Identification of self-growth-inhibiting compounds lauric acid and 7-(Z)-tetradecenoic acid from Helicobacter pylori

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Helicobacter pylori growth medium is usually supplemented with horse serum (HS) or FCS. However, cyclodextrin derivatives or activated charcoal can replace serum. In this study, we purified self-growth-inhibiting (SGI) compounds from H. pylori growth medium. The compounds were recovered from porous resin, Diaion HP-20, which was added to the H. pylori growth medium instead of known supplements. These SGI compounds were also identified from 2,6-di-O-methyl-β-cyclodextrin, which was supplemented in a pleuropneumonia-like organisms broth. The growth-inhibiting compounds were identified as lauric acid (LA) and 7-(Z)-tetradecenoic acid [7-(Z)-TDA]. Although several fatty acids had been identified in H. pylori, these specific compounds were not previously found in this species. However, we confirmed that these fatty acids were universally present in the cultivation medium of the H. pylori strains examined in this study. A live/dead assay carried out without HS indicated that these compounds were bacteriostatic; however, no significant growth-inhibiting effect was observed against other tested bacterial species that constituted the indigenous bacterial flora. These findings suggested that LA and 7-(Z)-TDA might play important roles in the survival of H. pylori in human stomach epithelial cells.

INTRODUCTION

Helicobacter pylori is a well-known pathogen that causes gastritis, peptic ulcers and gastric cancer in humans (Marshall, 1994; Nomura et al., 1994; Forman, 1996; Goodwin et al., 1997). H. pylori was first cultured by Warren & Marshall (1983) and requires a complex medium containing supplements including horse serum (HS), FCS, 2,6-di-O-methyl-β-cyclodextrin (CD), activated charcoal or starch (Buck & Smith, 1987; Morgan, et al., 1987; Coudron & Stratton, 1995).

Olivieri et al. (1993) found that H. pylori grew better in medium supplemented with CD than in medium containing FCS. As both CD and activated charcoal have no nutrient value, the authors inferred that these supplements might eliminate toxic compounds contained in the medium or metabolic products of H. pylori (Taneera et al., 2002). Supporting this theory, activated charcoal supplemented into nutrient medium enhanced the growth of Legionella pneumophila (Edelstein & Edelstein, 1993). The porous resin Diaion HP-20 could also be used in place of activated charcoal to trap the self-growth-inhibiting (SGI) substance elemental sulfur, which is produced by Legionella pneumophila (Inoue et al., 2002). However, little is known about the properties of the growth inhibitors recovered from the supplements added to the growth medium of H. pylori.

CD mimics the mechanism of BSA, absorbing fatty acids to reduce their toxic effect (Olivieri et al., 1993). This protective mechanism of BSA was also described by Hazell & Graham (1990). The fatty acids showed antibacterial activity (Nienan, 1954; Kabara et al., 1972; Knapp & Melly, 1986), and several studies established that commercially available medium-chain fatty acids and monoglycerides could inhibit the growth of H. pylori (Petschow et al., 1996; Sun et al., 2003).
Identifying growth-inhibiting compounds produced by *H. pylori* may help in the development of treatments for *H. pylori* infection, as well as provide information on the specific niche of *H. pylori*. In this study, we isolated SGI compounds from *H. pylori*cells. The SGI compounds, recovered from the porous resin Diaion HP-20, were supplemented into *H. pylori* cultivation medium, where they showed *H. pylori*-specific growth inhibition. Therefore, the SGI compounds identified here might be useful in the treatment of *H. pylori* infections.

**METHODS**

**Bacteria and growth conditions.** *H. pylori* strain ATCC 43504 was used for identification of SGI compounds in this study. *H. pylori* strains CPY3401 (Karita et al., 1991), N6 (Ferrero et al., 1992) and J99 (Occhialini et al., 2000) were used for partial purification of SGI compounds and/or measurement of SGI effects. *H. pylori* strain JCM12095 (The Japan Collection of Microorganisms, Tsukuba, Japan) was used for measurement of SGI effects.

Other bacteria used in this study were *Bacillus subtilis*, *Bacillus cereus*, *Corynebacterium bovis*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Myobacterium smegmatis*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enterica serovar Enteritidis*, *Serratia marcescens*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Escherichia coli*, all of which were obtained from the Microbial Chemistry Research Foundation (Tokyo, Japan). *Bacteroides distasonis*, *Bacteroides fragilis*, *Bacteroides merdae*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Clostridium difficile*, *Clostridium perfringens*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus plantarum* and *Porphyrimonas gingivalis* were obtained from the Japan Collection of Microorganisms (Tsukuba, Japan).

*H. pylori* strains were mainly grown in Brucella broth (Becton Dickinson) containing 3 % (w/v) charcoal to culture *H. pylori*-infected monolayers, as well as provide information on the specific niche of *H. pylori* infection, as well as provide information on the specific niche of *H. pylori*. The active solution was then extracted from the concentrated EtOAc extract was purified using a Sephadex LH-20 procedure is summarized in Fig. 1.

**Extraction and purification of growth-inhibiting compounds.** *H. pylori* was cultured in 600 ml Brucella broth, PPL0 broth or RPMI 1640 supplemented with Diaion HP-20, as described above. The Diaion HP-20 was collected by filtration, washed with 3 vols distilled water and eluted in 5 vols methanol (MeOH). Methanol fractions were collected and then concentrated to a volume of ~2.3 ml. Then, 1/100 vols of this concentrated MeOH fraction was added to the bioassay medium without adjusting the pH to test its ability to inhibit the growth of *H. pylori*. The active solution was then extracted from the MeOH fraction with an equal volume of ethyl acetate (EtOAc).

The EtOAc was collected and concentrated. The purification procedure is summarized in Fig. 1.

The concentrated EtOAc extract was purified using a Sephadex LH-20 (GE Healthcare) column and eluted with MeOH. The active fractions were collected and concentrated in vacuo to give a pale brown oil, which was run through a silica gel column and eluted with CHCl₃/MeOH at 39:1 and 19:1. The active component was eluted with toluene/acetone at 4:1 and 2:1, and was concentrated in vacuo to yield a colourless oil containing lauric acid (LA) and 7-(Z)-tetradecenoic acid [7-(Z)-TDA] (Fig. S1, available in the online Supplementary Material). The crude material was dissolved in 250 μl MeOH and treated with diazodiphenylethane (96.4 mg). The reaction mixture was incubated at 50 °C for 18 h and concentrated in vacuo. The residue was evaporated and then purified successively by HPLC (Shiseido Capcell Pak C18 UG120A; 10 × 250 mm, CH₃CN/H₂O, 85:15, flow rate 5 ml min⁻¹) to give LA diphenylmethyl ester and 7-(Z)-TDA diphenylmethyl ester (Fig. 1b). Each sample was treated with a mixture of 1 M NaOH and an equal volume of MeOH, which dissolved the eluted residue. Samples were then incubated at 85 °C with stirring for 1 h. The solutions were acidified to pH 2 with 1 M HCl following a hexane wash and were extracted with EtOAc. The EtOAc extract was concentrated to yield LA and 7-(Z)-TDA (Fig. 1a).

These were dissolved in 0.5 M NaOH and 1/100 vols of the bioassay medium (pH 7.0) was added. A bioassay was used during the purification of SGI compounds to monitor the growth-inhibitory effects of the purified fractions towards *H. pylori*. The active fraction was directly extracted from replicate cultures carried out in 600 ml PPL0 broth supplemented with 0.2 % CD, using the same volume of EtOAc. These EtOAc extracts were treated using the procedure described in Fig. 1.

**Structural determination of the SGI compounds.** High-resolution electrospray ionization (HRESI)-MS (LTQ Orbitrap XL mass spectrometer; Thermo Fisher Scientific) analysis and NMR (INN-EC600 spectrometer; JEOL) experiments were performed to determine the structure of the extracted LA and 7-(Z)-TDA. NMR analyses included 1H, 13C, distortionless enhancement by polarization transfer (DEPT), 1H-detected multiple quantum coherence spectrum (HMQC), 1H-13C correlation spectroscopy (COSY), totally correlated spectroscopy (TOCSY) and 1H-detected multi-bond heteronuclear multiple quantum coherence spectrum (HMBCC).

**MIC assay.** MICs were determined using the agar dilution method recommended by the Clinical Laboratory Standards Institute guidelines M11-A6 and M7-A7 (NCCLS, 1990). *H. pylori* strains were grown in the presence of each compound and the concentration of each compound was increased in an agar dilution plate. The MIC was defined as the lowest concentration of the compound that inhibited the visible growth of *H. pylori*.
for 48 h at 37 °C in Brucella broth containing 3 % HS under microaerophilic conditions. Colony replicators were used to spot broth cultures onto Brucella agar plates containing 3 % HS, resulting in ~10^5 c.f.u. per spot (~1 µl). Agar plates were incubated at 37 °C for 48 h under microaerophilic conditions. Other enteric bacterial strains were grown anaerobically for 18–20 h at 37 °C and then spotted onto agar as described above, using the following media: *Lactobacillus* and *Bifidobacterium*, lactobacilli MRS broth (Becton Dickinson); *Clostridium*, *Clostridium difficile* broth and modified *Clostridium difficile* agar base (Thermo Fisher Scientific); *Enterococcus* and *Escherichia coli*, Mueller–Hinton broth (Becton Dickinson); other enteric bacteria, ABCM broth (Nissui Pharmaceutical). All enteric bacteria were cultured on ABCM agar (Eiken) at 37 °C for 20 h under anaerobic conditions. Aerobic bacterial strains were grown for 18 h in Mueller–Hinton broth at 37 °C to achieve a final inoculum of ~10^4 c.f.u. µl^{-1} (or per spot) on the agar plates. Bacteria were cultured on Mueller–Hinton agar at 37 °C for 18 h.

**Live/dead staining.** Live/dead DAPI/propidium iodide (PI) staining was performed as described previously (Garren & Azam, 2010) with
some modifications. A 100 μl aliquot of each of the culture broths was suspended in 800 μl sterile saline (Otsuka Pharmaceutical). The saline-suspended samples were stained with 100 μl DAPI (1.0 μg ml⁻¹; Koyo Sangyo) for 2 min. Each stained cell sample was fixed on a polycarbonate membrane filter (Koyo Sangyo) by aspiration and then stained with 100 μl PI (2.5 μg ml⁻¹; Koyo Sangyo). Using this method, both live and dead cells take up the DAPI stain, whilst the PI only stains the dead cells. The number of living bacterial cells in each sample was calculated using Bioplorer (Matsushita Electric Industrial). Fluorescence resulting from DAPI reacting with DNA was detected at 460 nm following excitation with UV light (375 nm), whilst fluorescence from PI reacting with DNA was detected at 620 nm following excitation with green light (525 nm).

RESULTS

Isolation and structural elucidation of SGI compounds from H. pylori

H. pylori was cultured in Brucella broth, PPLO broth or RPMI 1640 supplemented with Diaion HP-20 to test SGI compound production in the different types of medium. Diaion HP-20 was collected by filtration, washed with distilled water and eluted in MeOH. Methanol fractions were collected and then concentrated as described in Methods, and 1/100 vols of this concentrated MeOH fraction was added to test its ability to inhibit the growth of H. pylori. Bioassays using the culture medium Diaion HP-20 MeOH extracts revealed an inhibitory effect for each of the H. pylori strains, with no effect noted for the extract of the H. pylori-minus medium (negative control). Cultivation without supplement did not show growth of H. pylori. Although no growth was observed when H. pylori was cultured with 3 % HS plus MeOH extracts from H. pylori cultures grown in Brucella broth supplemented with HP-20, supplementation of excess HS after 12 h enabled the growth of H. pylori. Representative results using H. pylori strain ATCC 43504 are shown in Fig. 2. The peak of SGI activity was also confirmed by bioassay during the purification process, the procedure for which is shown in Fig. 1. As shown in Fig. 3, SGI compounds from H. pylori were isolated by HPLC from Diaion HP-20 supplemented into PPLO broth or RPMI 1640 (Fig. 3b, c), and from CD supplemented into PPLO broth (Fig. 3d). The isolation of SGI compounds from PPLO broth with CD was done to show that addition of CD to the growth medium resulted in the production of the same fatty acid compounds and that the bacteria were growing in the absence of Diaion HP-20, suggesting that these lipids could also be absorbed by CD instead of Diaion HP-20 (but only if growth was comparable with that under the conditions described in Methods). The NMR and MS analyses, and the trimethylsilyldiazomethane reaction using the partially purified sample, suggested that there were two active compounds, both of which had aliphatic chains and carboxyl groups, indicative of fatty acids. Therefore, we decided to convert the mixture of fatty acids to less polar derivatives that were easily purified by HPLC. As carboxyl groups tend to give tailing peaks in various chromatographic techniques, the functional group is masked as diphenylmethyl ester. The HPLC analyses of the other three H. pylori strains, CPY3401, J99 and N6, are shown in Fig. S2.

Structural determination of the SGI compounds

The 1H-NMR spectrum indicated that compound 1 was a saturated fatty acid. The molecular formula was established as C12H24O2 based on NMR and HRESI-MS, which gave an (M−H) ion at m/z 199.1698 (Δ−0.52 mDa; calculated for C12H24O2). This identified compound 1 as LA (Fig. S3). The chemical shifts of 1H and 13C NMR analysis of LA were identical to those of purchased LA.

The molecular formula of compound 2 was determined to be C14H26O2, based on NMR and HRESI-MS, which gave an (M−H) ion at m/z 225.1854 (Δ−0.58 mDa; calculated for C14H26O2) (Fig. S4–S8). NMR analyses (1H, 13C, DEPT, HMQC, 1H−1H COSY, HMBC and TOCSY) of compound 2 were performed to determine the structure. Fig. S9 summarizes the NMR experiments. The 1H−NMR spectrum indicated that compound 2 was an unsaturated fatty acid. With a molecular formula of C14H26O2, compound 2 was predicted to be a C14 fatty acid with an olefinic double bond, which was indicative of 7-(Z)-TDA. DEPT, HMQC,
HMBC, HMQC/TOCSY and TOCSY revealed an olefinic double bond located between C-7 and C-8 (Fig. S4–S8). Extensive decoupling experiments with 7-(Z)-TDA clearly showed a coupling constant of $J = 11.3$ Hz and that the olefin adopted a Z geometry (Fig. S9).

**MICs of the SGI compounds against *H. pylori* and other micro-organisms**

The MICs of the SGI compounds for five *H. pylori* strains were determined using Brucella agar containing 3% defibrillated HS. LA and *syn*-7-(Z)-TDA showed similar results for all strains, with MIC values of 8–16 μg ml$^{-1}$ (Table 1). Antibacterial activities of LA and *syn*-7-(Z)-TDA towards other aerobic and anaerobic bacteria, including enteric bacteria, were markedly reduced compared with activity towards *H. pylori* strains (Table 1).

**Live/dead staining to determine the viable population versus the culturable population of *H. pylori***

To determine the effective concentrations of the SGI compounds against *H. pylori*, DAPI/PI staining of live/dead cells was analysed using Bioplorer (Fig. S10). The number of live cells was calculated by subtracting the number of cells stained with PI (dead cells) from the number of cells stained with DAPI (both live and dead). Based on c.f.u. analysis, in the presence of the SGI compounds at 1–4 $\times$ MIC in HS-free Brucella medium, the proliferation of *H. pylori* cells was greatly reduced in a dose-dependent manner. At a concentration of 4 $\times$ MIC, LA caused the number of *H. pylori* to decrease from $1.3 \times 10^8$ to $2.5 \times 10^5$ c.f.u., whilst *syn*-7-(Z)-TDA caused cell numbers to drop to undetectable levels after only 2 h (Fig. 4a). However, Bioplorer showed that the number of live *H. pylori*...
cells was only slightly reduced and was independent of dose. At 48 h post-inoculation in broth containing 4 × MIC of the SGI compounds, the number of live _H. pylori_ cells decreased from $2.0 \times 10^8$ to $1.2 \times 10^7$ c.f.u. for LA and to $8.0 \times 10^7$ c.f.u. for _syn-7-(Z)-TDA_ (Fig. 4b).

**DISCUSSION**

_H. pylori_ infection is one of the most common bacterial diseases in the world (Cave, 1997). Most _H. pylori_ infection occurs in infancy and continues throughout life without drug treatment (Brown, 2000). However, why this
organism can persistently exist in the mucosa is unknown, although a signalling factor might be involved.

In this study, we demonstrated that the resin Diaion HP-20 is a useful supplement for cultures of H. pylori because it adsorbs the SGI compounds LA and 7-(Z)-TDA. These SGI compounds were produced by H. pylori not only in Brucella broth with Diaion HP-20, but also in RPMI 1640 or PPLO broth with the same resin. Shimomura et al. (2009) cultured H. pylori in PPLO broth supplemented with 0.2 % CD; therefore, we examined the same growth conditions for isolation of SGI compounds from H. pylori. Our results indicated that H. pylori may produce SGI compounds in any growth medium and that a variety of supplements may be used to adsorb them. Although we have not directly isolated the SGI compounds from cholesterol, which can be supplemented into the medium instead of HS or CD, as reported by Jiménez-Soto et al. (2012), cholesterol might also be used to adsorb the SGI compounds.

LA and 7-(Z)-TDA have not previously been identified as being present in H. pylori (Geis et al., 1990), although 7-(Z)-TDA makes up ~15 % of the unsaturated fatty acid content of the E. coli cell envelope (Batchelor & Cronan, 1973). MIC values for the SGI compounds towards H. pylori on Brucella agar containing 3 % HS were much lower than those for the other bacterial species examined under both aerobic and anaerobic conditions, suggesting that the SGI compounds may specifically target H. pylori.

Results of the culturable versus viable population analysis suggested that SGI compounds have a bacteriostatic effect against H. pylori and that a significant proportion of the population might be in a viable but non-culturable state following exposure to SGI compounds. Our preliminary results shown in Fig. 2 also indicated that the extracts containing SGI compounds had a bacteriostatic and not a bactericidal effect because addition of excess HS after 12 h showed multiplication of the cells. One of the SGI compounds, LA, could inactivate H. pylori at the high concentrations, as determined by c.f.u. count. Petschow et al. (1996) previously showed that the MIC for this fatty acid towards H. pylori was 120 µM and a bactericidal effect was observed at 1.0 mM (Sun et al., 2003). However, the effective bactericidal concentration of LA during live/dead analysis in the current study was <32 µM. This concentration was confirmed by c.f.u. count, although many live cells were observed by live/dead staining for Bioplorer (Fig. S10). Therefore, we suggest that SGI compounds might be bacteriostatic at low concentrations.

Fatty acids have various functions in bacteria. The inhibitory effect of polyunsaturated fatty acids on the growth of H. pylori, particularly docosahexaenoic acid and linoleic acids, was reported previously by Thompson et al. (1994), Correia et al. (2012, 2013, 2014) and Obonyo et al. (2012). Docosahexaenoic acid pre-treated H. pylori cultures showed a reduction in IL-8 production, and a decrease of cyclooxygenase-2 and inducible nitric oxide synthase by adhesion to gastric epithelial cells. This evidence suggested that an n-3 polyunsaturated fatty acid impaired the interaction between H. pylori and gastric epithelial cells. Tanaka et al. (2011) demonstrated that branched fatty acids inhibit the biosynthesis of menaquinone, which is an essential component of the electron transfer pathway in H. pylori.

Fatty acids have also been partially characterized as diffusible signal factors that act as cross-kingdom communication signals in bacteria (Wang et al., 2004), although 7-(Z)-TDA has not been reported as such. However, 2-unsaturated fatty acids, such as 2-(Z)-11-methyldocosenoic acid has been identified as a diffusible signal factor in Xanthomonas campestris, which is a major bacterial pathogen of cruciferous plants worldwide (Barber et al., 1997). Beaulieu et al. (2013) characterized 2-(Z)-TDA, a positional isomer of 7-(Z)-TDA, produced by the xylem-limited plant pathogen Xylella fastidiosa as a diffusible signal factor, where it induces biofilm formation and promoter activity of hxA/B, which encodes haemagglutinin-like proteins Hx/A/B. 2-(Z)-TDA contains a conjugated double bond, whilst 7-(Z)-TDA in H. pylori only has an isolated one. It is not

![Fig. 4. Effect of LA and 7-(Z)-TDA on the culturable population versus the viable population of H. pylori.](image-url)
clear whether the position of the double bond or \( Z \) configuration is important for the growth-inhibiting function. The SGI compounds identified in the current study may also act as signalling molecules to control the cell density of \( H. \) pylori, as no homologue of the autoinducer AI-1-related gene has been found in the genome of \( H. \) pylori to date. However, \( H. \) pylori does produce a growth-phase-dependent signal molecule, AI-2, encoded by luxS (Forssyth & Cover, 2000; Joyce et al., 2000).

Complete eradication of \( H. \) pylori infection using antibiotics is the most common treatment; however, some problems still remain. Although three drug types (proton pump inhibitors, amoxicillin and clarithromycin) are used to treat \( H. \) pylori infection, the endemic enterobacterial flora might be influenced by the drugs. As a result of the serious side-effects of this kind of treatment, such as soft bowel movements or diarrhoea, patients are often forced to discontinue the therapy. In addition, the massive doses of antibiotics used may be responsible for the appearance of drug-resistant \( H. \) pylori. The SGI compounds identified in this study may be used for the development of new therapeutic products because they do not readily inhibit the growth of other enteric or indigenous bacteria and because fatty acids are thought to be less likely to induce bacterial drug resistance (Petschow et al., 1996; Obonyo et al., 2012). Further investigations should be carried out to determine the functions of the SGI compounds, particularly as to how and why they inhibit the growth of the producing bacterium, including the possibility of signalling factors. The findings of the current study might be of some help in the development of new therapies for gastritis caused by \( H. \) pylori infection and help avoid the appearance of drug-resistant strains.

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