to afford 356 mg of a brown-colored sticky solid (fraction-D).
<table>
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<tr>
<th>Fr.</th>
<th>Amount (mg)</th>
<th>IC99 (µg/ml)</th>
<th>Activity (Units/mg)</th>
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<th>Total activity (Units)</th>
<th>Recovery (%)</th>
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<td>16.1</td>
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<td>7.58</td>
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Figure 3: (A) Anti-\textit{Helicobacter pylori} activities of purified fractions. The concentration necessary to decrease by 99% the number of live \textit{Helicobacter pylori} (IC99) was determined and the inverse of IC99 was used to define activity unit. (B) Comparison of anti-\textit{Helicobacter pylori} activities of purified fraction-C and authentic methyl 4-hydroxybenzoate.

Figure 3A summarizes the purification steps with their activities and recovery rates. As shown in this figure, almost all of the anti-\textit{H. pylori} activity (96.0%) was extracted with ethyl acetate at pH2. The low polarity fraction-C contained only 0.377% of the total activity of supernatant (fraction-A), whereas the relatively high polarity fraction-D contained about 23.4% of that of the supernatant (fraction-A).

Since fraction-C appeared as a single spot as visualized by thin-layer chromatography (TLC), we determined its structure using NMR analysis performed in CDCl$_3$ solution. The $^1$H NMR spectral data revealed that fraction-C was a mixture of an aromatic compound and a saturated fatty acid in the molar ratio of 2:3. This aromatic compound showed $^1$H NMR signals for a para substituted benzene ring at 7.96 and 6.85 ppm with 2H doublet signal ($J = 8.7$ Hz). The presence of a methyl ester was confirmed by Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectral analyses. The methyl signal was observed at 3.88 (3H, s) ppm by $^1$H NMR and at 51.9 ppm by $^{13}$C NMR; this methyl signal and the low-field shifted 7.96 ppm signal showed long-range coupling correlations to the carbonyl carbon at 166.7 ppm in the HMBC spectrum. Other aromatic $^{13}$C signals were observed at 159.5, 131.9(2C), 122.9 and 115.1(2C) ppm. Based on these NMR data, the aromatic compound was identified to be methyl 4-hydroxybenzoate and the fatty acid, palmitic acid. Finally, we confirmed this identity by direct comparison of $^1$H and $^{13}$C NMR data to those of an authentic sample of methyl 4-hydroxybenzoate. Concerning fraction-D, we first tried to separate it by reverse-phase high-performance liquid chromatography (RP-HPLC). However, its anti-\textit{H. pylori} activity was widely distributed throughout the RP-HPLC chromatogram and identification by LC-MS/MS of active peak(s) on chromatogram was not successful to date.

Finally, we compared anti-\textit{H. pylori} activity of fraction-C with that of authentic methyl 4-hydroxybenzoate. As shown in Figure 3B, both “fraction-C” and “methyl
4-hydroxybenzoate” inhibited *H. pylori* with similar inhibition profiles. Furthermore, their spots on TLC coincided with each other (data not shown). These results confirmed that fraction-C was composed of mainly methyl 4-hydroxybenzoate.

**Methyl 4-hydroxybenzoate as natural anti-*H. pylori* agents**

In this study, we found that one of the active compounds in *B. subtilis* A81 culture supernatant was methyl 4-hydroxybenzoate. Parabens including methyl 4-hydroxybenzoate (methylparaben) are widely used as preservatives in cosmetics and pharmaceuticals. Anti-bacterial and fungal activities of methyl 4-hydroxybenzoate have been reported and its MICs against, for example, *Staphylococcus aureus, Escherichia coli, Aspergillus niger, Candida albicans, Saccharomyces cerevisiae* and *B. subtilis* are 4, 2, 1, 1, and 2 mg/ml, respectively (Aalto et al., 1953). Since the assay used to determine the MICs mentioned here was performed by culturing the bacteria on nutrient plates containing various concentrations of methyl 4-hydroxybenzoate, we determined the MIC of methyl 4-hydroxybenzoate against *H. pylori* using plate culture method. The resulting MIC was 0.128 mg/ml. For comparison, we also determined the MICs of methyl 4-hydroxybenzoate against other bacteria, that is, *S. aureus ATCC25923* and *E. coli ATCC25922*, the result being >1.024 mg/ml for both bacteria. Therefore, *H. pylori* can be thought to be relatively more susceptible (10-fold or more) to methyl 4-hydroxybenzoate than other bacteria.

Although parabens including methyl 4-hydroxybenzoate are manufactured by chemical synthesis to be mainly used as preservatives, some natural compounds with the 4-hydroxybenzoate structure have been reported. A marine bacterium of genus *Microbulbifer* (strain A4B-17) was reported to produce 4-hydroxybenzoate and 3 different alkyl esters of it (that is, butyl heptyl and nonyl esters) (Peng et al., 2006). In *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the two main pathogenic mycobacteria in humans, specific long chain β-diols have been reported to be important virulence cell-surface factors. In its biosynthesis, 4-hydroxybenzoate was found to play an important role as the intermediate structure to which mono or poly saccharide(s) and lipid structures are combined to become final cell-surface antigens (Constant et al., 2002; Staedhagen et al., 2005). Although some natural 4-hydroxybenzoate ester compounds have been reported, as earlier mentioned, to the best of our knowledge, this is the first report describing methyl 4-hydroxybenzoate as a natural product derived from *B. subtilis*.

Due to the fact that the amount of methyl 4-hydroxybenzoate produced by *B. subtilis* A81 is relatively small and its anti-*H. pylori* activity is relatively weak, it cannot be thought to be the main agent responsible for the anti-*H. pylori* activity of this bacterium. However, it might be possible that there exists other *B. subtilis* strains producing larger amounts of methyl 4-hydroxybenzoate.

**Conclusion**

Novel bacilli producing anti-*Helicobacter pylori* compounds were screened from natural sources and a new strain, *B. subtilis* A81, was found to have strong anti-*Helicobacter pylori* activity both in vitro and in vivo. To determine the molecular structure of the anti-*Helicobacter pylori* compounds in *B. subtilis* A81 cultures, we fractionated the activities with solvent extraction and silica gel chromatography. By analyzing with 1H and 13C nuclear resonance analyses, one compound was identified to be methyl 4-hydroxybenzoate. To our knowledge, this might be the first report describing methyl 4-hydroxybenzoate as a natural anti-*H. pylori* product derived from *B. subtilis*.

**ACKNOWLEDGEMENTS**

The authors are grateful to Kenji Takahashi and Akira Hasegawa for culturing *B. subtilis* A81 and preparing freeze-dried powder of its culture supernatant and also Dr. Takemichi Nakamura for performing LC-MS/MS analyses.

**Author Contribution**

YA. and T.H. equally contributed to this study.

**REFERENCES**


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