Early Propagation of Catalase-proficient *Proteus vulgaris* is Associated with Robust Increase in *Escherichia coli* Population in DSS-treated Mouse Colitis model

Yaeseul Kim

Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Sang Sun Yoon

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Yaeseul Kim

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YONSEI UNIVERSITY

This certifies that the Master's Thesis of

Yaeseul Kim is approved

Thesis Supervisor : Sang Sun Yoon

Thesis Committee Member#1 : Dongeun Yong

Thesis Committee Member#2 : Jaehee Cheon

The Graduate School

Yonsei University

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ABSTRACT

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On the onset of ulcerative colitis (UC), the microbial dysbiosis is induced which promotes disease progression. Here, we attempted to identify the commensal microbe that propagates under the hyper-inflammatory condition to understand the mechanism underpinning the ability of bacteria to survive in stressful conditions. Since hyper-inflammation is strongly associated with oxidative stress, we aimed to identify bacterial species with high resistance to reactive oxygen species (ROS). Through our investigation, the relative abundance of *Escherichia coli* and a *Proteus* sp. were highly increased during dextran sulfate sodium (DSS)-induced colitis in mice. Interestingly, *Proteus vulgaris* isolated from inflammation induced mouse feces

exhibited an outstanding catalase activity. Further analysis with whole genome sequencing revealed an extra alkyl hydroperoxide reductase (*AhpD*) that was found from the ROS-resistant *P. vulgaris* strain only. In addition, the ROS-sensitive *E. coli* remained viable when grown together with *P. vulgaris* strain, in lethal concentration of hydrogen peroxide. Combining all above, the outstanding activity of the extra ROS-degrading enzyme is somehow linked to the propagation of *P. vulgaris* in hyper-inflammatory condition, which might provide the selective advantage for both *P. vulgaris* and ROS-sensitive *E. coli* in colitogenic environment

Key words: *P. vulgaris*, DSS-induced colitis, *E. coli*, ROS resistance

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I. INTRODUCTION

Previously, we postulated that a robust catalase activity expressed in an atypical *E. coli* (at*Ec*) is critical for its clonal expansion in streptomycin-treated condition [1], for which large quantities of reactive oxygen species (ROS) (•OH, •$O2^-$, and H_2O_2) are released by stress responses in host intestine [2]. The commensal microbes possessing excellent ROS removal capability may be useful for the treatment of various diseases associated with high level of ROS production. For this purpose, it is reasonable to accommodate an inflammatory experimental mouse model, since inflammation is strongly associated with ROS stress [3-5].

ROS release during inflammation is more than just a defensive mechanism against bacteria including pathobionts, potential disease-causing commensals that, under normal circumstances, live as symbionts [6]. ROS-induced oxidative stress distorts wide range of signaling processes and promotes tissue injury [7-9]. Any changes in microenvironment meant that an alternation in microbial communities is induced. Intestinal dysbiosis, also defined as a shift in bacterial communities, are strongly associated with several diseases such as Type 1 Diabetes, Liver diseases, inflammatory bowel diseases and typically known brain diseases including Alzhemier's and Parkinsons diseases. Therefore, it is outermost important to identify bacteria that survive in altered microenvironment because the mechanism if revealed, can be used for therapeutic purposes for ameliorating diseases caused by microbial disruptions. In this study, we were specifically interested in identifying the bacteria that survive in ROS-rich colitogenic environment which mimics inflammatory bowel diseases in humans.

For this, Dextran sulfate sodium (DSS) salts were used to induce intestinal dysbiosis. A dramatic shift in the ratio of facultative anaerobes has been reported. Together, the dominant obligate anaerobes are notably reduced, suggesting the changes in oxygen contents within the niche. Likewise, major bacteria dominated following dysbiosis are Proteobacteria, which are facultative anaerobes. In addition, a distinct shift in population of *Faecalibacterium prausnitzii* and *Enterobacteriaceae* were reported. This matches with our findings as our illumina sequencing data indicated an explosive proliferation of *Enterobacteriaceae* groups (17%). We identified that *E. coli* are the most dominant species found in chronic colitis mouse

model. Interestingly, *E. coli* are not propagated in the initial stage of colitis. However, when colonic tissues collapse and become extremely hostile, *E. coli* take an advantage of chaos to maintain their unequaled colonization. In parallel, *Proteus* sp. undergo consistent propagation in hyper-inflammatory condition during colitis. Normally, the entry of *P. mirabilis* is strictly restricted by specific surface marker expressed on healthy colon tissue namely Lypd8, known for trapping the flagellated bacteria, particularly *P. mirabilis* and *E. coli*, but upon disruptions, *Proteus* sp. swarms over readily to reach the destination and start colonizing from the very beginning stage of colitis development.

In this study, we aimed to induce hyper-inflammatory condition using effective but toxic chemical molecules called DSS to accommodate an environment with increased oxygen tension and hence ROS levels. We sought to believe that bacteria bearing high resistance to both hydrogen peroxide and superoxide are critical in maintaining their survival within such complex and disarrayed surroundings.

II. MATERIALS AND METHODS

1. Animal strains and testing

7-9 week old male C57BL/6 mice were purchased from Orient Bio (Seoungnam, Korea). All mice were maintained in a specific pathogen free (SPF) facility. Mice were kept in separate cages (4 per cage) and secured for the period of acclimatization before any treatments provided. To induce colitis in mice, we used 5% Dextran sulfate sodium salts (DSS; 40kDa) mixed in water and provided for a week. DSS was changed at least twice in a week to prevent any contamination. Mouse body weights were recorded everyday to assess the process of inflammation development. Also, the fresh mice fecal samples (10 feces per cage) were collected for microbial analysis. All mice were sacrificed on day 7 after DSS treatment. Colon tissues extracted on the day of sacrifice were widely used for microbial analysis, histology and qRT-PCR.

2. Bacterial strains and media

The *Proteus vulgaris* and *Proteus mirabilis* strains used in this study is described in supplementary Figure 2. *Proteus* species were grown and maintained in Luria-Bertani and Tryptic Soy Broth. Media were usually solidified with 1% Agar unless otherwise stated.

3. Quantitative real-time PCR

Bacterial DNA was extracted from mouse feces and colons using Fast DNA Spin Kit for Feces (MP Bio) and quality was assessed by Nanodrop before further use. For dye mixture, 7.6ul DW, 10ul SYBR Green, 0.4ul ROX I dye, 1ul mixture of both forward and reverse primers and 2ul DNA templates were used. The details for the information of primers are listed in supplementary table 1

4. Biochemical tests

For indole test, *Proteus* strains were inoculated in 5ml Trypton Broth (BD Bacto™ Trypton) and incubated for 24 hr at 37°C in a light-protected, humidity-controlled incubation shaker. 0.5ml Kovac's reagent (Sigma) was added to the bacterial culture, without shaking. The presence of decarboxylases can be tested by inoculating bacteria in 5ml of ornithine decarboxylase broth at two different time points, 24 and 48hrs at 37°C shaking incubator. The pH drop in the media turns the broth to purple, indicating the presence of ornithine decarboxylase enzyme. If bacteria cannot utilize the sugars in the media, the color remains yellow, inferring the negative result.

5. Catalase activity assay

The protocols for catalase activity assay was adapted from T. Iwase et al.[10] The prepared bacterial protein extracts were incubated with Triton-X-100 in 1 to 1 ratio before any exposure to hydrogen peroxide. Once hydrogen peroxide is added into the tube, the bubble formations are visualized within a minute

6. Viability after exposure to hydrogen peroxide

All bacterial strains were grown overnight in 5 mL Luria-Bertani Broth (LB) (BD Bacto™) in 50 mL conical tube for 16-18 h at 37°C in a light-protected, humidity-controlled incubation shaker. Bacterial cells were diluted 1:100 in 10 mL LB and further incubated for 2 hr until reach to early-exponential phase. Bacterial cultures were treated with 0, 2, 4, 6, 10, 20, 30, 40 and 50 M H_2O_2 and incubated for 4hrs. The viability was measured by counting the numbers of colonies (CFU/ml) on LSW^- plate. The composition in LSW^- agar was adapted from R. Belas et al (1991).[11]

7. Viability after exposure to superoxide

Overnight cultures were diluted to 1:100 in 5 mL Luria-Bertani Broth (LB) (BD Bacto^{TM}). The media were freshly changed and incubated for 2hrs at 37^{o}C in a light-protected, humidity-controlled incubation shaker. The OD_{600} before exposure to Plumbagin and Phenazine methosulfate (Tocris) were approximately 0.1. The bacterial cells was treated with various concentrations of Plumbagin ranging from 0 to 300 ug/ml. The viability was measured by counting the numbers of colonies (CFU/ml) on LSW^{-} plate.

8. Native gel assay

The protocol on native gel was adapted from Wayne & Diaz (1986) and W. Woodbury et al (1971).[12,13] Overnight grown bacterial cultures were diluted to -100 fold in fresh LB and incubated at 37^{o}C in a light-protected, humidity-controlled incubation shaker until OD_{600} reach at late exponential phase (~0.8). For washing step, 10mL Dulbecco's phosphate-buffered saline (DPBS) was gently poured on top of bacterial culture then centrifuged down at 4000rpm for 30 minutes at 4 ^{o}C. The supernatants were discarded and the pellets were suspended in 300ul of 50mM Tris-HCl (pH7.4). Bacterial lysis was preceded by sonication in which the instrument was set at 3-9 seconds routine (cell breaking-resting) for at least 1 minute. When it become clear, the cells were further centrifuged down at 13000rpm for 30 minutes at 4 ^{o}C. Protein quantities from the supernatants were measured by Bradford assay. After normalization, the protein extracts were loaded on the freshly prepared 7.5% acrylamide gel. Electrophoresis was done at 100V for approximately 5 hours. The gel was stained with two different substrates. Initial staining was preceded by rocking the

gel with 3'3- diaminobenzidine for 20 minutes. Further staining with ferricyanide mixtures (2% ferric chloride and 2% potassium ferricyanide) dyes the gel and intensifies the band.

9. Whole genome sequencing

KCTC2566 (*P. mirabilis* type strain), KCTC2579 (*P. vulgaris* type strain) and the isolated *P. vulgaris* strain were used for whole genome sequencing analysis. To extract DNA, we used G-Spin^{TM} Genomic DNA Extraction Kit. The protocols in the kit was modified to enhance the quality and efficiency. The quality of extracted DNA was initially tested by A_{260}/A_{280} ratio greater than 1.8. To run PacBio 20kb service, more than 16ug DNA were required. The sequencing service was done by ChunLab.

10. DNA Extraction from fecal and colon samples

Fast DNA Spin Kit for Feces (MP Bio) were used for DNA extraction from both mouse feces and colons. The freshly collected feces (n=10) and colon tissues were finely homogenized before further treatments are given. For colon tissues, the homogenized samples were centrifuged at 4^{o}C in 3000rpm for 5 minutes. The supernatants were used for further centrifugation at 13000 rpm for 5 minutes. The pellets are then washed with sodium phosphate buffer provided by DNA extraction kit.

III. RESULTS

1. Establishing a mouse colitis model by DSS treatment.

To establish colitis in murine model, we used Dextran Sulfate Sodium (DSS) salts as it is the most potent and effective chemical used for inducing hyper-inflammation. Here, we used 7-9 weeks old male C56BL/6 mice as an inflammatory murine model. After a week of acclimatization, the mice were weighted, mixed and separated into 2 groups (control and 5 % DSS) (n=6), to minimize errors caused by variations in body weight. Mice were treated with DSS by drinking water containing DSS for 7 days (Fig. 1A). The severity of DSS is often measured by scoring index that estimates three major criteria (body weight loss, stool consistency, Hemoccult positivity) set by researchers [10].

Effects of DSS treatment were first evaluated by monitoring mouse body weights. Body weight loss was obviously more significant with 5 % DSS. At 5 days post-treatment, body weight was reduced by 10 %, after which body weight loss was accelerated. After 7 days of treatment, body weight was reduced by 25 % (Fig. 1B), while ~10 % body weight loss was observed with 2.5 % DSS (data not shown). None of mice appeared to be ill or suffered until 4~5 days after the treatment. Since we modulate chronic inflammation in colon, the colons from all groups were extracted following sacrifice and subjected for further analysis. Mouse colons were substantially shortened, in 5 % DSS treated groups (Fig. 1C). Together with this, the cecum becomes smaller in size (data not shown). The distal region of colon was fixed in 10 % formalin before H&E staining. A severe ulceration in colonic tissues was observed in 5 % DSS treated mouse, further indicating that colonic tissues are highly

inflamed by DSS treatment (Fig. 1D). We next homogenized colonic tissues and stained them with PO-1 fluorescent dye, known to specifically probe the presence of H_2O_2 [1]. As shown in Fig. 1E, fluorescent signals detected from colonic homogenates prepared from 5 % DSS treated mice were ~1.5-fold more intense than those of control group.

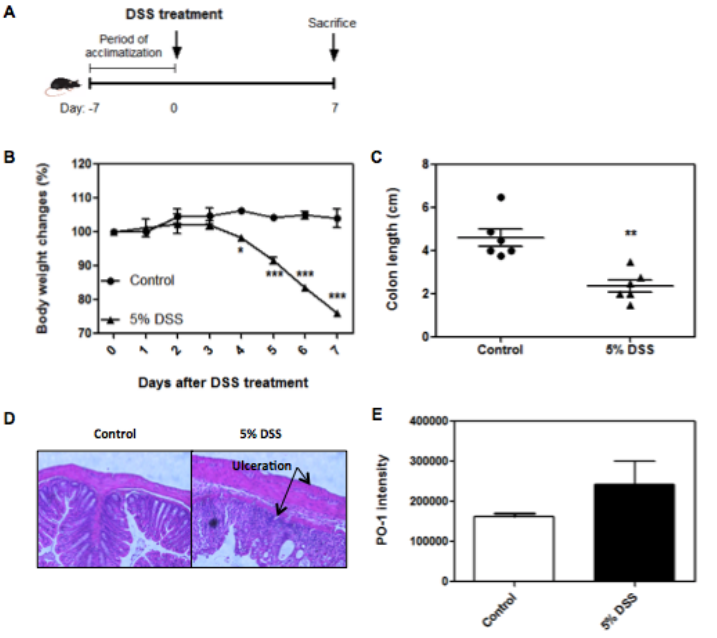


Figure 1. DSS-induced colitis in 7 weeks old C57BL/6J male mice. (A) A schematic diagram illustrating the design of experiment. All mice (n=8 per group) were sacrificed a week after DSS treatment. (B,C,D) The progression and severity of colitis was assessed by measuring (B) body weight changes (C) the differences in colon lengths (n=6 for control and 5% DSS, n=4 for 2.5% DSS group) and (D) Histology. (E) Hydrogen peroxide concentration within the inflamed colon was measured using Peroxy-orange 1 dye. Values are expressed as means±s.e.m. ***<0.001, **<0.005, *<0.05.

2. Propagation of *Proteus vulgaris* in response to the DSS treatment.

When fecal pellets, collected at day 1, were suspended and inoculated for growth in LB agar plates, larger numbers of bacterial colonies were detected in 5 % DSS treated vs. control group (Fig. 2A). Of note is that the colonies of robust swarming motility were increased in DSS-treated group (Fig. 2A). Although a limited portion of fecal microbes can grow in LB media, these results strongly suggest that under perturbed circumstances in the gut, the population change might have been induced among commensal microbes during the first 24 hrs of the treatment. The 16S rRNA gene sequencing suggested that colonies of hyper-swarming phenotype are *Proteus vulgaris* (data not shown).

In accordance to this, bacterial DNA extracted from both mouse feces and colons were subjected to quantitative real-time PCR to determine if *P. vulgaris* undergo population expansion through treatments. In this study, 2 different primers were used; 16S rRNA gene and nitrate reductase gene that specifically amplify the corresponding genes in *P. vulgaris*. To confirm the specificity of primers, we ran PCR with DNA templates from 3 different *P. vulgaris* strains and one *P. mirabilis* type strain. The detailed information of primers used is listed in Table S1. With this, approximately 10-fold and 20-fold increase in *P. vulgaris* population was observed in mouse feces and colon samples, respectively (Fig. 2B).

3. Characterization of *P. vulgaris* species by biochemical tests.

It was found that *P. vulgaris* isolated from mouse gut exhibits different morphology compared to *P. vulgaris* type strain. As shown in figure 3A, the swarming is actively expressed in *P. mirabilis* but not in *P. vulgaris* type strain. Unlike to this however, the isolated *P. vulgaris* strain formed a clear and distinctive bull's eye pattern. Through our whole genome sequencing analysis, we found that the ANI values of *Proteus* species were >98% (Fig. 5C). Therefore, it was difficult to soley rely on the results of 16S rRNA sequencing when we came to bacterial characterization. To enhance the accuracy and reliability for bacterial characterization, we carried out indole and ornithine decarboxylase tests (Fig. 3B and 3C) in parallel with 16s rRNA sequencing, as these two tests are most widely used for differentiating *Proteus* at species level. For tryptophase assay, also known as indole test, the Kovac's reagent was introduced in overnight grown *Proteus* in Trypton broth. The red ring formation on top of broth indicates the positive result for indole production (Fig. 3B). Further, we showed that *P. mirabliis*, when inoculated in ornithine decarboxylase broth, the color of broth turns into purple from yellow, following the catalytic process activated by ornithine decarboxylase in *P. mirabilis* (Fig. 3C).

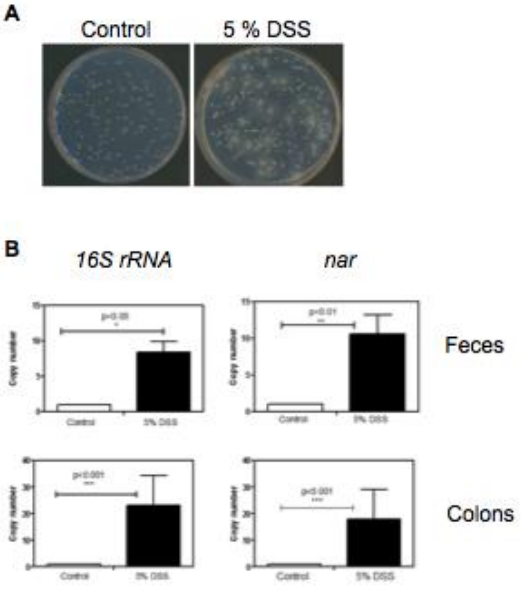


Figure 2. Propagation of *P. vulgaris* after DSS treatment. (A) Fecal samples (n=10) were collected on day 1 after DSS treatment and spread on LB plate. Bacterium forming swarming colony were proliferated in 5% DSS treated mice. This was also measured by quantitative PCR using bacterial DNA extracted from mouse feces (n=3), using primers specific for each bacterial species. (B) Quantitative real-time PCR using bacterial DNA extracted from colitis induced mice colons (n=4), using primers for *Proteus* genus, Nitrate reductase (specific for *P. vulgaris*) and isolated *P. vulgaris*. All values were normalized by primer '16S rRNA for all bacteria'. Values are expressed as means±s.e.m. ***<0.01, **<0.01, *<0.05.

4. An isolation of *P. vulgaris* with outstanding activities in ROS degradation.

To verify our hypothesis that ROS-resistant bacteria are the chosen survivals under inflammatory circumstances, the overnight grown *P. vulgaris* was sub-cultured, then treated with hydrogen peroxide and superoxide. To ensure that isolated *P. vulgaris* is highly responsive to oxidative stresses, we assessed the viability test after exposure to a wide range of ROS stresses, from mild to lethal. The isolated *P. vulgaris* strain remained viable after treatment with H_2O_2 at concentrations as high as 10 mM for 4 hrs (Fig. 4A). Even after the treatment with 20 mM H_2O_2, significant numbers of viable cells were detected. The *P. vulgaris* reference strain was highly sensitive to the treatment and complete loss of viability was observed with 4 mM H_2O_2 treatment (Fig. 4A). A *P. mirabilis* strain, phylogenetically close to *P. vulgaris*, was resistant to the H_2O_2 treatment. Viability loss was not observed until 10 mM H_2O_2 was used, whereas its viability was completely abrogated with 20 mM H_2O_2 (Fig. 4A). Likewise, the isolated *P. vulgaris* strain also exhibited strong resistance to the treatment with plumbagin that induces ROS stress[11]. Its viability was not substantially affected as plumbagin concentrations increased up to 140 μg/ml (Fig. 4B). The reference *P. vulgaris* strain, on the other hand, was highly sensitive to the treatment. Similar to what was observed with the H_2O_2 treatment, the *P. mirabilis* strain was relatively resistant to the plumbagin-mediated oxidative stress (Fig. 4B).

Next, we sought to measure catalase activities of tested strains to examine whether the ROS resistant phenotypes mirror with strains' H_2O_2 degrading capabilities. Protein extracts, prepared from exponential phase cells, were mixed with 100ul of Triton X-100 and 8.8M H_2O_2 in 1:1 ratio (Fig. 6A). As proteins contact with H_2O_2, a rise in

bubble formation was observed, depending on the catalase activity of each strain. Consistent with our results in viability assay, the catalase activity of the isolated *P. vulgaris* strain was the highest among 3 tested strains, while the reference *P. vulgaris* strain exhibited the weakest activity (Fig. 6A).

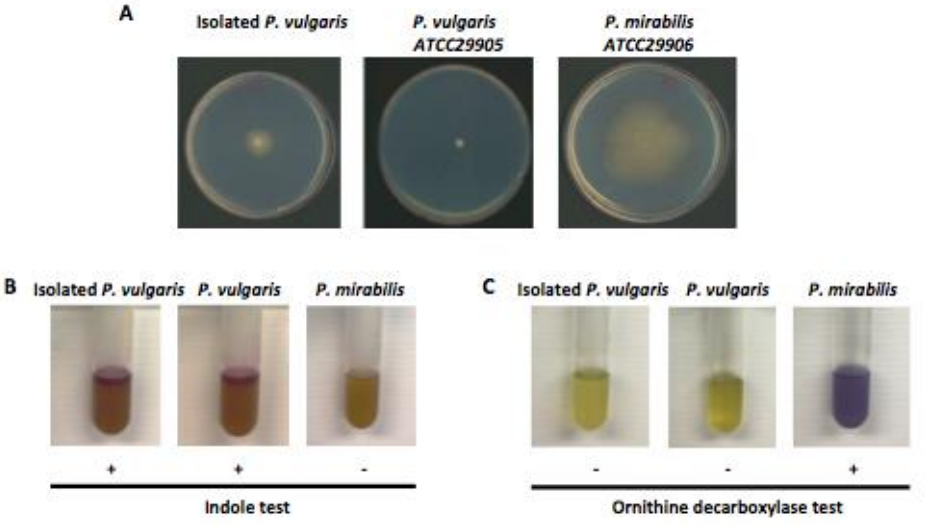


Figure 3. Characterization of *Proteus* sp by biochemical tests. (A) The figure illustrates the morphology of Isolated *P. vulgaris*, ATCC29905 (*P. vulgaris* type strain) and ATCC29906 (*P. mirabilis* type strain), accordingly. (B) The result of Indole test and (C) ornithine decarboxylase tests in Isolated *P. vulgaris*, ATCC29905 (*P. vulgaris* type strain) and ATCC29906 (*P. mirabilis* type strain). The red ring formation in (B) indicates indole positive and yellow solution in (C) indicates negative for ornithine decarboxylase activity.

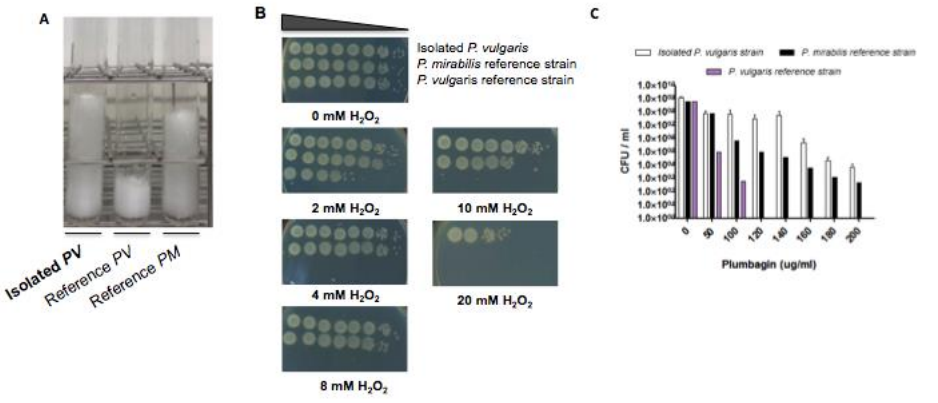


Figure 4. An isolated *P. vulgaris* strain exhibits outstanding activity in ROS degradation. (A) Bacterial cell extracts were treated with Triton-X-100 before exposure to Hydrogen peroxide. The height of bubbles indicate the activity of catalases (B) Non-denaturing 7.5% NATIVE Gel assay was performed to visualize the intensity of catalase present in the following cell extracts. (C) Viability assay after exposure to high concentration of hydrogen peroxide and superoxide.

5. The summarized result of whole genome sequencing.

To better understand the outstanding activity of the isolated *P. vulgaris* strain against high concentrations of hydrogen peroxide, we sequenced the whole genome of 3 *Proteus* strains including our isolated *P. vulgaris* and *Proteus* type strains *(*ATCC29905 and ATCC29906). The genome size and GC contents present in the isolated *P. vulgaris* strain was not significantly different when compared with two reference strains (Fig. 5A). This was further validated through our whole genome alignment analysis and ANI (Average nucleotide identity) values (Fig. 5B).

(a) Genome information

Strain	Genome size	G+C ratio	CDS	Chromosomes	Plasmids	Contigs
ATCC29906 (*P. mirabilis*)	4.09Mb	38.78%	3,678	0	0	1
ATCC29905 (*P. vulgaris*)	3.92Mb	37.97%	3,503	0	0	1
Isolated *PV*	3.85Mb	37.89%	3,358	0	0	1

(b) Whole genome alignment by Mummer dot plot

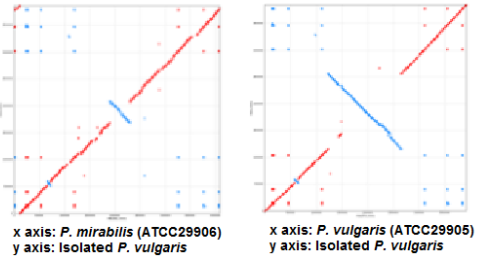


Figure 5. The summarized result of whole genome sequencing. (A) Genome information of three *Proteus* strains was compared according to genome size and GC contents. (B) The forward and reverse strands of isolated *Proteus vulgaris* vs Proteus *mirabilis* and isolated *Proteus vulgar*is vs *Proteus vulgaris* were aligned by Mummer dot plot.

6. Alkyl hydroperoxide reductase like gene is uniquely expressed in the *P. vulgaris* isolates.

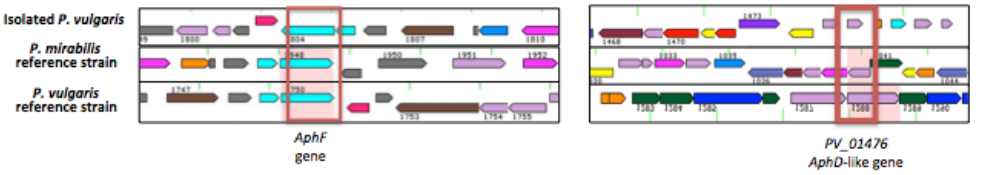
Using the whole genome data, we aimed to identify a single gene that might be responsible for high catalase activity. Our initial approach was to sort out the candidate genes involved in oxidative stress and from this, approximately 300 different proteins present in the isolated *P. vulgaris* strain were found. These were then BLASTed to identify if any amino acid sequences match with 'catalase' or 'peroxidase' on the UniProt database. This led us with 18 different proteins with similar sequences in either catalase or peroxidase. For accuracy, these candidate genes were further BLASTed in NCBI protein database. We came into conclusion that one catalase gene and two peroxidase genes were present in the isolated *P. vulgaris* strain. A catalase showed 99 % similarity with *KatA* but not with *KatE* or *eKatE* [1]. Moreover, *KatA* is a catalase expressed within all *Proteus* sp, indicating that different hydrogen peroxide targeting enzyme may be more important than *KatA*. Two peroxidases found in the isolated *P. vulgaris* strain were alkyl hydroperoxide reductase F (*AhpF*) and an uncharacterized protein (*PV1476*) (Fig. 6A and 6B). Like *KatA*, *AhpF* is highly conserved in all *Proteus sp*. Accordingly, 98 % and 96 % similarity in amino acid sequences in two type strains of *P. vulgaris* and *P. mirabilis* were seen (Fig. 6A). An extra alkyl hydroperoxide reductase (*PV1476*) with conserved CXXC motif in amino acid sequence (Fig. 6C) was identified from the isolated *P. vulgaris* strain only. In addition, CXXC motif has been reported to be strongly involved in redox function. Through our comprehensive studies using CLgenomics and NCBI protein database, we revealed that this hypothetical protein predicts to function as *AhpD*, an antioxidant

protein with alkyl hydroperoxidase activity. The BLAST results indicated that alkyl hydroperoxide reductase has a conserved domain of YciW, showing the greatest homology to carboxymunolactone decarboxylase family protein in *Cosenzaea myxofaciens* (80% amino acid identity) but low identities with *Proteus* sp (>30% amino acid identity). Although catalase/peroxidase related studies were not investigated in *Proteus* species, the importance of peroxidases such as alkyl hydroperoxide reductases in *Mycobacteria* and *E. coli* are highly acknowledged [12,13]. Based on our BLAST results and genome studies, we can speculate that an alkyl hydroperoxide reductase (*AhpD*) take a role in reducing cysteine residues at alkyl hydroperoxide C, thereby enhancing ROS degrading activity.

A

Peroxidase genes	*P. mirabilis* type strain (ATCC29906)	*P. vulgaris* type strain (ATCC29905)
AphF genes	96%	98%
PV1476 (*AphD*-like gene)	48%	25%

B



C

>PV_01476

MNSRVSLSKKNAKAYSQLITMSNNIDEQAILAGLEEGFIHLLKLRLSQINGCAFCVRLHT
QDAQKCGVSIDKIALVATWKETEYFSEKEKSALSLAESVNLIHNEYAPDDIYQESAKYWD
EAQLASIEWISIIIGAFNRVAITSRYSVKP

Figure 6. The newly found alkyl hydroperoxide reductase (*AhpD*)-like gene is present in the isolated *P. vulgaris* strain only. (A) The table compares the amino acid similarities of both alkyl hydroperoxide reductase gene found in isolated *P. vulgaris* strain with two *Proteus* reference strains. (B) The gene map indicates the position where both *AhpF* and PV_1476 gene is located. (C) The amino acid sequence of PV_1476 gene was colored with blue and red. The blue region is highly conserved with alkyl hydroperoxidase reductase found in all *Proteus* strains while CXXC motif colored in red are mostly found in catalytic domain, indicating that it is responsible to hydrogen peroxide stress.

7. *E. coli* undergo explosive propagation in DSS-treated mice.

Here, we observed a rise in the population of *E. coli* forming metallic green sheen on EMB plate, as a response to DSS treatment (Fig. 7A). It is important to note that, compare to *Proteus* species, *E. coli* are not propagated at initial exposure to DSS. Throughout the days after treatments however, the growths of *P. vulgaris* and *E. coli* were increasing in sync (Fig. 7B). It is not surprising to state that *E. coli* strains proliferate throughout DSS treatment [14-16]. However, it is new to deliver that *E. coli* strains proliferate only at the later stages of enteritis, the point where a complete dysbiosis is induced. Since we focused in oxidative stress during hyper-inflammatory condition, we tested if proliferated *E. coli* colonies bear high resistant to ROS, just like *P. vulgaris* (Fig. 4), as a strategy to enhance bacterial survival. However, we realized that *E. coli*, when exposed to hydrogen peroxide, become very sensitive and fail to maintain their viability (Fig. 7C). Interestingly, when co-cultured with ROS-resistant *P. vulgaris* strain, the viability was rescued, even at high concentration of H_2O_2 (Fig. 7C). Taken together, we suggest an involvement of *P. vulgaris* in ROS scavenging process, especially in the beginning stage of DSS treatment and this somehow is associated with the following ROS-sensitive *E. coli* propagation in colitogenic environment.

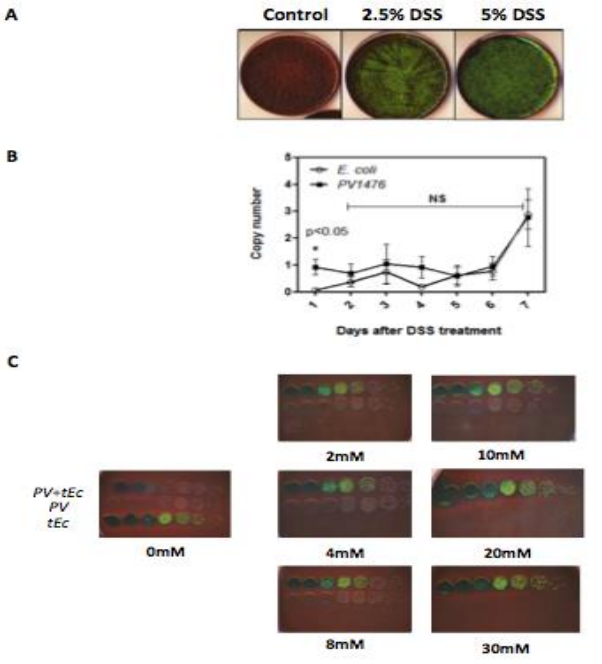


Figure 7. ROS-sensitive *E. coli* were rescued by ROS-resistant *P. vulgaris* after exposure to ROS (hydrogen peroxide). (A) Fecal samples (n=10) were collected on day 7 after DSS treatment and spread onEMB plate. The metallic green sheen colonies indicate *E. coli* strains. (B) Consequential proliferation of both *P. vulgaris* and *E. coli* during inflammatory development process. *P. vulgaris* is initially propagated after DSS is being treated. All values were normalized by primer '16S rRNA for all bacteria'. Values are expressed as means±s.e.m. ***<0.001, **<0.005, *<0.05. (C) ROS-sensitive *E. coli* strains maintained viability in high concentration of hydrogen peroxide when co-cultured with ROS-resistant *P. vulgaris* strain.

Table 1. Primers used in this study

Target group	Primer	Sequence (5'-3')	Source or reference
Proteus	16S *Proteus*_F	GTTATTCGTGATGGTATGGG	
	16S *Proteus*_R	ATAAAGGTGGTTACGCCAGA	[14]
Nitrate reductase	NR_F	CGAACTGGGAGGACGATTTA	In this study
	NR_R	CGGCATTTATCTTGGTCGAT	
PV_1476	*PV1476*_F	ATGGAGTATGTATGAATAGCCG	In this study
	*PV1476*_R	CCAGCCAATATTGCTTGCTC	
E. coli	*E. coli*_F	CAGCCACACTGGAACTGAGA	
	*E. coli*_R	GTTAGCCGGTGCTTCTTCTG	[1]
Bacterial 16S rRNA	Bac 16S_F	CGG TGAATACGTTCCCGG	
	Bac 16S_R	TACGGCTACCTTGTTACGACTT	[14]

F: Forward; R: Reverse

IV DISCUSSION

When DSS is administered, a huge impact is delivered to switch the healthy gut bacterial communities into harmful ones. The mice neither bleed nor lose weights unless the treatment continues to day 5 after DSS treatment [32]. This reflects that gradual alteration in bacteria is associated with the progression of colitis. Likewise, we observed an interesting change induced in bacterial communities during colitis development. A noticeable propagation of *Proteus* sp. were seen on day 1 after DSS treatment and population consistently rise in number as long as colitis is induced.
As mentioned previously, hyper-inflammation induced by DSS is strongly associated with rise in oxidative stress [2,15]. Thus, we hypothesized that ROS-resistant bacteria might be more proficient at colonization resistance in DSS-treated mouse. Since we found out that the proliferation of *Proteus* sp. are highly promoted, we isolated the colonies and exposed at extremely high concentration of hydrogen peroxide and superoxide (Fig. 4). Through our investigation, we realized that our isolated *Proteus* sp, namely *P. vulgaris* remained to be viable at 20mM of hydrogen peroxide. In parallel, the type strains of *P. vulgaris* and *P. mirabilis* (ATCC29905 and ATCC29906, accordingly) were treated identically as above, but these could not maintain viability.

We found that the isolated *P. vulgaris* strain possess a uniquely expressed alkyl hydroperoxide reductase gene, *AhpD*. According to the genome database, *AhpD* may be required for the reduction of the *AhpC* active site cysteine residues and for the regeneration of the *AhpC* enzyme activity. The presence of *AhpD* has not been demonstrated in *Proteus* sp. but other subunits of alkyl hydroperoxide reductases were investigated from many bacteria, including *Vibrio parahaemoviticus*, *Pseudomonas*

aeruginosa, *Campylobacter jejuni* and *Escherichia coli* [16-19]. The relevant studies indicated that an effective degradation of ROS molecules is facilitated by the presence of alkyl hydroperoxide reductases.

As to our observation in *P. vulgaris* proliferation, *E. coli* undergoes explosive propagation in response to DSS too. However, unlike to *P. vulgaris*, which increase in number at the early stage of DSS treatment, *E. coli* expand their population at the late stage of colitis (Fig. 7). The cultivated *E. coli* strains formed metallic green sheen on EMB plate. This finding was interesting as our previous study found a colorless colony forming *E. coli* strain that was propagated in response to antibiotic treatment [1]. This was named as atypical *E. coli* due to their outstanding activity in ROS degradation. We believed that the proliferation of atypical *E. coli* was due to their advantageous feature in ROS resistance as large amounts of ROS were released by an increased LPS following antibiotics treatment.[3] Therefore, we assumed, as the following context, that the colorless colony forming atypical *E. coli* would be the dominant species as the result of DSS treatment. Unlike to this assumption however, the ROS-sensitive *E. coli* strains were propagated. In terms of growth rates, the ROS-sensitive *E. coli* and ROS-resistant *P. vulgaris* were not significantly different, except that P. vulgaris underwent an early expansion by DSS treatment (Fig. 7). Together with our *in vitro* result (Fig. 7C), we speculate that an initial propagation of *P. vulgaris* makes the favorable condition for *E. coli* expansion in the later stages.

V. CONCLUSION

Inflammatory bowel disease (IBD) is one of common diseases occurring in worldwide. Both Crohn's disease (CD) and Ulcerative colitis (UC) are two major types of IBD and are distinguished by the extent of inflammation within the digestive system [20]. UC is associated with hyper-inflammation confined in the distal colon and is easily inducible in murine models by using either DSS or 2,4,6-Trinitrobenzenesulfonic acid (TNBS) [21,22]. The etiology of UC occurrence is not fully understood but its development is known to be facilitated by certain genes, environmental factors (diet, stress, etc) and gut microbiota [23-25]. Most importantly, the implicative roles mediated by bacterial communities are highly appreciated, as good and bad bacteria in colitis-induced gut had been determined [26-28]. The studies acquired data from Next Generation Sequencing (NGS) platform in order to analyze the microbial contents within colitogenic gut. To add on, the proliferation of certain *Firmicutes* including *Escherichia* and *Clostridia* were expanded in number [29,30] while Butyrate producing bacteria (BPB) and *Lactobacialles* were significantly reduced [31]. Studies have also modulated these bacteria to investigate the effectiveness in ameliorating colitis [31-34]. In this respect, certain groups of bacterial genus had been designed for use of hallmarks in IBD [35]. However, data obtained from NGS provides the big image of what had been altered according to DSS treatment but do not necessarily infer us about the changes in bacteria, especially in species level. Thus, researchers are putting an effort to identify and reveal the bacteria that play essential roles during colitis development.

Here, we focused 'hyper-inflammatory condition' mediated in DSS-induced

colitis. Although, other potential factors are strongly associated with the process of inflammation, our attempt was to isolate the bacterial species harboring the high antioxidant activity. In this investigation, we perceived an interesting phenomenon with obvious increase in swarming morphology forming bacteria in day 1 after DSS treatment. Through 16S rRNA sequencing, this bacterium was designated as *P. vulgaris*, which was very similar to *P. mirabilis*. Despite the fact that *P. mirabilis* is closed to *P. vulgaris*, it is the first time to demonstrate the association of *P. vulgaris* with IBD. Further, *P. mirabilis* was reported to elicit colitis in murine models by facilitating the host immune responses [36,37] but none had reviewed their activity in ROS degradation. Our extensive research in catalase-focused studies has found that the isolated *P. vulgaris* exhibits an extraordinary enzyme that targets hydrogen peroxide. So far, we did not knock-out the potential candidate gene that is believed to be important in ROS degradation, but through out analysis in whole genome sequencing, we identified a unique alkyl hydroperoxide reductase (PV1476) that was only expressed in our isolates (Fig. 6B). This bacterium remained to be viable at extremely high concentration of hydrogen peroxide and superoxide stress. Coincidentally, the ROS-sensitive *E. coli* strain, when co-cultured with ROS-proficient *P. vulgaris*, the lethality was rescued at 30mM hydrogen peroxide (Fig. 7C), which is detrimental to all known bacterial strains. Interestingly, it was found that the initial growth rate of both ROS resistance *P. vulgaris* and ROS sensitive *E. coli* strains in DSS treated mouse feces were not any different (Fig 7B). Our speculation based on this data is that the initial proliferation of ROS-resistant *P. vulgaris* brings beneficial effects for *E. coli* propagation in DSS-induced intestinal

dysbiosis circumstances.

In summary, when an environment within the gut is exacerbated and a distinct shift in microbial communities is induced, colitis develops [25]. The host tissues lose their proximity that the restricted area down to colonic epithelial cells are exposed to exogenous sources [27]. Extremely high oxidative stress is induced and this promotes inflammation.0 At the same time, the ROS-resistant *P. vulgaris*, a pathobiont that reside within mouse gut has undergone explosive propagation, compared to ROS-susceptible *E. coli* in the very early stage of DSS treatment. Still, we lack our evidence to state an existence of interaction between *P. vulgaris* and *E. coli*, but at least, we revealed an unraveled functions in *P. vulgaris* to improve our knowledge in their survivals upon DSS treatment. Further investigations with gene knockout will provide even more reliable and comprehensive insights in understanding the importance of ROS degradation for bacterial survival in hyper-inflammatory condition.

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ABSTRACT (IN KOREAN)

대장염이 유도된 마우스에서 뛰어난 카탈라제 활성을 보인 프로테우스 불가리스 균의 초기증식과 대장균 증식의 연관성

<지도교수 윤 상 선>

연세대학교 대학원 의과학과

김 예 슬

대장염이 유도됨에 따라 박테리아 분포가 변화되고 이와 같은 현상은 대장염 발전 및 진행 과정에 영향을 준다. 대장염이 진행되면 초염증 상태가 유도되며 산화 스트레스의 농도가 높아진다. 따라서, 본 연구진은 대장염이 유도되었을 때 동반되는 초염증 상태에서 활성산소에 뛰어난 저항성을 보이며 성장에 영향을 받지 않는 균을 동정하는데 목적을 두고 연구를 시작하였다. 대장염이 유도된 마우스 모델의 분변을 분석하여 봤을 때, *프로테우스 불가리스* 균의 특이적인 초기 증식 및 대장균의 성장이 목격되었다. 실험 결과에선, 폭발적인 증식을 보인 *프로테우스 불가리스* 균이 고농도의 활성산소가 노출된 환경에서도 뛰어난 저항성을 보이며 생존을 유지 시키는 것을 확인할 수 있었다. 또한, 유전체 분석을

통해 알아본 결과, 다른 *프로테우스* 균에 비해 동정된 *프로테우스 불가리스* 균에서만 특이적으로 발현되는 alkyl hydroperoxide reductase (*AhpD*) 가 있다는 것을 알 수 있었다. 더 나아가, 대장염 모델에서 증식한 *대장균*의 경우 활성산소에 대해 매우 민감하게 반응하였으나, 활성산소 저항성이 뛰어난 *프로테우스 불가리스* 균과 같이 배양되었을 때는 고농도의 활성산소가 처리되어도 성장에 영향을 받지않는다는 것을 알 수 있었다. 따라서, *프로테우스 불가리스* 균의 뛰어난 활성산소 저항성은 초염증이 유도되었을 때 목격된 *프로테우스 불가리스* 균의 초기성장에 도움을 주었을 것이라고 추측할 있다.

핵심되는 말 : 대장염, 초염증, 활성산소 저항성, *프로테우스 불가리스*, *대장균*