Adsorption of extracellular proteases and pyocyanin produced by *Pseudomonas aeruginosa* using a macroporous magnesium oxide-templated carbon decreases cytotoxicity

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**A R T I C L E   I N F O**

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**A B S T R A C T**

*Pseudomonas aeruginosa* is one of the most common pathogens isolated in clinical settings and produces a wide range of extracellular molecules that contributes to the virulence. Chemotherapy options to prevent and treat *P. aeruginosa* infections are limited because this pathogen is highly and innately resistant to some classes of conventional drugs. Alternative methods to conquer *P. aeruginosa*, including multidrug resistant strains, are being investigated. This study showed that a macroporous magnesium oxide (MgO)-templated carbon material (MgOC\(^{150}\)) attenuates the toxicity of this bacterium in human epithelial cells. A proteomic analysis revealed that MgOC\(^{150}\) adsorbs some extracellular proteases, including elastase (LasB) and alkaline protease (AprA), and pyocyanin, which is another molecule involved in its pathogenesis, but is a nonprotein small-sized molecule. These results suggest a potency of MgOC\(^{150}\) that suppresses the virulence of *P. aeruginosa*. MgOC\(^{150}\) has been used for industrial purposes, as an electrode catalyst and a bioelectrode and for enzyme immobilization. Thus, MgOC\(^{150}\) could be beneficial for developing novel anti-*Pseudomonas* therapy.

1. Introduction

*Pseudomonas aeruginosa* is a clinically important pathogen. This bacterium is frequently isolated with nosocomial infections, and it causes serious infections in immunocompromised individuals and patients who have chronic respiratory diseases, such as cystic fibrosis (CF) and diffuse panbronchiolitis (Murray et al., 2014, Mittal et al., 2009, Zaborina et al., 2006). Although a major site of *P. aeruginosa* infection is the lungs, a considerable number of fatal systemic infections occur when it becomes bacteremic (Turner et al., 2014, Mittal et al., 2009, Zaborina et al., 2006). This bacterium is not a common intestinal pathogen in healthy hosts however, *P. aeruginosa* colonizes in the gut of hospitalized, immunocompromised, antibiotic-treated patients or patients with cancer, and it disrupts the intestinal epithelial barrier, leading to bloodstream infections (Adlard et al., 1998, Gomez-Zorrilla et al., 2015, Balzan et al., 2007, Okuda et al., 2010, Markou and Apidianakis, 2014). *P. aeruginosa* infection is generally difficult to treat because it is innately resistant to many classes of antimicrobial agents and there are multidrug-resistant strains (Poole, 2011, Hoiby, 2011, Aloush et al., 2006, Obritsch et al., 2004). For these reasons, developing any strategies to prevent and/or ameliorate infections caused by this pathogen is necessary.

Porous carbon can adsorb certain organic molecules that fit its internal pore. Activated charcoal is the most well-known substrate of porous carbons. It is used to remove toxic substances in industrial applications while several activated charcoal materials can be used for medical purposes as oral medicines, such as treating intoxications and certain circulatory diseases by adsorbing body wastes (Hegade et al., 2015, Scaldarferri et al., 2013, Zellner et al., 2019). For instance, AST120 (Kremenez) is used to treat progressive chronic kidney disease by adsorbing and removing uremic toxin precursors produced by gut microorganisms (Niwa, 2011). Pyocyanin is the most-characterized non-protein molecule that contributes to virulence of *P. aeruginosa*, and it impairs host cells by generating reactive oxygen species (Hall...
et al., 2016). We previously found that AST-120, adsorbs pyocyanin produced by *P. aeruginosa*, and attenuated pyocyanin-associated toxicity to intestinal epithelial cells (Hirakawa et al., 2021b). This finding leads to the focus of potency of porous carbon as an anti-Pseudomonas option. The average pore size of AST-120 is ~2 nm, and this pore size is suitable for the high adsorption of the pyocyanin molecule. However, it is presumably too small to adsorb relatively large-sized protein molecules. Virulence of *P. aeruginosa* is supported by not only certain non-protein molecules, including pyocyanin, but also various extracellular protein molecules, such as elastase and alkaline protease (Gellatly and Hancock, 2013, Hauser, 2009, Jenkins et al., 2004, Kipnis et al., 2006, O’Callaghan et al., 2019).

Magnesium oxide (MgO)-templated carbon (MgOC) is another recently industrialized porous carbon. Its pore producing process is supported by not only certain non-protein molecules, including pyocyanin, but also various extracellular protein molecules, such as magnesium citrate and magnesium gluconate, into a carbon matrix, and the pore is produced by removing the MgO molecule (Morishita et al., 2010). The pore size of the resulting MgOC can be precisely predesigned according to this method. This study aims to develop a strategy that suppresses the virulence of intestinal *P. aeruginosa*. It relies on adsorbing extracellular molecules required for bacterial virulence. MgOC material with an average pore size of 150 nm, named MgOC<sub>150</sub>, was utilized because this size is predicted to adsorb protein molecules >30 kDa (Funabashi et al., 2017). This study demonstrated that the MgOC material could adsorb extracellular proteases and pyocyanin produced by *P. aeruginosa*, including clinical isolates that are resistant to antimicrobials, such as multidrug-resistant *P. aeruginosa* (MDRP), and attenuate the toxicity to host intestinal epithelial cells.

2. Materials and Methods

2.1. Bacterial strains, host cells, and culture conditions

We used *P. aeruginosa* PAO1 strain and several non-PAO1 antibiotic-resistant clinical isolates (including the MDRP strains) as listed in Table 1. Ps.a-885, Ps.a-890, Ps.a-1016 and RPs.a-884 were isolated from the feces. Ps.a-1200, Ps.a-1205 and RPs.a-914 were isolated from the sputum while RPs.a-946 was isolated from the urine. The bacteria were aerobically grown at 37°C. Caco-2 (ATCC HTB-37) cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% HyClone FetalClone III serum (Hyclone Laboratories, Inc., Logan, UT, USA) at 37°C, 5% CO<sub>2</sub>.

| Strains   | Specimen | Resistance to: |
|-----------|----------|----------------|
| Ps.a-885  | Feces    | Levofoxacin    |
| Ps.a-890  | Feces    | Ceftazidime    |
| Ps.a-1016 | Feces    | Imipinem       |
| Ps.a-1200 | Sputum   | Levofoxacin    |
| Ps.a-1205 | Sputum   | Aztreonam, Meropenem, Levofoxacin |
| RPs.a-884 | Feces    | Ceftazidime, Imipinem, Meropenem, Cefepime |
| RPs.a-914 | Sputum   | Gentamicin, Amikacin, Levofoxacin |
| RPs.a-946 | Urine    | Imipinem, Meropenem, Gentamicin, Amikacin, Levofoxacin |

2.2. Cytotoxicity assay

Cytotoxicity of bacterial cultures to human intestinal epithelial cells was estimated in a cell viability assay as previously described (Hirakawa et al., 2021b). Bacteria were grown in LB medium for 24 h. A total of the 50 μL of bacteria-free culture supernatants was diluted into 50 μL DMEM containing 10% HyClone FetalClone III serum, and added to cultured Caco-2 cells in a 96-well plate. As a control, twofold diluted LB medium in DMEM containing 10% HyClone FetalClone III serum was added to the host cells. After incubation for 24 h, the cell viabilities were determined by measuring intracellular ATP levels with the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp., Madison, WI, USA). The cell viabilities were represented as relative light units (RLUs) by their ratios (%) to the RLU of the control sample. To estimate cytotoxic effects of MgOC<sub>150</sub> to human intestinal epithelial cells, Caco-2 cells were incubated with MgOC<sub>150</sub> in 150 μL DMEM containing 10% HyClone FetalClone III serum for 24 h and the cell viabilities were determined.

2.3. Protease assay

Protease activity was determined by measuring a proteolytic digestion of azocasein, a protease substrate. To measure the extracellular protease level in a bacterial culture, bacteria were grown in LB medium for 24 h. In addition, 7.5 μL of bacteria-free culture supernatant was added into a 50 mM phosphate buffer (pH 7.5) containing 1 mg azocasein. To determine protease activity for purified recombinant proteins, 5 μg of each protein was incubated with 1 mg azocasein in a 50 mM phosphate buffer (pH 7.5) containing 1 mM CaCl<sub>2</sub> and 0.1 mM ZnCl<sub>2</sub>. After incubation for 1 h at 37°C, 3 % trichloroacetic acid (TCA) was added, and the protease activity was quantified by measuring the absorbance at 366 nm.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry analyses

The PAO1 strains were grown with and without 30 mg MgOC<sub>150</sub> in 5 mL of LB medium for 8 h, and separated by centrifugation and filtration. Extracellular proteins were precipitated from 1350 μL supernatants with 10% trichloroacetic acid (TCA) and dissolved in 60 μL of the Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA). A total of 30 μL of the protein solutions were separated by SDS-PAGE with a 10% gel and stained with Coomassie brilliant blue (CBB). Two protein bands, corresponding to approximately 35 and 20 kDa, respectively, were excised and subjected to in-gel digestion using trypsin (Promega Corp., Madison, WI, USA) at 37°C overnight. Peptides were desalted with C18 StageTips. LC-MS/MS analysis was performed using a Dionex Ultimate 3000 nano HPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled to a LTQ Orbitrap Velos (Thermo Fisher Scientific). Before injection into the mass spectrometer, peptides were loaded online in a trap column Acclaim<sup>™</sup> PepMap<sup>™</sup> 100 C18 LC column (5 μm particle size, 100Å pore size, 0.1 mm×20 mm; Thermo Fisher Scientific) and separated by a nanoscale C18 capillary LC column (NTCC-360/75-3-125; Nikkyo Technos, Tokyo, Japan) by a linear gradient of acetonitrile (4-33%) in 0.1% formic acid for 120 min at a flow rate of 0.3μL/min and directly electrosprayed with electrospray ionization (ESI) source in a positive ion mode. The mass spectrometric conditions were as follows: m/z range, 350-1500; spray voltage, 2.5 kV; capillary temperature, 250°C; normalized collision energy, 35.0%; isolation width, 1 m/z; activation time, 10 ms; activation Q,0.25; dynamic exclusion, 60 s; resolution, 30,000; and data dependent mode with product ion scans for the 15 most intense ions in the full-scan mass spectrum. Peptide identification and label-free quantitation (LFQ) were performed using MaxQuant software (ver.1.6.2.10, Cox and Mann, 2008) using default settings (1% overall peptide false discovery rate) with slight modifications as follows: main search peptide tolerance, 6 ppm; and variable modifications, Oxidation (M), Acetylation (Protein N-term), and
Carbamidomethylation (C). The proteomics data have been deposited to the ProteomeXchange Consortium via the jPOST (Moriya et al., 2019) with dataset identifier PXD032870.

2.5. Plasmid construction

To construct IPTG-inducible C-terminally histidine-tagged LasB, AprA, PA0423 and LolA expression plasmids pET28a-lasB-His, pET28a-aprA-His, pET42c-PA0423-His and pET42c-lolA-His, respectively, we PCR-amplified the lasB, aprA, PA0423 and lolA genes with the primer pairs listed in Table 2. These PCR products were digested with NcoI and HindIII for the lasB and aprA genes and NdeI and XhoI for the PA0423 and lolA genes, and ligated into pET28a (for the lasB and aprA genes) and pET42c (for the PA0423 and lolA genes) plasmids. All constructs were confirmed by DNA sequencing.

2.6. Protein purification

The C-terminally histidine-tagged LasB, AprA, PA0423 and LolA proteins were expressed and purified from *Escherichia coli* Rosetta (DE3). Bacteria carrying each expression plasmid were cultured at 37°C to an OD_{600} of 0.4 in LB medium and then chilled to 16°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and culture growth was continued at 16°C for 18 h. Cells were harvested by centrifugation, and the cells were resuspended in BactYeastLysis reagent (ATTO, Tokyo, Japan) and lysed by sonication. An equal volume of purification buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 1 mM CaCl₂, 2 mM dithiothreitol, and 10% glycerol) was added to the lysate, and the mixture was centrifuged. The resulting supernatant was mixed with nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA, USA) for 1 h. The agarose was washed twice with 10 mM imidazole and once with 50 mM imidazole, and protein was eluted with 200 mM imidazole as described previously (Hirakawa et al., 2018). The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

2.7. Protein adsorption assay

To test the adsorption of target proteins to MgOC₁₅₀, each protein (10 µg) was incubated with and without MgOC₁₅₀ for 2 h at 4°C. Non-adsorbed protein in the supernatant was quantified in a Bio-Rad protein assay according to the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Pyocyanin assay

The pyocyanin level was determined as previously described (Essar et al., 1990; Hirakawa et al., 2021b). Bacteria were grown in LB medium for 24 h and the cell cultures were centrifuged to remove the cell pellets. Pyocyanin in the culture supernatants was extracted with chloroform and then re-extracted into 0.2 M HCl. Pyocyanin was quantified by measuring the absorbance of this solution at 520 nm.

2.9. Quantitative real-time PCR

The transcript levels of *lasI*, *rhlA*, *aprA* and *lasB* were measured in quantitative real-time PCR assays. Bacteria were grown with and without 30 mg MgOC₁₅₀ to 5 ml of LB medium to the early stationary phase (OD₆₀₀ =2.0). Total RNA was extracted using a Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer’s instructions. cDNA synthesis and real-time PCR were carried out as previously described (Hirakawa et al., 2021a, Hirakawa et al., 2020a). The constitutively expressed 16S rRNA and rpoD genes were used as internal controls. The primers used for real-time PCR are listed in Table 2.

2.10. Promoter assay

The promoter activities of *lasI* and *rhlA* were measured as previously described (Hirakawa et al., 2021b). The PA01 strains carrying each reporter plasmid, pBBRlasI (PAO1)-P or pBBRrhlA (PAO1)-P, were grown in the presence and absence of 30 mg MgOC₁₅₀ in 5 mL of LB medium to the early stationary phase (OD₆₀₀ =2.0). The chemiluminescent signal in the cell lysates was generated using a Tropix Galacto-Light Plus kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA, USA). Their β-galactosidase activities corresponding to LacZ expression were determined as the signal value normalized to an OD₆₀₀ of 1.

2.11. Adsorption assays for antimicrobial agents

To test the adsorption of antimicrobial agents to MgOC₁₅₀, 1.25 mg levofloxacin, aztreonam, piperacillin, cefepime and meropenem, or 5 mg amikacin, fosfomycin and colistin in 5 mL aqueous solution were incubated with and without 30 mg MgOC₁₅₀ for 2 hours. Drug amounts were calculated as described previously (Hirakawa et al., 2020b).

2.12. Antimicrobial susceptibility test

To estimate the susceptibility to antimicrobial agents, MIC assay was performed according to a serial agar dilution method with the standard method of the Clinical and Laboratory Standards Institute (CLSI). The MICs were determined as the lowest concentration at which growth was inhibited. Amikacin, fosfomycin and colistin were incubated with and without MgOC₁₅₀ for 2 hours. After removal of MgOC₁₅₀ by centrifugation at 15,000 g, the supernatants were used for the MIC assay.

2.13. Statistical analysis

The p-value in each assay was determined using the unpaired t-test for two-group comparison between control and MgOC₁₅₀-treated samples and the one-way ANOVA for multigroup comparison among samples treated with different amounts of MgOC₁₅₀ / AST-120. An alpha value of 0.05 was chosen as our significance cut-off for all analyses.

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Table 2: Primers used in this study.

| Primer | DNA sequence (5'-3') | Use |
|--------|---------------------|-----|
| pET-lasB-F | gcgcaggaggggggtccggtagg | pET28a-lasB-His construction |
| pET-lasB-R | gcgcagcttcgctcgggtagg | pET28a-lasB-His construction |
| pET-aprA-F | gcgcctggagcagctggtcggg | pET28a-aprA-His construction |
| pET-aprA-R | gcgcagcttgcgctggtcggg | pET28a-aprA-His construction |
| pET-PA0423-F | aagctctacttgaagagagacttg | pET42c-PA0423-His construction |
| pET-PA0423-R | gcgcctggagcagctggtcggg | pET42c-PA0423-His construction |
| pET-lolA-F | gcgcctggagcagctggtcggg | pET42c-lolA-His construction |
| pET-lolA-R | gcgcctggagcagctggtcggg | pET42c-lolA-His construction |
| 16SRNA-qPCR-F | ggccggatcacgatcaga | Real-time PCR |
| 16SRNA-qPCR-R | ggccggtcgggctggtcggg | Real-time PCR |
| rpoD-qPCR-F | gcgcaggaggggggtccggtagg | Real-time PCR |
| rpoD-qPCR-R | gcgcagcttcgctcgggtagg | Real-time PCR |
| lasI-qPCR-F | gcgcctggagcagctggtcggg | Real-time PCR |
| lasI-qPCR-R | gcgcagcttgcgctggtcggg | Real-time PCR |
| rhlA-qPCR-F | gcgcctggagcagctggtcggg | Real-time PCR |
| rhlA-qPCR-R | gcgcagcttcgctcgggtagg | Real-time PCR |
| aprA-qPCR-F | gcgcctggagcagctggtcggg | Real-time PCR |
| aprA-qPCR-R | gcgcagcttcgctcgggtagg | Real-time PCR |
| lasB-qPCR-F | ggccggtcgggctggtcggg | Real-time PCR |
| lasB-qPCR-R | gcgcaggaggggggtccggtagg | Real-time PCR |
3. Results

3.1. MgOC\textsubscript{150} attenuates cytotoxicity of \textit{P. aeruginosa} in intestinal epithelial cells

\textit{P. aeruginosa} produces many extracellular protein molecules, and some of them target and impair host epithelial cells. First, to evaluate an efficacy of MgOC\textsubscript{150} that attenuates virulence of \textit{P. aeruginosa} against intestinal epithelial cells, the toxicity of supernatants from \textit{P. aeruginosa} PAO1 cultured with and without MgOC\textsubscript{150} to human colon adenocarcinoma Caco-2 cells was determined. The addition of supernatant from the PAO1 culture killed ~98% of Caco-2 cells, while more than 60% and 90% of the host cells survived even after incubated with supernatant from PAO1 cultured in the presence of 5 and 30 mg MgOC\textsubscript{150}, respectively (Fig. 1A). The capability of MgOC\textsubscript{150} to neutralize cytotoxicity in culture supernatants from drug-resistant clinical isolates, including multidrug-resistant \textit{P. aeruginosa} (MDRP) strains, was also tested. Supernatants from Ps.a-885, Ps.a-890, Ps.a-1016, Ps.a-1200, Ps.a-1205, Rps.a-884 and RPs.a-946, but not from RPs.a-914, exhibited the strong cytotoxic activity against Caco-2 cells. The addition of MgOC\textsubscript{150} into their cultures highly reduced the cytotoxicity (Fig. 1B). MgOC\textsubscript{150} did not impair bacterial growth because there was no significant difference in bacterial CFUs when PAO1 was cultured in the presence and absence of MgOC\textsubscript{150} (Fig. 2A). These results suggest that MgOC\textsubscript{150} attenuates the toxicity of \textit{P. aeruginosa} culture to intestinal epithelial cells, but it does not affect bacterial growth.

To confirm that MgOC\textsubscript{150} does not disturb Caco-2 cells, the host cells were incubated with the MgOC\textsubscript{150} material for 24 h. No significant reduction in the number of viable cells was observed after incubation with MgOC\textsubscript{150} (Fig. 2B).

3.2. MgOC\textsubscript{150} decreases extracellular proteolytic activity in \textit{P. aeruginosa} cultures

A subset of extracellular proteases, produced by \textit{P. aeruginosa} are involved in pathogenesis of this bacterium. Attenuated cytotoxicity of a culture supernatant from \textit{P. aeruginosa} cultured with MgOC\textsubscript{150} may be due to a reduced extracellular proteolytic activity. To test this hypothesis, the proteolytic activity in a supernatant from the bacterial culture

Fig. 1. Cytotoxicity of supernatants from \textit{P. aeruginosa} PAO1 strain and drug-resistant clinical isolates. (A) Survival of Caco-2 cells after incubation with culture supernatants from PAO1 cultured with MgOC\textsubscript{150}. (B) Survival of Caco-2 cells after incubation with culture supernatants from the indicated \textit{P. aeruginosa} strains cultured in the presence or absence of 30 mg MgOC\textsubscript{150}. The survival rates are presented as the percentage of the RLU value for the cells after incubation with each supernatant relative to that after incubation without supernatant. Data plotted are the means of three biological replicates, and error bars indicate the standard deviations. Asterisks denote significance for values (p < 0.05) of survival rates in CaCO-2 cells after incubated with supernatant samples from bacterial cultures in the presence of MgOC\textsubscript{150} relative to those after incubation with supernatant samples from bacterial cultures in the absence of MgOC\textsubscript{150}. The experiments were repeated twice with similar results.

Fig. 2. Toxicity of MgOC\textsubscript{150} in bacterial and host cells. (A) Growth of the PAO1 strains cultured with and without 30 mg MgOC\textsubscript{150}. Bacterial growth was estimated by measuring the CFUs. (B) Survival of Caco-2 cells after incubation with and without MgOC\textsubscript{150}. MgOC\textsubscript{150} was added into 150 μL Caco-2 cell cultures. Amounts (mg) of MgOC\textsubscript{150} on the x-axis were represented as those corresponding to 5 mL cultures. The survival rates are presented as the percentage of the RLU value for the cells after incubation with MgOC\textsubscript{150} relative to that after incubation without MgOC\textsubscript{150}. Data plotted are the means of three biological replicates, and error bars indicate the standard deviations. The experiments were repeated twice with similar results. No statistical significance for values of samples treated with and without MgOC\textsubscript{150} was detected.
was assessed by measuring their abilities to degrade azocasein as a proteolytic substrate. A degradation product of azocasein was detected at an absorbance of 366 nm when incubated with a supernatant from the PAO1 culture. On the other hand, when azocasein was incubated with a supernatant from bacteria co-cultured with 30 mg MgOC$_{150}$, its degradation product was undetectable (Fig. 3A). To compare the capability of MgOC$_{150}$ with that of AST-120 used as an oral adsorbent medicine, the PAO1 strain was cultured with 30 mg AST-120. In contrast to MgOC$_{150}$, a significant level of the proteolytic activity was still observed in the supernatant from the bacteria cultured with AST-120 (Fig. 3A). In addition to PAO1, supernatants from Ps.a-885, Ps.a-890, Ps.a-1016, Ps.a-1200, Ps.a-1205, Ps.a-884, and Ps.a-946 cultures exhibited proteolytic activities, and their activities was reduced by adding MgOC$_{150}$ into cultures although a moderate proteolytic activity remained (Fig. 3B). Unlike those from PAO1, Ps.a-885, Ps.a-890, Ps.a-1016, Ps.a-1200, Ps.a-1205, Ps.a-884, and Ps.a-946, the proteolytic activity of the supernatant from RPs.a-914 was below the limit of detection even when cultured in the absence of MgOC$_{150}$. Therefore, low cytotoxicity of the RPs.a-914 culture may be due to its deficient proteolytic activity. To estimate the ability of MgOC$_{150}$ to adsorb proteolytic activity-associated proteins, the bacteria-free supernatant from a 24 h culture of PAO1 was incubated with MgOC$_{150}$, and the residual proteolytic activity in the supernatant after removal of MgOC$_{150}$ was determined. The proteolytic activity decreased by ~90% after incubation with 30 mg MgOC$_{150}$ (Fig. 3C). The bacteria-free supernatant was also incubated with AST-120. The proteolytic activity in the supernatant did not decrease (Fig. 3C). Altogether, these results suggest that MgOC$_{150}$ adsorbs some extracellular proteins of _P. aeruginosa_, including extracellular proteases, and decreases their proteolytic activity in bacterial culture.

3.3. Addition of MgOC$_{150}$ decreases levels of certain extracellular proteins, including LasB elastase and AprA alkaline protease, in _P. aeruginosa_ culture

To identify extracellular proteins of _P. aeruginosa_, that are adsorbed by MgOC$_{150}$, PAO1 strains were cultured in the presence and absence of MgOC$_{150}$, and proteins in culture supernatants were analyzed using a proteomics technique. Proteins were separated by SDS-PAGE and stained with CBB. Two major protein bands, corresponding to ~35 and 20 kDa, respectively, had intensities that were remarkably lower in the supernatant from PAO1 grown with MgOC$_{150}$ compared to the supernatant from PAO1 grown without MgOC$_{150}$ (Fig. 4). Proteins contained in these bands were identified (The dataset ID is PXD032870 in the ProteomeXchange Consortium). Table 3 and Table 4 show the top 35 highest-scoring candidate proteins, which were identified from either 35 or 20 kDa bands detected by SDS-PAGE from the culture supernatant of PAO1. Firstly, we note that some proteins were identified in both fractions that differ in molecular weight. We guess that these proteins were detected as partial degradation products. Two extracellular proteases involved in the pathogenesis of _P. aeruginosa_, predicted LasB elastase and AprA alkaline protease (Czyz and Iglewski, 1980; Kessler and Safrin, 2014), were detected for the 35 kDa band (Table 3). LasB is produced as a 54 kDa precursor protein and it is processed to a 33 kDa active form when secreted into the extracellular space (Kessler and Safrin, 2014), were detected for the 35 kDa band (Table 3). LasB and AprA peptides corresponding to this protein were detected in the 35 kDa fraction. In contrast, AprA was only identified from the supernatant of PAO1 grown without MgOC$_{150}$, and the LFQ intensity of AprA from the 35 kDa band from the supernatant of PAO1 grown with MgOC$_{150}$ was approximately 13-fold lower than that grown without MgOC$_{150}$ (Table 3). In contrast, AprA was only identified from the supernatant of PAO1 grown without MgOC$_{150}$, and the LFQ intensity of AprA from
PAO1 grown with MgOC\textsubscript{50} was nearly zero (Table 3). These results suggest that levels of extracellular LasB and AprA decreased when cultured with MgOC\textsubscript{50}.

LasB was also detected from the 20 kDa band, which may be processed by partial degradation, and showed 4.2-fold lower in PAO1 grown with MgOC\textsubscript{50} than that grown without it (LFQ intensity: 7.23×10\textsuperscript{7} for bacteria grown without MgOC\textsubscript{50}), although LasA was identified by very low score (score 22) and not listed in the top 35 protein candidates (See PXD032870 in the ProteomeXchange Consortium). LasA is a known elastase involved in the pathogenesis of \textit{P. aeruginosa}, and it is processed to a 22 kDa active form by LasB (Li and Lee, 2019). Thus, the decreased level of active LasA in bacteria grown with MgOC\textsubscript{50} may be associated with a reduction of LasB.

Several intracellular and outer membrane-associated proteins, including GroEL, DnaK, FliC and FliD, were also detected from the 35 and 20 kDa bands (Table 3 and Table 4). Since these proteins are abundantly expressed in bacteria, significant amounts of proteins derived from lysed cells might be undesirably detected even if the lysed cell populations were low (Roncarati and Scarlato, 2017, Zhang et al., 2007).

Extracellular flagella-associated proteins of \textit{P. aeruginosa} damage epithelial cells by inducing inflammatory responses (Haiko and West-erlund-Wikstrom, 2013). We found that FlgK protein was detected from both 35 and 20 kDa fractions in PAO1 grown without MgOC\textsubscript{50} while the

### Table 3

| Uniprot ID | Proteins                                      | Score | LFQ intensity None | LFQ intensity MgOC\textsubscript{50} |
|-----------|-----------------------------------------------|-------|-------------------|-------------------------------------|
| P30718    | Chaperonin (GroEL)                            | 259.55| 1.03.              | 1.22.E+08                           |
| P72151    | B-type flagellin (FltG)                       | 238.66| 1.60.              | 8.01.E+07                           |
| Q94F3     | Flagellar hook-associated protein (FlgK)      | 222.17| 2.06.              | 0.00.E+00                           |
| P21175    | Branched-chain amino acid transport protein (Boc) | 176.33| 3.10.              | 1.45.E+08                           |
| Q9402     | L-glutamate/L-aspartate-binding protein (PA1342) | 161.24| 3.78.              | 6.34.E+07                           |
| Q91XG8    | Uncharacterized protein (PA3836)              | 156.33| 8.59.              | 1.34.E+08                           |
| Q93DI     | Dihydrolipoyl dehydrogenase (IpdG)           | 155.15| 8.33.              | 2.51.E+07                           |
| Q91O47    | Tranmaslase (Tel)                            | 154.3 | 4.43.              | 7.20.E+07                           |
| Q9HIV3    | Chaperone protein (DnaK)                      | 151.49| 1.99.              | 1.07.E+07                           |
| P14756    | Elastase (LasB)                               | 150.19| 2.23.              | 1.75.E+08                           |
| Q9K3C5    | B-type flagellar hook-associated protein (FltD) | 143.91| 2.38.              | 6.99.E+04                           |
| Q9HI18    | C4-dicarboxylate-binding protein (DcpP)       | 126.13| 3.13.              | 4.94.E+07                           |
| Q9HIVA2   | Ketal-acid reductoisomerase (IvC)             | 124.36| 3.01.              | 9.08.E+07                           |
| Q9I0D3    | Cysteine synthase (CysK)                      | 124.14| 3.51.              | 9.66.E+07                           |
| Q9H2Q8    | Aminopeptidase (Lap)                         | 122.34| 9.27.              | 0.00.E+00                           |
| Q993C5    | Chaperone protein (IhpG)                      | 121.69| 1.64.              | 0.00.E+00                           |
| Q9XAD8    | Phosphate-binding protein (PstS)              | 118.77| 2.37.              | 3.28.E+07                           |
| Q59653    | Aspartate carboxamoyltransferase (PyR)        | 116.56| 9.58.              | 1.67.E+07                           |
| P13794    | Outer membrane protein F (OmpF)               | 109.31| 2.11.              | 4.88.E+07                           |
| Q9HTX3    | Iron transporter (PAS217)                     | 107.35| 8.89.              | 1.90.E+08                           |
| Q9JX05    | Bacteriophagpe protein (PA0618)               | 105.36| 2.09.              | 1.50.E+08                           |
| Q9ISD1    | AmpDh3                                        | 101.07| 0.00.              | 1.64.E+07                           |
| Q9HZ48    | ABC sugar transport protein (PAS190)         | 102.02| 8.33.              | 9.45.E+06                           |
| Q9I5W4    | Immunomodulating metalloprotease (ImpA)       | 101.99| 1.04.              | 1.01.E+07                           |
| Q9HZ31    | 30S ribosomal protein S1 (RpsA)               | 100.1 | 1.10.              | 0.00.E+00                           |
| Q9HN5     | Chaperone protein (ClpB)                      | 99.932| 2.89.              | 0.00.E+00                           |
| Q9HT29    | Uncharacterized protein (PAS545)              | 99.795| 8.54.              | 2.67.E+07                           |
| Q9I4T7    | LysM domain-containing protein (PA0202)       | 96.521| 3.13.              | 1.05.E+07                           |
| Q9ISQ3    | Tyrrosine-tRNA ligase 2 (TyrS2)               | 95.356| 1.48.              | 0.00.E+00                           |
| Q86428    | Branched-chain-amino-acid aminotransferase (IvE) | 92.5 | 6.74.              | 9.91.E+07                           |
| Q93023    | Alkaline protease (AprA)                      | 90.26 | 2.50.              | 0.00.E+00                           |
| Q9H456    | Outer membrane protein (PA1288)              | 81.569| 4.94.              | 1.18.E+07                           |
| Q9H650    | Virginiacumin B hyase (PA0681)                | 79.946| 1.24.              | 2.08.E+07                           |
| Q9IS89    | Chitin-binding protein (CbpD)                 | 77.539| 1.38.              | 1.70.E+07                           |
| Q120851   | Elongation factor Ts (Tsf)                    | 76.463| 1.47.              | 1.14.E+07                           |

\*1: Proteins from a supernatant of PAO1 grown without MgOC\textsubscript{50}  
\*2: Proteins from a supernatant of PAO1 grown with MgOC\textsubscript{50}

Fig. 4. Extracellular proteins from the PAO1 strain cultured in the presence and absence of MgOC\textsubscript{50}. Proteins were separated by SDS-PAGE using a 10% acrylamide gel, and stained with Coomassie brilliant blue. Locations of molecular mass standards (in kDa) are indicated on the left. Asterisks indicate protein bands which levels are remarkably low in the strain cultured in the presence of MgOC\textsubscript{150} compared to the strain cultured in the absence of MgOC\textsubscript{50}.
Table 4

Top 35 protein candidates in scores for the 20 kDa fraction from the culture supernatant of PAO1

| Uniprot ID | Proteins                                      | Score   | LFQ intensity | LFQ intensity |
|------------|-----------------------------------------------|---------|---------------|---------------|
| Q9H4V3     | Chaperone protein (DnaK)                       | 239.49  | 1.73          | 0.00.E-00     |
| Q9K3C5     | B-type flagellar hook-associated protein (FliD) | 204.01  | 2.53          | 3.18.E-06     |
| P27151     | B-type flagellin (FlgC)                        | 183.15  | 6.00          | 2.54.E-07     |
| P30718     | Chaperonin (GroEL)                             | 181.64  | 2.71          | 3.54.E-07     |
| Q9690      | Extracellular protease (PA0423)                | 162.39  | 7.75          | 1.89.E-09     |
| P14756     | Elastase (LasB)                                | 147.53  | 2.09          | 4.93.E-07     |
| Q90M4      | Outer membrane lipoprotein carrier protein (LoLA)| 141.31  | 1.76          | 1.77.E-08     |
| O82853     | Ribosome-recycling factor (Frr)                | 131.5   | 7.45          | 1.02.E-07     |
| Q9S5D1     | AmpDhl3                                        | 128.06  | 2.78          | 1.02.E-08     |
| P17794     | Outer membrane porin F (OprF)                  | 126.26  | 8.62          | 1.23.E-07     |
| Q92F8      | D-Ribose/D-allose-binding protein (Rhdh)       | 120.15  | 6.37          | 4.19.E-07     |
| G3XD74     | Arginine/ornithine binding protein (AonI)      | 107.83  | 2.10          | 2.55.E-07     |
| Q9H4Y4     | Pyocin SS (PysS)                               | 106.84  | 3.93          | 3.00.E-00     |
| Q9H7M5     | Uncharacterized protein (PA5350)               | 101.42  | 4.72          | 3.21.E-07     |
| Q9I1Q8     | Aminopeptidase (Lap)                           | 99.454  | 7.51          | 1.00.E-08     |
| Q9S3D1     | Dihydroylipoyl dehydrogenase (LpdG)           | 98.982  | 3.16          | 2.53.E-07     |
| Q9402      | L-Glutamyl/L-aspartate-binding protein (PA1342)| 98.45   | 4.99          | 2.45.E-07     |
| P21175     | Branched-chain amino acid transport protein (BraC)| 98.406  | 2.36          | 3.18.E-07     |
| Q9H7A8     | Ferric iron-binding protein (IIBA)             | 96.294  | 2.87          | 1.21.E-07     |
| P08308     | Ornithine                                     | 94.575  | 1.95          | 1.35.E-07     |
| Q9H6L0     | Aromatase (Arom)                               | 91.92   | 3.26          | 2.08.E-08     |
| Q9P3D8     | Uncharacterized protein (PA3775)               | 89.453  | 1.62          | 7.23.E-07     |
| Q9S456     | Outer membrane protein (PA1288)                | 76.379  | 5.59          | 8.98.E-06     |
| G3XD39     | Bacteriophage protein (PA0622)                | 75.407  | 2.48          | 1.72.E-07     |
| Q9H1V7     | LPS export protein (LptA)                      | 75.126  | 2.09          | 1.50.E-07     |
| Q9H7Y1     | Alkyl hydroperoxide reductase C (PA3529)       | 72.492  | 2.80          | 6.63.E-05     |
| Q9P43      | Flagellar hook-associated protein (FlgK)       | 68.059  | 1.33          | 0.00.E-00     |
| G3XD52     | MacC-like protein (PA3302)                     | 66.561  | 3.27          | 3.04.E-07     |
| Q9H8U1     | ABC transporter periplasmic binding protein (PA1553) | 63.569  | 1.26          | 1.45.E-07     |
| Q9H1R3     | ABC transport protein (PA2204)                 | 62.183  | 1.02          | 5.00.E-06     |
| Q9H6J1     | Putrescine-binding protein (SpBD)              | 61.376  | 3.67          | 5.48.E-06     |
| Q9S5W4     | Metalloprotease (ImpA)                         | 61.116  | 1.17          | 9.64.E-06     |
| Q9H1W1     | UFPS234 protein (PA4395)                       | 59.911  | 3.33          | 1.58.E-06     |
| Q9H457     | Glutathione peroxidase (PA1287)                | 59.694  | 1.08          | 1.14.E-08     |
| Q9H4V6     | Transcription elongation factor (GreA)         | 58.009  | 1.26          | 4.30.E-06     |

*1: Proteins from a supernatant of PAO1 grown without MgOC150
*2: Proteins from a supernatant of PAO1 grown with MgOC150

LFQ intensity of FlgK exhibited zero in PAO1 grown with MgOC150 (Table 3 and Table 4). These observations indicate that MgOC150 can also reduce the level of extracellular FlgK.

3.4. LasB and AprA proteolytic activities disrupt intestinal epithelial cells and MgOC150 adsorbs these proteins

Reduced levels of extracellular LasB and AprA in P. aeruginosa grown with MgOC150 may be due to adsorption of these proteins. To test this hypothesis, the ability of MgOC150 to adsorb LasB and AprA was estimated using the C-terminally histidine-tagged recombinant proteins. First, this study demonstrated that the purified recombinant LasB and AprA proteins have protease activity and cytotoxicity in Caco-2 cells (Fig. 5A and data not shown). A proteolytic product from azocasein was observed after incubation with the LasB and AprA proteins (data not shown) and administration of these proteins significantly reduced the viability of Caco-2 cells (Fig. 5A). The LasB and AprA proteins were incubated with different amounts of MgOC150 for 2 h, and the residual amount in the supernatant was measured after MgOC150 removal. More than 85 % of the LasB protein (10 µg) in a solution could be removed with MgOC150 when incubated with 100 µg of this adsorbent while approximately 70 % of the AprA protein was removed by adsorption to MgOC150 (Fig. 5B). These results suggest that MgOC150 adsorbs the LasB and AprA proteins and reduces the proteins-associated proteolytic activity and cytotoxicity. For the negative control, LoLA and PA0423 were used from the 20 kDa fraction because their LFQ intensity was not reduced even when MgOC150 was added in the culture (Table 4). The C-terminally histidine-tagged LoLA and PA0423 proteins were purified and adsorption of these proteins to MgOC150 was evaluated. Consistent with the data from the LFQ intensity, adsorption of neither the recombinant LoLA nor PA0423 protein to MgOC150 was observed (Fig. 5B). To compare the ability of AST-120 to adsorb LasB and AprA proteins with that of MgOC150, these recombinant proteins were incubated with AST-120. In contrast to MgOC150, no adsorption of LasB and AprA to AST-120 was observed (Fig. 5B).

3.5. MgOC150 adsorbs pyocyanin and reduces its level in P. aeruginosa culture

Pyocyanin is another molecule that contributes to the virulence of P. aeruginosa. Its cytotoxic function has been defined in airway epithelial cells, such as A-549, but it is known to disrupt intestinal epithelial cells (Hall et al., 2016, Hirakawa et al., 2021b). Unlike extracellular proteases, pyocyanin is a non-protein small-sized molecule. We previously found that AST-120 highly adsorbs the pyocyanin molecule (Hirakawa et al., 2021b). To compare the adsorption ability of MgOC150 to pyocyanin with that of AST-120, the bacteria-free supernatant from a 24 h culture of PAO1 was incubated with MgOC150 and AST-120 for 2 h. After removal of these adsorbents, residual pyocyanin in the supernatant was quantified. Similar to AST-120, MgOC150 adsorbed pyocyanin in the PAO1 culture supernatant. More than 50 % and 90 % of pyocyanin could be eliminated when incubated with 5 mg and 30 mg MgOC150 respectively (Fig. 6A). The PAO1 strain was cultured in the presence and absence of AST-120 or MgOC150, and the pyocyanin level in the supernatants was measured. The pyocyanin level was below the limit of detection when cultured with the 30 mg of adsorbents (Fig. 6B). Pyocyanin was undetectable even when 5 mg MgOC150 was present although a significant level of pyocyanin was still observed when grown with 5 mg AST-120. Thus, MgOC150 can adsorb pyocyanin more effectively than AST-120.

3.6. MgOC150 does not affect the activity of LasI-LasR and RhlI-RhlR-mediated quorum sensing

P. aeruginosa controls subsets of genes via quorum sensing (QS) that is one manner of bacterial signal transduction. This bacterium possesses...
two major systems mediated by LasI-LasR and RhlI-RhlR (Schuster et al., 2003). The production of LasB, AprA and pyocyanin is induced by these QS systems (Smith and Iglewski, 2003). LasI-LasR and RhlI-RhlR primarily activate the transcription of \( lasI \) and \( rhlA \) genes, respectively. The levels of \( lasI \) and \( rhlA \) transcripts were measured by qPCR analyses to estimate LasI-LasR and RhlI-RhlR activities in PAO1 grown with and without MgOC\(_{150}\). No significant difference in these transcript levels in the PAO1 strains grown with and without MgOC\(_{150}\) was seen (Fig. 7A). The levels of \( lasI \) and \( rhlA \) promoters were also measured. Consistent with the qPCR results, there was no significant difference in \( \beta \)-galactosidase levels corresponding to the promoter activities of \( lasI \) and \( rhlA \) between bacteria grown with and without MgOC\(_{150}\) (Fig. 7B). Thus, MgCO\(_{150}\) does not affect the activities of LasI-LasR and RhlI-RhlR-mediated QS systems associated with the production of LasB, AprA and pyocyanin. There was also no significant difference in the \( aprA \) transcript level in PAO1 grown with and without MgOC\(_{150}\). However, the \( lasB \) level in PAO1 grown with MgOC\(_{150}\) was moderately lower than that in PAO1 grown without MgOC\(_{150}\) (Fig. 7A).

### 3.7. MgOC\(_{150}\) does not impair antibacterial activity of amikacin, fosfomycin and colistin

To investigate whether MgOC\(_{150}\) can be used together with antimicrobial agents, we estimated the capability of MgOC\(_{150}\) to adsorb some classes of drugs that are commonly used for treatment of \( P. \) aeruginosa infections. Levofloxacin, aztreonam, piperacillin, cefepime and meropenem were highly adsorbed by MgOC\(_{150}\) because these drugs were eliminated more than 60% after incubation with MgOC\(_{150}\) (Fig. 8). In contrast, amikacin, fosfomycin and colistin retained more than 75% even after incubation with MgOC\(_{150}\) (Fig. 8). We determined that MICs of amikacin, fosfomycin and colistin were 2, 64 and 32 mg/L in PAO1 and >64, 64 and 16 mg/L in RPs.a-884, respectively (Table 5). After incubation with MgOC\(_{150}\), MICs of amikacin, fosfomycin, and colistin exhibited 2, 64 and 32 mg/L in PAO1 and >64, 64 and 32 mg/L in RPs.a-884, respectively (Table 5). These results suggest that MgOC\(_{150}\) does not impair the activities of amikacin, fosfomycin and colistin.

### 4. Discussion

*Pseudomonas*-caused bacteremia is commonly hospital-acquired and is a problem worldwide. It can result from some sources including primary infection in the lungs, gastrointestinal tract, urinary tract, skin, and soft tissue (Turner et al., 2014, Mittal et al., 2009, Zaborina et al., 2006). It is extremely hard to prevent bacteremia caused by intestinal *P. \) aeruginosa* because they cannot be selectively eliminated without killing other normal gut bacteria.

Several adsorbents have been approved as oral medicines, such as colestyramine and AST-120, which are used for the treatment of the

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Fig. 5. Cytotoxicity and adsorption of the recombinant LasB and AprA proteins. (A) Survival of Caco-2 cells after incubation with the purified recombinant LasB and AprA proteins. (B) The recombinant proteins (10 \( \mu \)g) were incubated with and without MgOC\(_{150}\) / AST-120. Relative (Non-adsorbed) protein levels are presented as the percentage of the value for samples after incubation with MgOC\(_{150}\) / AST-120 relative to that after incubation without MgOC\(_{150}\) / AST-120. Data plotted are the means from three independent experiments; and error bars indicate the standard deviations. Asterisks denote significance for values (\( p < 0.05 \)) of the protein level after incubation with MgOC\(_{150}\) relative to that after incubation without MgOC\(_{150}\)-
hypercholesterolemia and the pruritus in individuals with liver failure and treatment of progressive chronic kidney disease, respectively (Scaldaferri et al., 2013, Hegade et al., 2015, Niwa, 2011). However, no adsorbent medicine is approved for treatment of infectious diseases. MgOC$_{150}$ is used for industry purposes, including an electrode catalyst, a bioelectrode, and enzyme immobilization (Funabashi et al., 2017, Tsumujima et al., 2014, Mazurenko et al., 2018). This study proposed a potential benefit of MgOC$_{150}$ that attenuates the toxicity of intestinal $P$. aeruginosa (Fig. 1). Some extracellular molecules produced by $P$. aeruginosa, including LasB elastase, AprA alkaline protease and pyocyanin, disrupt intestinal epithelial cells serving as an intestinal barrier to prevent bacterial entry into the bloodstream (Fig. 5A, Hirakawa et al., 2021b). MgOC$_{150}$ confers protection to the intestinal barrier function against intestinal $P$. aeruginosa, including strains that are resistant to conventional antimicrobial agents, by adsorbing these molecules (Figs. 1 and 3-6, Tables 3 and 4).

MgOC$_{150}$ is predicted to highly adsorb molecules that are more than 30 kDa (Funabashi et al., 2017). Therefore, the effective adsorption of LasB and AprA by MgOC$_{150}$ is reasonable. In contrast to MgOC$_{150}$, AST-120 poorly adsorbed the LasB and AprA proteins (Figs. 3 and 5B).

The average pore size of AST-120 is approximately 2 nm, and this pore size may be too small to adsorb the LasB and AprA proteins, but it can highly adsorb pyocyanin (Fig. 6, Hirakawa et al., 2021b). Similar to AST-120, MgOC$_{150}$ adsorbed pyocyanin, although the size of the pyocyanin molecule is much smaller than the pore size of MgOC$_{150}$ (Fig. 6). The pore of MgOC$_{150}$ is produced from an Mg-containing template substrate. If some hydroxyl groups derived from this substrate molecule remain even after the pyrolysis process, a significant amount of a small-sized compounds may be bound by this hydroxyl group. For these reasons, MgOC$_{150}$ is a superior adsorbent compared to AST-120 for the suppression of $P$. aeruginosa virulence.

On the other hand, the adsorption ability of MgOC$_{150}$ for LolA and PA0423 was relatively low (Table 4 and Fig. 5B). Molecular sizes of the LolA and PA0423 monomers are 23 kDa and 21 kDa, respectively, and they are smaller than the pore size of MgOC$_{150}$. The LolA protein was shown to form a complex with lipoproteins in the periplasmic space (Takeda et al., 2003) while one zymography data showed that the PA0423 protein is secreted into the extracellular space where it forms a tetramer (Tang et al., 2009). These protein complexes may fit pores from MgOC$_{150}$. However, the results of the proteomics analysis and adsorption experiment imply that significant amounts of the LolA and PA0423 monomers may exist in the extracellular fraction under this study’s
culture conditions, which may explain the low adsorption ability of MgOC\textsubscript{150} relative to the drug amount (mg) after incubation with MgOC\textsubscript{150}. Data plotted are the means from three independent experiments; error bars indicate the standard deviations. Asterisks denote significance for values ($p < 0.05$) of drug amount after incubation with MgOC\textsubscript{150} relative to that after incubation without MgOC\textsubscript{150}.

![Fig. 8. Drug adsorption by MgOC\textsubscript{150}. Each drug was incubated in an aqueous solution with and without 30 mg MgOC\textsubscript{150} for 2 hours. The y axis shows the percent of drug amount (mg) after incubation with MgOC\textsubscript{150} relative to the drug amount (mg) after incubation without MgOC\textsubscript{150}. Data plotted are the means from three independent experiments; error bars indicate the standard deviations. Asterisks denote significance for values ($p < 0.05$) of drug amount after incubation with MgOC\textsubscript{150} relative to that after incubation without MgOC\textsubscript{150}.](image)

**Table 5** Amikacin, fosfomycin, and colistin MICs of *P. aeruginosa* PAO1 and RPs.a-884 strains

| Drugs         | MICs (mg/L) | PAO1 | RPs.a-884 |
|---------------|-------------|------|-----------|
| Amikacin      | 2 > 64      |      |           |
| Amikacin + MgOC\textsubscript{150} | 2 > 64      |      |           |
| Fosfomycin    | 64 64       |      |           |
| Fosfomycin + MgOC\textsubscript{150} | 64 64       |      |           |
| Colistin      | 32 16       |      |           |
| Colistin + MgOC\textsubscript{150} | 32 32       |      |           |

* Drugs were pre-treated with 30 mg of MgOC150 in 5 mL aqueous solutions for 2h, and used for MIC assays.

LasB is the most abundant extracellular protease produced by *P. aeruginosa* and its role on the pathogenesis has been characterized (Beaufort et al., 2013). This protease degrades mucins, cadherin, and surfactant proteins, which induce tissue injury and bacterial dissemination (Alcorn and Wright, 2004, Golovkine et al., 2014, Kuang et al., 2011, Mun et al., 2009). The cytotoxicity of the LasB protein is well defined in respiratory epithelial cells. This study demonstrated that the recombinant LasB protein impairs intestinal epithelial cells (Fig. 5A). The LasB protein also degrades IgG, IgA, several cytokines (including IFN-γ and IL-6), and protein molecules involved in the complement system (Bainbridge and Fick, 1989, Bastaert et al., 2018, Heck et al., 1990, Parmely et al., 1990, Saint-Criq et al., 2018). AprA is another extracellular protease contributing to the virulence of *P. aeruginosa*. Similar to LasB, AprA has the ability to modulate immune responses because it degrades TNF-α and IFN-γ together with the C3 and C2 protein complexes composing the complement system (Hong and Ghebrehiwet, 1992, Laarman et al., 2012, Parmely et al., 1990). Thus, adsorption of the LasB and AprA proteins using MgOC\textsubscript{150} decreases its cytotoxicity in the gut and the ability of bacteria to evade the host immune system. AprA activity has been also shown to sustain the production of pyocyanin (Iiyama et al., 2017). Therefore, adsorption of the AprA protein may lead to a decreased pyocyanin production.

Pyocyanin is a major virulence molecule associated with the progression of chronic respiratory infections, such as in patients with CF (Wilson et al., 1988). We suggest that this molecule also has an important role in the pathogenesis of intestinal *P. aeruginosa* for the following reasons. Pyocyanin damages intestinal epithelial cells because the phzA1/B1 mutant that does not produce pyocyanin is much less toxic in Caco-2 cells than the pyocyanin-producing parent PAO1 strain (Hirakawa et al., 2021b). This molecule also contributes to anaerobic survival, iron acquisition, and biofilm development (Costa et al., 2015, Glasser et al., 2014, Wang et al., 2011). Available oxygen and iron for intestinal *P. aeruginosa* could be limited in the gut by microorganism competitors while biofilm formation enables *P. aeruginosa* to evade many antimicrobial compounds and intestinal immune systems (Giofu and Tolker-Nielsen, 2019). Therefore, pyocyanin may support the colonization and survival of *P. aeruginosa* in the gut. MgOC\textsubscript{150} can eliminate the benefits conferred by pyocyanin for intestinal *P. aeruginosa*. Additionally, pyocyanin has a bactericidal activity against many gut microbiota which act as an intestinal barrier. One study suggested that *E. coli* protects mice from intestinal *P. aeruginosa* (Christofi et al., 2019). Thus, adsorption of pyocyanin by MgOC\textsubscript{150} may protect the host from intestinal *P. aeruginosa* by alleviating damage to intestinal epithelial cells and disturbance of gut microbiota, including *E. coli*, contributing to the homeostasis of the intestinal barrier.

The production of LasB, AprA and pyocyanin is induced by LasI-LasR and RhlI-RhlR-mediated QS systems because strains that lack these QS activities do not produce these molecules adequately (Gambello et al., 1993, Pearson et al., 1997, Van Delden and Iglewski, 1998). LasB, AprA and pyocyanin-associated virulence can be attenuated by disrupting these QS systems. For this reason, interference of QS is noticed as a promising alternative antimicrobial strategy (Hirakawa and Tomita, 2013, Whiteley et al., 2017). Since MgOC\textsubscript{150} does not affect the QS systems (Fig. 7), it may be incorporated with novel approaches to prevent and treat *P. aeruginosa* infections.

Undesirably, MgOC\textsubscript{150} adsorbed levofloxacin and some of β-lactams despite of small-sized molecules (Fig. 8). These drugs might be trapped by some residual hydroxyl groups within the MgOC\textsubscript{150} material as well as pyocyanin. In contrast, the adsorption capability for amikacin, fosfomycin, and colistin was low (Fig. 8). For this reason, MgOC\textsubscript{150} did not interfere with activities of amikacin, fosfomycin, and colistin (Table 5). Fosfomycin and colistin are well known as last resort agents in the treatment of MDRP strains (Michalopoulos et al., 2011, Montero et al., 2009). We suggest that MgOC\textsubscript{150} may offer a benefit by assisting fosfomycin and colistin therapy.

Since MgOC\textsubscript{150} is currently used for only industrial purposes, it is important to note that its safety must be carefully validated for medical applications, although this study provided evidence that MgOC\textsubscript{150} does not impair human intestinal epithelial cells (Fig. 2B). Some extensive studies are necessary to be addressed whether MgOC\textsubscript{150} adsorb any other molecules including beneficial compounds and fully understand the impact on host. However, microporous carbon adsorbents may open the door to the development of novel antimicrobial therapy.

**Author Contributions**

H. Hirakawa, A. Kimura, K. Tanimoto, and H. Tomita designed the research, and wrote the manuscript. H. Hirakawa, A. Kimura, and H. Tomita analyzed the data. H. Hirakawa, A. Kimura, A. Takita and S. Chihara, K. Tanimoto performed the research.

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