이학박사학위논문

Comparison of virulence and immunogenicity of *Vibrio cholerae* cultured in aerobic and anaerobic condition

호기배양과 혐기배양 콜레라균의 병독성과 면역원성의 비교

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자연과학대학 협동과정 유전공학전공

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2018년 12월

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Abstract

Comparison of virulence and immunogenicity of *Vibrio cholerae* cultured in aerobic and anaerobic condition

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Objective

Cholera is an acute intestinal infectious disease caused by *Vibrio cholerae*. *V. cholerae* is endemic in many low-income countries, particularly in areas with inadequate sanitation and poor food hygiene practices. To date, three oral cholera vaccines (Dukoral, Shanchol and Euvichol) have been approved from the WHO and licensed in several countries. Although the pathogenesis caused by *V. cholerae* occurs in the intestine (an anaerobic environment), the above oral

vaccines are formulated with *V. cholerae* cultured in aerobic conditions. Moreover, anaerobiosis-induced microbial gene regulation and virulence are not fully elucidated in *V. cholerae*. The objective of this study is to examine the effect of anaerobic growth of *V. cholerae* as the aspect of immunogenicity and protectivity.

Methods

To investigate immunogenicity and protectivity of *V. cholerae* grown under anaerobic conditions, a clinical strain *V. cholerae* O1 El Tor T19479 was cultured in an anaerobic chamber maintained by circulating a 5% H_2/ 5% CO_2/ 90% N_2 gas mixture. Human epithelial cell lines, Caco-2 and HT-29 were exposed to *V. cholerae* for 1 h to analyze colonization of *V. cholerae*. The mRNA expression of *tcpA-F, ompA, ompU, tolC, falB, HA/P, acfA, gbpA, toxTR, tcpPH, aphAB,* and *CRP* was determined using real-time polymerase chain reaction (PCR). *V. cholerae*'s colonization in mouse intestine was assessed via oral inoculation under anesthesia with ketamine and xylazine. Infectivity of *V. cholerae* was determined by oral challenge in mice treated with streptomycin in drinking water or intranasal challenge that causes pneumonia. Mice were injected intranasally with heat-killed *V. cholerae* vaccine twice to induce immunogenic responses and

then followed by the challenge with live *V. cholerae* to test the protective effect of those vaccines. Vaccine-induced expression of Immunoglobulin (Ig) G, IgM, and IgA were determined using enzyme-linked immunosorbent assay (ELISA) and bactericidal antibodies were determined by vibriocidal assay. Expressions of TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, and IL-17A were analyzed through cytometry bead array.

Results

In this study, the colonization, infectivity and immunogenicity of *V. cholerae* grown under anaerobic conditions were investigated. In an anaerobic environment, *V. cholerae* displayed significantly increased bacterial adhesion along with up-regulation of *tcpA, tcpF* and *ompU*. This enhancement was limited to some strains which were capsule-deficient (O1 T19479, O1 N6961 and O139 CIRS134) and at a mid-log growth phase. Interestingly, the enhanced colonization ability was not linked to the virulence of *V. cholerae* in terms of mouse infectivity. Moreover, heat-killed cholera vaccine that was prepared in aerobic conditions had increased levels of anti-bacterial IgG in bronchoalveolar lavage fluids, lungs and sera when administered via a nasal route. An increase of Th1 and Th17 biased cytokines were also detected in mice administered with

vaccine prepared aerobically. These results were linked to a more efficient protection against cholera infections.

Conclusions

Overall, the anaerobic culture condition makes *V. cholerae* less infectious and low immunogenic while it appeals to increase the ability of *V. cholerae* to colonize the intestines.

Key words: *Vibrio cholerae*, anaerobic culture, vaccine, adhesion, infection

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Chapter I. Introduction

1.1. *Vibrio cholerae*

Vibrio cholerae is a member of the family Vibrionacease. *V. cholerae* is a Gram-negative, rod-shaped, facultative anaerobe which can grow with aerobic and anaerobic respiration. It naturally inhabits within an aquatic environment, especially brackish or salt water by forming microcolonies, biofilm or being planktonic. Growth of *V. cholerae* is stimulated by the addition of sodium chloride, however it is able to distinctively grow in nutrient broth without added sodium chloride differing itself from other *Vibrio* spp. *V. cholerae* has a single flagellum and three sets of chemotaxis signaling proteins (Che proteins) which make it a highly mobile and chemotactic bacterium. *V. cholerae* is generally classified according to the sugar composition of the O antigen of lipopolysaccharide (LPS); as of now, the bacterium consists of 206 O serogroups [1].

1.2. Cholera

V. cholerae is mainly transmitted to humans orally through contaminated food or drinks, which causes the acute intestinal disease, cholera. Most cases (95%) are asymptomatic or mild, but 5% have severe symptoms, such as diarrhea, vomiting and abdominal cramps. The onset of diarrhea and vomiting begin abruptly from within several hours to 2~3 days of ingestion, and the disease typically lasts 5 days. Because of severe dehydration through diarrhea, cholera is particularly dangerous to children and pregnant women.

Cholera has caused seven pandemics in the past 200 years. The last pandemic originated in Indonesia in 1961. Moreover, there have been many reported cholera outbreaks such as the 2010 Haitian cholera outbreak and the 2016 Yemen cholera outbreak. The first cholera pandemic (1817-24) began in the Bengal region of India and spread to Southeast Asia, the Middle East, Europe and Eastern Africa through trade routes. The second pandemic (1826-37) reached from India across to Europe, Great Britain and North America. The third pandemic erupted in 1846 in India and persisted until 1860 by extending to North Africa and South America, and for the first time specifically affecting Brazil. The fourth pandemic (1863-75) spread from India to Italy (The epidemic claimed 30,000 of 90,000 pilgrims in its first year), and Spain. The fifth pandemic (1881–1896) started in India and spread to Europe (Germany was the only major European outbreak; about 8,600 people died in Hamburg in 1892) Asia, and South America. The sixth pandemic started in India where it killed more than 800,000 people,

and spread to the Middle East, North Africa, Eastern Europe and Russia [1] from 1899–1923 [2]. The seventh pandemic originated in 1961 in Indonesia and is marked by the emergence of a new strain, which still persists as of 2018 in developing countries.

Among 206 serogroups, O1 and O139 serogroups are exclusively associated with worldwide cholera disease. Since 1884 when *V. cholerae* was formally identified, cholera remains endemic in many low-income countries, particularly in areas of inadequate sanitation and poor food hygiene practices. Indeed, the global burden of cholera in 2013 was estimated at 1.4 to 4.3 million cases and 28,000 to 142,000 deaths per year [3]. The O1 serogroup is divided into two biotypes, classical and El Tor; antigenic factors allow further differentiation into three serotypes; Ogawa, Inaba and Hikojima. Strains of the Inaba express only the A and C antigens whereas the Ogawa strains express the A and B antigens and a small amount of C antigen. The Hikojima serotype expresses all three antigens but is rare and unstable. The O139, a previously unrecognized serogroup of *V. cholerae,* was identified as the cause of cholera in 1992. The O139 is identical to the *V. cholerae* O1 El Tor biotype, except for substitution of genes encoding the LPS and the ability to produce capsule [4-6].

1.3. Pathophysiology of *V. cholerae*

V. cholerae generally survives the acidic environment of the stomach and then can pass through to possibly colonize the intestines to form a microcolony and secret cholera toxin (CT), a causative agent of severe diarrhea. The organism colonizes the epithelium of the small intestine by means of the toxin-coregulated pili (TCP) [7] and possibly other colonization factors which are thought to play a role. The adherent vibrios secret cholera enterotoxin that consists of an A subunit and 5 identical B subunits [8]. The B subunit serves to bind the toxin to GM1 ganglioside on intestinal epithelial cells. This binding is enhanced by neuraminidase. Binding of B subunits to epithelial cells allows the internalization of A subunit, in turn, cytosolic A subunit activates the $G_s\alpha$ protein via ADP-ribosylation, thereby stimulates adenylate cyclase to produce cAMP. The high level of cAMP results in a dramatic efflux of ions into the lumen by the activation of a cystic fibrosis trans-membrane conductance regulator (CFTR**)** [9]**.**

The major pathogenic effects of cholera are the result of cholera toxin exposure. Moreover, other virulence factors support the *V. cholerae* in its colonization, toxin action and coordinated expression of virulence factors. These additional proteins include zona occludens toxin (Zot, involved in *V. cholerae* invasion by acting to decrease intestinal tissue resistance) [10, 11], accessory cholera toxin (Ace, increases fluid secretion) [12], toxin co-regulated pilus (TcpA, essential colonisation factor and receptor for the CTXϕ phage) [13], outer membrane proteins (OmpU and OmpT, a potential adherence factor) [14], hemagglutinin/Zn-metalloprotease (HapA, penetrate intestinal mucus layer)[15]

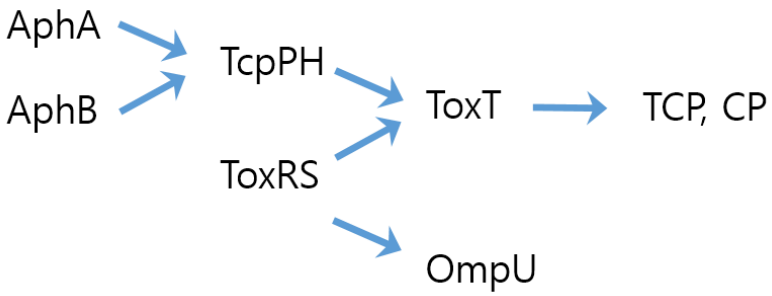
and flagellum (movement toward the epithelial surface) [16].

The mechanisms in intestinal colonization of *V. cholerae* have remained more elusive than cholera toxin action. Per Almagro-Moreno *et. al*., in 2015 [17]; *V. cholerae* may be infected as free-living cells, microcolonies or biofilm. By avoiding a bile-containing environment, it contacts first with the mucus layer covering the epithelium [18]. When *V. cholerae* reaches the intestinal epithelium, noncommitted (reversible) attachment occurs by adhesions such as GbpA [19] or Mam7 [20], OmpU [14], FrhA [21] and flagellum (in addition to motility) [22]. Subsequently *V. cholerae* produces the specific attachment adhesin, but this stage is hypothetical (no adhesions involved in committed attachment are known in *V. cholerae*). When the bacterial number reaches a certain concentration, the toxin coregulated pilus is produced and it allows a microcolony formation to occur [23] and toxin production to begin [24].

Mobility and chemotaxis of *V. cholerae* with flagella and some chemotaxis-related proteins have been implicated in pathogenicity [16, 25, 26]. Although motility and chemotaxis are believed to lead *V. cholerae* to colonize into its preferred site, the precise role of each in the infection process is still controversial.

The expression of virulence factors is transcriptionally controlled by multiple systems [27]. The primary, direct transcriptional activator of the genes encoding CT and TCP in *V. cholerae* is ToxT [28]. Transcription of *toxT* is regulated by the ToxR and TcpP

proteins [29]. The expression of *tcpP* is activated by AphB cooperating with AphA at the *tcpPH* promotor [30], and the expression of *toxR* is constitutive and can be activated by AphB [31]. ToxR and TcpP can interact with each other to regulate ToxT and OmpU [32, 33], but the exact mechanism is unclear.



The ToxR regulon

1.4. Immune response to *V. cholerae*

V. cholerae is thought to be a prototypical non-invasive, non-inflammation inducing bacteria. Therefore, it often causes no severe changes to the intestinal mucosa or the structural integrity of the small bowel. However, there is upregulation of pro-inflammatory cytokines (including interleukin-1β and tumor necrosis factor), the expression of diverse bactericidal proteins (lactoferrin) and increased infiltration of neutrophils to the epithelial cell and lamina propria during acute cholera [34-36]. It is also reported that LPLUNC1 (long palate, lung and nasal epithelium carcinoma-associated protein 1) is expressed in Paneth cells and likely plays a role in modulating host inflammatory responses to *V. cholerae* infection by attenuating innate immune responses to LPS [37]. Moreover, comparative proteomic analysis reveals the increased expression of S100A8 and WARS (cytoplasmic tryptophanyl-tRNA synthetase), in lamina propria cells during acute-stage cholera [38].

1.5. Vaccines; Cholera prevention

To date, three oral cholera vaccines (Dukoral, Shanchol and Euvichol) are approved from the WHO and are licensed for use in several countries [9]. These vaccines contain commonly inactivated O1 serogroup *V. cholerae* with either cholera toxin B or an additional O139 serogroup *V. cholerae*. They follow a similar vaccine strategy administrating more than 2 doses of vaccines to above 1 to 2 years of age. Additionally, they are needed for booster vaccine after 2 years for Dukoral (not specified in Shanchol and Euvichol). Even though Dukoral and Shanchol give about 57% of protective efficacy at 2 years and 65% at 5 years after vaccination, respectively, the protection is worse in children younger than 5 years old compared to older children.

Although those vaccines are immunogenic, they give relatively short-term protection. This results in the need for repeated vaccination programs. Therefore, continuous development of new vaccine candidates which show full protection with low cost is highly encouraged.

Table 1. Composition of vaccines

DUKORAL®	ShanChol®, Euvichol-Plus®/Euvichol®
• *V. cholerae* O1 Inaba Cairo 48 classical biotype, Heat inactivated • *V. cholerae* O1 Inaba Phil 6973 El Tor biotype, Formalin inactivated · • *V. cholerae* O1 Ogawa Cairo 50 classical biotype, Formalin inactivated • *V. cholerae* O1 Ogawa Cairo 50 classical biotype, Heat inactivated • Recombinant cholera toxin B subunit (rCTB)	• *V. cholerae* O1 Inaba classic strain, heat inactivated • *V. cholerae* O1 Inaba El Tor strain, formalin inactivated • *V. cholerae* O1 Ogawa classic strain, heat inactivated • *V. cholerae* O1 Ogawa classic strain, formalin inactivated • *V. cholerae* O139 4260B, Formalin inactivated

Table 2. Characteristics of oral cholera vaccines

Manufacturer	Valneva SE, France	Shantha, A Sanofi company, India	Eubiologics, Korea
Composition	Recombinant cholera toxin B subunit 1 mg Plus killed whole cells of the following Vibrio cholerae 01 organisms	Killed whole cells of O1 classical and El Tor biotypes plus O139	
Regimen	Age > 6 years: two doses given at least 1 week apart 2–6 years old: three doses given at least 1 week apart	Age 1 year and older: two doses 14 days apart	
Booster dose	Age > 6 years: every 2 years 2–6 years old: every 6 months	Every 2 years (may be subject to change)	
Administration	Administer with oral buffer (sodium hydrogen carbonate solution)	No oral buffer required	
Licensure	WHO prequalified since October 2001, licensed in ~60 countries [WHO, 2013b]	WHO prequalified since September 2011, licensed in India, Philippines, Nepal, Malaysia and Ivory Coast [WHO, 2010b, 2013b]	

1.6. Immunogenicity of cholera vaccine

Since those three vaccines are prepared from the combination of heat- and formalin-inactivated whole *V. cholerae* and cholera toxin B subunit. The immune response to cholera vaccines is directed largely against surface molecules of *V. cholerae* and cholera toxin. The response includes serum IgA, IgG and vibriocidal antibodies [39], intestinal–mucosal secretory IgA (sIgA) [40, 41] gut homing β7-positive antibody-secreting cells [42], T cells [43], and memory B cells [44] for long-term protection.

In general, serum vibriocidal and antitoxic antibodies have been detected in vaccinated subjects. Vibriocidal assay is the method to examine the bactericidal capacity of antibody (mainly IgM) with complement. For vibriocidal assay, *V. cholerae* O1 strain T19479 (El Tor Inaba), X25049 (El Tor Ogawa) and O139 strain CIRS 134 were generally used to evaluate clinical vaccine studies [45-47]. The seroconversion rate is calculated from vibriocidal assay and 4-fold rise of it is considered as immunogenic.

However, despite worldwide use of vibriocidal assay, there is a poor correlation between serum vibriocidal antibody response and protection [48]. Indeed, some study corroborated that in spite of the decline in vibriocidal antibodies at one year, the vaccine maintained its efficacy for at least five years post-vaccination [49, 50]. Since intestinal anti-cholera toxin IgG and IgA antibody levels are increased in vaccine recipients [51], antitoxin immunoglobulins (especially intestinal secretory IgA) which differ from the serum vibriocidal antibodies, are believed to mediate the protection after vaccination.

A booster dose of vaccine elicits an anamnestic response indicative of an immune memory. Therefore, the most feasible explanation for long-lasting protection is a rapid anamnestic response upon re-exposure despite waning serum antibody tiers [52].

1.7. *V. cholerae* cultured in an anaerobic environment

V. cholerae encounters oxygen-limited conditions when it infects host intestine [53]. And following pathogenic process of *V. cholerae* in host intestine is under anaerobic conditions.

In 2004, Kan *et. al*., have compared the proteomes of *V. cholerae* cultured in aerobic and anaerobic conditions [54] and revealed that some stress response proteins are found more abundant and flagellin B subunit was decreased in the anaerobic culture condition. In similar settings, anaerobic growth promotes synthesis of colonization factors (TcpA, TcpC, TcpQ, TcpS, TcpF and AcfA) in the El Tor *V. cholerae* C7258 strain, whereas the increased accumulation of TCP proteins is not coupled to high levels of CT [55]. The enhanced dimerization of AphB and interaction of TcpP and ToxR is verified to activate TcpP in turn, to increase *V. cholerae* virulence genes under oxygen-limiting conditions [56, 57]. Anaerobic growth of *V. cholerae* is promoted when trimethylamine N-oxide (TMAO) is used as an alternative electron acceptor. Moreover, cholera toxin production is markedly increased during anaerobic respiration in presence of TMAO [58]. In *V. cholerae* O395, classical strain however anaerobic conditions reduce the expression of *ctxAB* and do not induce CT at a detectable level. And the expression of the major regulatory genes, *tcpP, toxR* and *toxT* are not changed as in *tcpA* expression [59].

1.8. Model for cholera infection

One major barrier of cholera study is that only humans are naturally susceptible to cholera infection. Therefore, preclinical evaluation of new vaccine candidates has easily encountered these obstacles. Since, *V. cholerae* is rapidly cleared from the intestine of adult mice [60, 61], several mouse models have developed to study cholera. The most widely used model is neonatal mice[62]. Since presumably their microbiota is different from adult mice, they generally can be infected with cholera [63]. However, this model infection is often lethal and did not induce sufficient immunogenicity; therefore, it is more applicable for passive immune responses. Although germ-free mice is susceptible to cholera infection because of the absence of intestinal microbiota [64], the immune systems are undeveloped for the same reason. Moreover, the cost for maintaining the animal is higher than those for other animal models. In another model, streptomycin was used to deplete the intestinal flora, which makes conventional mice susceptible to *V. cholerae'*s colonization into mouse intestine [65]. This model allows direct evaluation of colonization of *V. cholerae*. However, antibiotics induced effects are not fully understood in the model. Murine pulmonary infection model is also developed by Fullner *et. al.*, in 2002 [66] and applied in a licensed cholera vaccine (Dukoral) experiment in 2013 [67]. The premise of this model is that the bronchial tree is similar to the intestine in terms of their mucosal lining with relevant characteristic [68]. Indeed, this model allows acute inflammatory responses through IL-6, MIP-2 expression resulting in pneumonia [66]. Ligated ileal loops are practical models to quantitate the intestinal fluid secretion caused by *V. cholerae* [69]. Dysenteric fluid secretion, however,

is mostly caused by cholera toxin, not by immunogenic properties of *V. cholerae*. Therefore, this model is quite restricted to investigate the toxin induced fluid accumulation. Lastly, ketamine anesthesia model was proposed in 2009 by Olivier *et. al*., [70]. Under anesthesia with ketamine-xylazine and neutralization of stomach acid, *V. cholerae* colonized the mice intestine successfully without streptomycin.

Table 3. Models for cholera infection

Model	Description	Reference
Germ free mouse	germfree mice are readily colonized by *V. cholerae* and develop systemic and mucosal immune responses to antigens	Infect Immun. 1996 Oct;64(10):4373-7
Suckling (neonatal) mouse	*V. cholerae* colonizing the suckling mouse small intestine	Infect Immun. 1999 Aug;67(8):3733-9 Infect Immun. 2014 Jun; 82(6): 2434–2447
Streptomycin treated mouse	long-term colonization and growth of *V. cholerae* in the guts of Sm-treated adult mice	Infect Immun. 2009 Aug;77(8):3475-84
Pulmonary model	*V. cholerae* is highly infectious to pulmonary system leading to a diffuse pneumonia	J Exp Med. 2002 Jun 3;195(11):1455-62. Mucosal Immunol. 2013 Jul;6(4):826-37
Ligated ileal loops	a closed loop system allows direct and accurate quantitation of the intestinal fluid secretion	PLoS Negl Trop Dis. 2013 Jun; 7(6)
Ketamine Anesthesia model	successful colonization upon anesthesia with ketamine-xylazine and neutralization of stomach acid	PLoS One. 2009 Oct 8;4(10)

1.9. Aim of study

V. choerae is a pathogenic bacterium which is transmitted through gastric uptake. Although *V. cholerae* is invasive, it colonizes the intestine and secrets cholera toxin causing diarrhea. Therefore, the intestine is considered as the site of infection where *V. cholerae* initiates the pathogenic actions. This pathogenic process of *V. cholerae* in the host intestine is under anaerobic conditions. It is reported that when *V. cholerae* passes through the human gut, it had greatly enhanced infectivity compared to *in vitro* grown strains by Andrew Camilli and colleagues [71]. In addition, previous reports suggest anaerobic growth conditions will stimulate *V. cholerae* to be more virulent by producing its virulence factors. However, oral vaccines mentioned above are derived from aerobically cultured *V. cholerae*. Moreover, the anaerobiosis of *V. cholerae* is largely elusive. Here, the effect of anaerobic growth of *V. cholerae* was primarily examined as aspects of immunogenicity and protectivity.

Chapter II. Materials and Methods

2.1. Reagent and chemicals

Luria broth (LB) and agar were purchased from Conda (Madrid, Spain), and Junsei chemical (Koshigaya, Japan), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin) and CFDA-SE Cell Tracer were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Bacteria strains

V. cholerae O1 El Tor Inaba strain T19479 and O139 CIRS 134 and 4260B were kindly provided by Prof. Jan Holmgren (Gothenburg University, Sweden). *V. cholerae* O1 N16961 were kindly provided by Prof. Dong Wook Kim (Hanyang University, Korea). For *tcpA* and *tcpF* mutant strains, DNA fragments were amplified using primers, *tcpA*-XbaI-F (CGG TCT AGA CCG CCT AGA TAG TCT GTG), *tcpA*-SacI-R CGG GAG CTC TAG CTG ACG ACT TGT TTT), *tcpF*-XbaI-F (CGG TCT AGA CGG TGT TAC CCA AGC ACT), *tcpF*-SacI-R (CGG GAG CTC TAA TAG ATC TTT ATT TTC) respectively by PCR and inserted into a suicide plasmid, pCVD442. The resulting plasmid was used to delete the *tcpA* and *tcpF* genes on a chromosome of *V. cholerae* strain T19479 by allelic exchange. *V. cholerae ompU*-deficient (Δ*ompU*) strain was obtained as described previously [72]. Both wild-type and mutant strains were cultured at 37℃ in LB broth. *V. cholerae* was cultured in a shaking incubator and an anaerobic

chamber that could supply a 90% N_2/5% H_2/ 5% CO_2 atmosphere to make the aerobic (A) and anaerobic (N) environment respectively. For microaerobic condition, *V. cholerae* was incubated at 37℃ without shaking. At mid-log phase and early-stationary phase, *V. cholerae* was centrifuged and resuspended with 15% glycerol-PBS and stored below -70℃. Each stock was thawed and plated on LB/agar plates to count the colony forming unit. Frozen bacterial stock was directly used in the following experiments.

2.3. Human epithelial cell lines

Human epithelial cell lines, HT-29 and Caco-2, were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10 percent of FBS (Gibco, Burlington, ON, Canada) at 37℃ in a 5% CO_2 incubator. To prepare a polarized monolayer of epithelial cells, HT-29 and Caco-2 were grown on a polycarbonate 12-transwell plate (Corning, NY, USA) and their media were changed every two days for three weeks. The integrity of cell monolayers was assessed through trans-epithelial electrical resistance (TER). The human intestinal epithelial cells, SNU-407 and SNU-61 were purchased from the Korean Cell Line Bank (Seoul, Korea).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated per manufacturer's procedures using Trizol reagent (Invitrogen). Complementary DNA was transcribed from the isolated RNA using reverse transcriptase (Promega Madison, WI, USA) and random primer (Takara). Then, the cDNA was

amplified through real-time PCR reaction using SYBR Premix Ex Taq™ (Takara) and analyzed with QuantStudio™ 7 (Applied Biosystem). Each gene expression was normalized against endogeneous expression of recA from *V. cholerae*.

2.5. Adhesion assay

Caco-2 and HT-29 cells (2×10^5/ml) were plated in a 24-well plate and incubated at 37℃ in 5% CO_2 for 24 h. The confluent cells were washed twice with PBS and then exposed to *V. cholerae* in an antibiotics-free media. Cells were treated with *V. cholerae* at an MOI of 5 unless otherwise specified. The plate that held the *V. cholerae* was centrifuged for 5 minutes at 200 x g for the forced interaction of the *V. cholerae* and the cells. After an hour incubation at 37℃, the unbounded bacteria were washed away three times with chilled PBS. In certain experiments, host cells and the bound *V. cholerae* were further incubated for additional time as indicated. Then the cells were harvested with 1% of Triton X-100 for 10 min and diluted serially in PBS. Each diluted cell lysate was plated on an LB/agar plate and the colony forming unit of *V. cholerae* was counted. The percentage of adhesion was calculated by dividing the number of adherent *V. cholerae* by the number of *V. cholerae* initially treated.

2.6. Flow cytometry

V. cholerae was pre-stained with CFSE and co-incubated with Caco-2 cells for 1 h. Unbound *V. cholerae* was removed through repeated washing (three times) with PBS

containing 2% FBS. CFSE-labeled *V. cholerae* that bound to Caco-2 cells was analyzed through flow cytometry using LSR II (BD) and FlowJo software (TreeStar, San Carlos, CA, USA).

2.7. Mouse intestinal colonization

Mouse intestinal colonization was performed under International Vaccine Institute IACUC approval (PN 2015-003). Specific pathogen-free 6 week old BALB/c were purchased and held in barrier housing until the mouse intestinal inoculation. The mice were inoculated as previously described [70]. Briefly, the mice were intraperitoneally anesthetized with 60–70 mg/kg ketamine and 12–14 mg/kg xylazine. Under anesthesia, the mice were orally administered with 50 μl of 8.5% (w/v) $NaHCO_3$ which was immediately followed with 50 μl of bacterial suspension. The intestines were removed 20 h after inoculation and homogenized in PBS to assess intestinal colonization. The colonization index was calculated as Col. Index = $CFU_{recovered}$/ $CFU_{inoculated}$.

2.8. Immunization and challenge

Mice were immunized with heat-killed *V. cholerae* O1 T19479 (1 x 10^7 CFU) prepared in aerobic and anaerobic conditions. The *V. cholerae* suspension was applied via intranasal route at 50 μl volume per mouse. Two weeks after initial immunization, a booster immunization was provided. For the challenge experiments, the mice were intranasally or orally administered with live *V. cholerae* (CFU as indicated in the figure)

cultured in aerobic and anaerobic environments.

2.9. Measurement of antibody production

A microwell plate (Nunc) was coated with killed *V. cholerae (*10^8 CFU/ml) or LPS (4 μg/ml) and incubated overnight. The plate was washed three times with a washing buffer containing 0.05% Tween 20. To prevent non-specific binding, 200 ml of 1% bovine serum albumin (BSA) in PBS was added and incubated for 1 h. After washing the plate, 100 ml of pre-diluted sera in 1% BSA/PBS was added and incubated for 1 h. After washing the plate, 100 ml of alkaline phosphatase (AP)-conjugated anti-mouse IgG, IgM, or IgA (1:2000) (Jackson ImmunoResearch Laboratories,West Grove, PA) was added and incubated. The plate was washed three times with washing buffer. Then, 100 ml of 4-nitrophenylphosphate substrate (1 mg/ml) in 1 M Tris-HCl supplemented with 3 mM $MgCl_2$ at a pH 9.8 was added and incubated for 30 min at room temperature. Fifty microliters of 3 M NaOH was added to stop the reaction and the optical density (OD) was measured at 405-490 nm using a microplate reader. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution giving an OD 0.1 higher than the background sample [73].

2.10. Cytometric Bead Array (CBA)

CBAs were performed using bronchoalveolar lavage (BAL) fluids, lung extracts and sera from mice immunized with heat-killed *V. cholerae* O1 T19479 cultured in aerobic

and anaerobic conditions. Mouse Th1/Th2/Th17 cytokine were analyzed per the manufacturer's procedure (BD, San Jose, CA). Briefly, Unknown samples were plated on 96-well plate. Mouse TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, and IL-17A capture bead were mixed together and loaded into unknown samples containing plate. Mouse Th1/Th2/Th17 PE detection reagent were added and incubated for 2 h at room temperature, protected from light. After washing, samples were acquired on the flow cytometry and analyzed using FCAP array software.

2.11. Vibriocidal assay

Heat-inactivated BAL fluids, lung extracts and sera were loaded in the 96-well plate and 2-fold serial dilution were made in saline (0.85% v/v NaCl). A mixture of live *V. cholerae* O1 T19479 (1x 10^6 CFU/ml) and 0.5% (v/v) guinea pig complements were added into plate. The reaction mixture was incubated at 37°C for 1 h by shaking at approximately 50 rpm. BHI medium was added and followed another incubation for 3 to 5 h at 37°C without shaking. Bacterial growth was determined through optical density measured at 600nm spectrophotometrically. Vibriocidal titer was determined as the reciprocal $\log_2$ of the last dilution giving an optical density at 600 nm higher than mean of background +0.02.

2.12. Measurement of cholera toxin

Culture supernatant of *V. cholerae* in LB broth was used for cholera toxin measurement.

A microwell plate was coated with GM-1 ganglioside (0.5 μg/ml) and incubated overnight. The plate was washed three times with a washing buffer containing 0.05% Tween 20. To prevent non-specific binding, 200 ml of 1% BSA in PBS was added and incubated for 1 h. After washing the plate, 100 ml of culture supernatant or cholera toxin (100 ng/ml to 0.78 ng/ml) in 1% BSA/PBS was added and incubated for 1 h. After washing the plate, 100 ml of rabbit anti-CT IgG (1:5000) was added and incubated for 1 h. After washing the plate, 100 ml of alkaline phosphatase (AP)-conjugated anti-rabbit IgG (1:5000) (Jackson ImmunoResearch Laboratories,West Grove, PA) was added and incubated. After washing the plate, 100 ml of 4-nitrophenylphosphate substrate (1 mg/ml) in 1 M Tris-HCl supplemented with 3 mM $MgCl_2$ at a pH 9.8 was added and incubated for 30 min at room temperature. Fifty microliters of 3 M NaOH was added to stop the reaction and the OD was measured at 405-490 nm using a microplate reader. The concentration of CT in the culture supernatant was calculated by comparison to the standard curve.

2.13. Biofilm assay

V. cholerae O1 T19479 cultured in LB media at 37°C for O/N with shaking was inoculated in 100 μl LB media to make OD_{600} = 0.1 in 96 well plate. The plate was incubated in aerobic and anaerobic conditions for 4 to 24 h. At the indicated point of time, the plate was washed two times with distilled water twice. A 0.1% crystal violet solution was added and incubated for 30 min. After wash with distilled water, 100 μl of

95% ethanol containing 0.1% acetic acid was added to solubilize crystal violet from bacteria. The formation of biofilm was analyzed by measuring OD at 595 nm.

2.14. Preparation of sample

Blood, lungs and BAL fluids were collected from non-immunized and immunized mice on day 21. Sera were separated from blood samples by centrifugation 5,000 x g, 20 min following clotting for 2 h at room temperature. BAL fluids were prepared from trachea with PBS (1 ml per mouse) injection/retrieval by using an intravenous catheter. Lungs were homogenized in PBS (1 ml per mouse) and centrifuged 200 x g, 5 min to remove remaining debris. The sera, BAL fluids, and lung extracts were kept at -80°C until use.

2.15. Bone marrow-derived dendritic cells

The tibias and femurs were removed from 8- to 12-week BALB/c mice under sterile conditions. Bone marrow flew out from the bone cavity with RPMI-1640 medium which was supplied trough needle of a 1-mL syringe. The cell suspension was collected and centrifuged at 2000 x g for 5 min, and the red blood cells were lysed by RBC lysis buffer. Following the second centrifugation, the cell pellet was washed with PBS and collected. The cells were suspended in RPMI-1640 medium supplemented with 10% FBS, 20 ng/mL GM-CSF, and 10 ng/mL IL-4 and incubated at a density of 1×10^6 cell/ ml at 37℃ in an incubator containing 5% CO_2. The fresh culture medium was added on day 3 and day 6. On day 8, the semi-suspended cells and loosely attached cells were collected by

gently pipetting. The cells were plated into 24-well plates for an additional incubation with vaccines prepared from *V. cholerae* grown in aerobic and anaerobic conditions.

2.16. Statistical analysis

The mean values ± standard deviations were obtained from samples in triplicate at a minimum. Statistical significance was determined through the Student's *t*-test. Differences between the experimental groups and control group were considered statistically significant at $P < 0.05$.

Chapter III. Results

3.1. Anaerobic conditions affect the growth of *V. cholerae*, but not morphology, cholera toxin secretion and biofilm formation.

It is unclear whether anaerobic culture conditions *in vitro* influence the general physiology of *V. cholerae*. So, the growth of *V. cholerae* was primarily compared between anaerobic (N) and aerobic (A) conditions (Fig. 1A). When *V. cholerae* was cultured in LB medium of anaerobic conditions, it grew slowly and reached a stationary phase with low cell density, compared to the aerobically cultured counterpart (Fig. 1A). However, the general morphology (size and shape) of *V. cholerae* was not affected by different culture conditions (Fig. 1B). Next, the amount of CT which is a major virulence factor secreted by intestinally colonized *V. cholerae* [9] was assessed. The secretion of CT is quite low in growth condition, at 37°C/pH 7.0/ LB medium. However, it is known that the pH and temperature of growth medium influence CT production [74]. Furthermore, there is a report about anaerobic environment reducing the CT production of *V. cholerae* O395 in a low pH [59]. Contrary to the previous report, the levels of CT from aerobic and anaerobic conditions were comparable, regardless of culture time (Fig. 1C). Finally, it was observed that the anaerobic growth conditions had no effects on the biofilm formation of *V. cholera*e (Fig. 1D). Therefore, these data indicate that anaerobic conditions impaired growth kinetics of *V. cholerae* without any change of morphology, CT secretion, and biofilm formation.

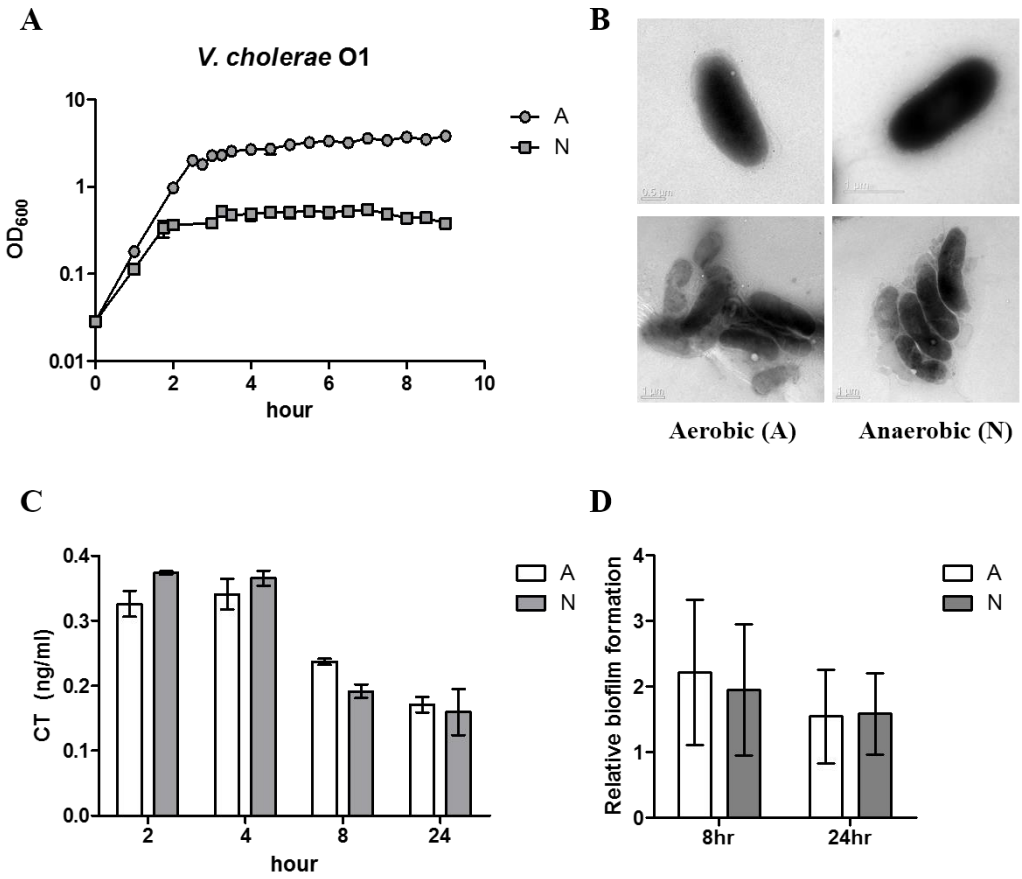


Figure 1. Effect of anaerobiosis on growth, phenotype, cholera toxin expression and biofilm formation of *V. cholerae*. (A) *V. cholerae* was cultured in aerobic (circle) and anaerobic (square) conditions and the growth was observed by spectrophotometry at OD 600 nm. (B) *V. cholerae* was stained with phosphotungstic acid to observe the morphology through a transmission electron microscope. (C) The expressions of cholera toxin in the *V. cholerae* culture supernatant were analyzed by ELISA. (D) Biofilm formation of *V. cholerae* cultured in aerobic and anaerobic conditions were matched with their growth.

3.2. Anaerobiosis improves the adhesive ability of *V. cholerae* on human intestinal epithelial cells.

V. cholerae should eventually adhere to the apical surface of the intestinal epithelial cells to exert the following virulence process. The assessment of whether anaerobic culture conditions regulate the adhesiveness of *V. cholerae* on human colonic epithelial cells was made. To assess the binding of *V. cholerae* to human colonic epithelial cells, CFSE-labeled *V. cholerae* was treated on Caco-2 cell line for 1 h and washed three times to remove unbound or weakly bound *V. cholerae*. Then, CFSE signals of the Caco-2 cell line were measured using flow cytometry. Intriguingly, *V. cholerae* cultured in anaerobic conditions attached to the colonic epithelial cells more than aerobically cultured one (Fig. 2A). Indeed, the adhesion assay showed greater CFU from the anaerobically cultured *V. cholerae*. The higher adhesion is quite constant (about 70%) in different MOI (5, 10 and 50) regardless to type of intestinal cell line (Fig. 2B). Considering the slow growth kinetics of *V. cholerae* under anaerobic conditions (Fig. 1A), the data indicates the enhanced adherence of anaerobically cultured *V. cholerae*. This was further confirmed by using the differentiated (polarized) Caco-2 cells and human primary colonic epithelial cells (SNU-407 and SNU-61), which mimic *in vivo* intestinal environment of the host (Fig. 2C). Together, these results suggested that anaerobic culture environment rendered *V. cholerae* more adhesive to intestinal epithelial cells.

To enlarge the study, the adhesion of *V. cholerae* to mouse lung macrophages and lung epithelial cells were tested. Anaerobic growth increases the adhesion ability of *V. cholerae* to mouse lung macrophages MH-S, lung epithelial cells LA-4 (Fig. 3A, B).

Moreover, *V. cholerae* adhered even higher in anaerobic conditions to an abiotic surface that is a polystyrene plate without cells (Fig. 3C).

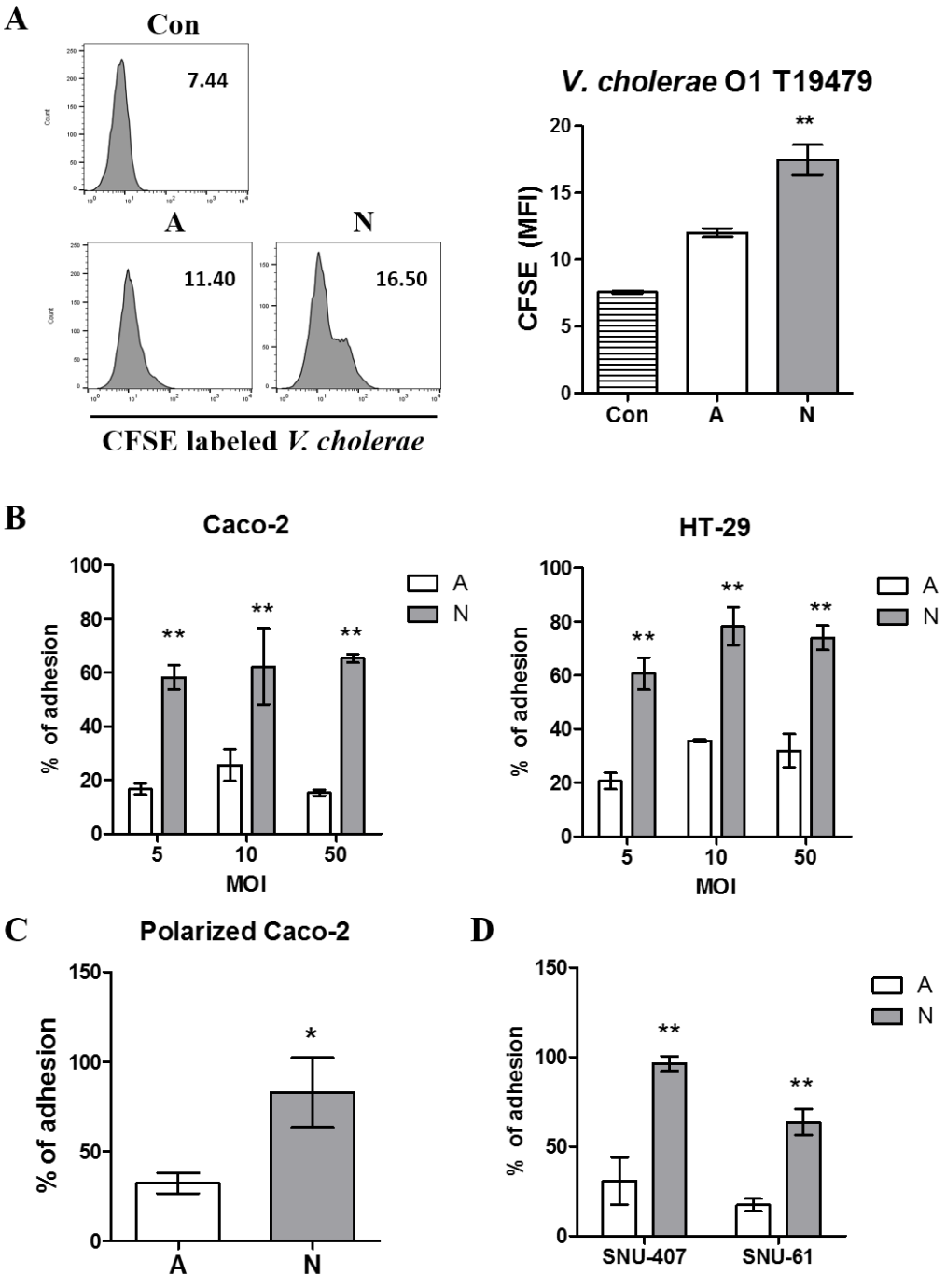


Figure 2. Anaerobic growth increases the adhesion ability of *V. cholerae* to human

epithelial cells. The human epithelial cells were treated with *V. cholerae* O1 El Tor Inaba strain T19479 cultured in aerobic (A) and anaerobic (N) conditions. (A) Caco-2 cells were co-cultured with CFSE-labeled *V. cholerae* for 1 h and then washed by PBS three times. The binding of *V. cholerae* to Caco-2 cells ($CFSE^+$ Caco-2 cells) were measured by flow cytometry. MFI, Mean Fluorescence Intensity. Number in histogram indicates MFI of CFSE. (B~D) The adhesion of *V. cholerae* was numerated by adhesion assay, (B) Caco-2 and HT-29 cells were treated with *V. cholerae* at an MOI of 5, 10 and 50 for 1 h. (C) Polarized monolayer Caco-2 cells and (D) human primary epithelial cells SNU 407 and SNU-61 were treated with *V. cholerae* at an MOI of 5 for 1 h. An asterisk indicates $P < 0.05$.

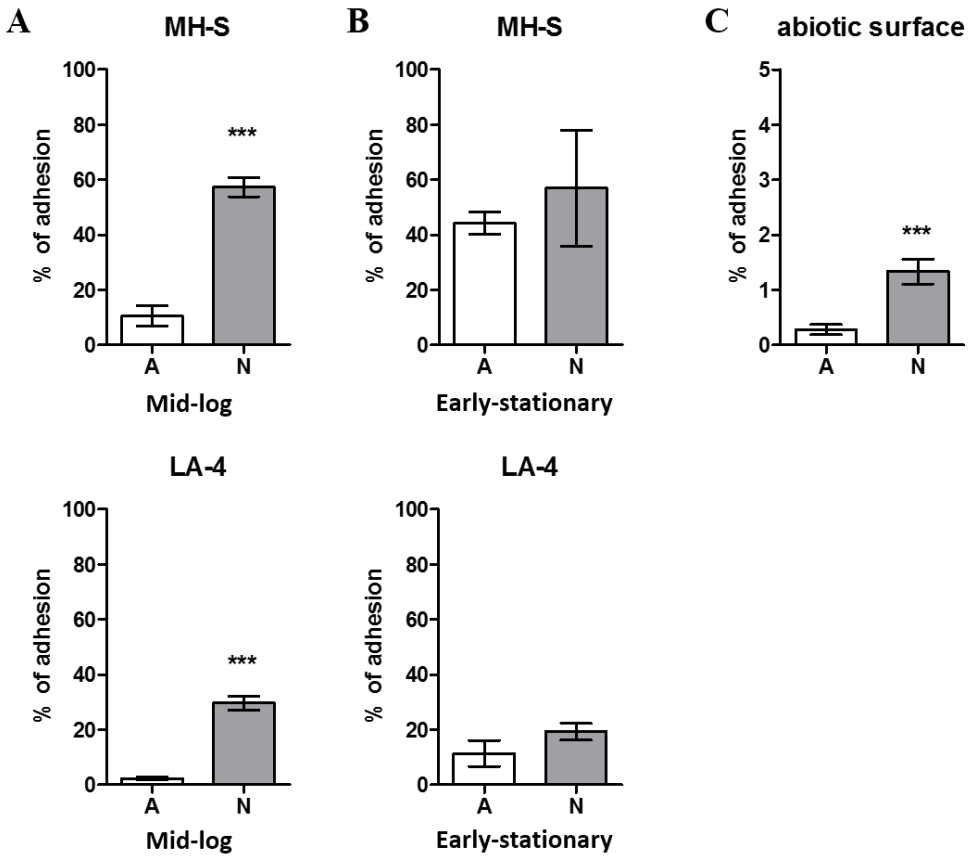


Figure 3. Anaerobic growth increases the adhesion ability of *V. cholerae* to mouse lung macrophages, lung epithelial cells and abiotic surfaces. The adhesion of *V. cholerae* O1 T19479 were numerated by adhesion assay. (A, B) The mouse lung macrophages (MH-S) and mouse lung epithelial cells (LA-4) were treated with *V. cholerae* O1 El Tor Inaba strain T19479 at an MOI of 5. *V. cholerae* cultured in aerobic and anaerobic conditions was applied when it was at mid-log phase or early-stationary phase. (C) The adhesion of *V. cholerae* was numerated in a plastic culture plate without cells.

3.3. The anaerobic environment induces higher expressions of *tcpA, tcpF* and *ompU* genes in *V. cholerae.*

Next, adherence factors of *V. cholerae* which are involved in the increased colonization in an anaerobiosis were determined. Expression of genes encoding adherence factor were assessed from *V. cholerae* cultured until the mid-log phase through quantitative real-time PCR. Interestingly, among toxin coregulated pilus (TCP) gene cluster, only *tcpA* and *tcpF* were considerably more expressed in anaerobic atmosphere. (Fig. 4A). In addition, when the gene expressions of three outer membrane proteins (*ompU, ompA* and *tolC*) reported as an adherence protein were analyzed, *ompU* was highly detected in the anaerobic environment, whereas *ompA* and *tolC* were slightly lower (Fig 4B). On the other hand, there was no significant change in gene expression of flagellin B (*flaB*), hemagglutinin/protease (*HA/P*), accessory colonization factor A (*acfA*) and N-acetylglucosamine-binding protein A (*gbaA*) between the anaerobic and aerobic environment (Fig. 4C). Therefore, *tcpA*, *tcpF* and *ompU* were identified as a highly upregulated adherence factor in an anaerobiosis.

The expression of these three genes were traced after 1 h incubation with Caco-2 cells (Fig. 4D). It is worth noting that the mRNA level of *tcpA* in the colonized *V. cholerae* from anaerobic condition was as greater as 15-fold than that of the colonized one from aerobic condition (Fig. 4D); before colonization, the extent of difference was about 4.5-fold between anaerobic and aerobic conditions (Fig. 4A). However, the difference of *tcpF* and *ompU* between two culture conditions became lesser after colonization (10 to 5 and 3 to 1.3, respectively). These suggested that *tcpA* might be an important factor that

confer adhesive capacity on anaerobically cultured *V. cholerae* in the present experimental setting.

Moreover, it was observed that an anaerobic environment induced relatively higher expression of *tcpPH*, t*oxRS* and *aphAB* (Fig. 4E), all of which are reported to regulate the expression of TCP and outer membrane proteins [56, 75], presumably suggesting the involvement of these regulatory circuits in upregulation of adherence factors. Taken together, these results demonstrate *tcpA*, *tcpF* and *ompU* might be involved in *V. cholerae* adhesion to intestinal epithelial cells under an anaerobic environment.

In addition, it is confirmed that TCP protein existed in anaerobic but not in aerobic conditions by using liquid chromatography-mass spectrometry (Fig. 5D). Proteins that are responsible for catalytic activity (Fig. 5A) or that are components of cell parts (Fig. 5B) are reduced in anaerobic environment.

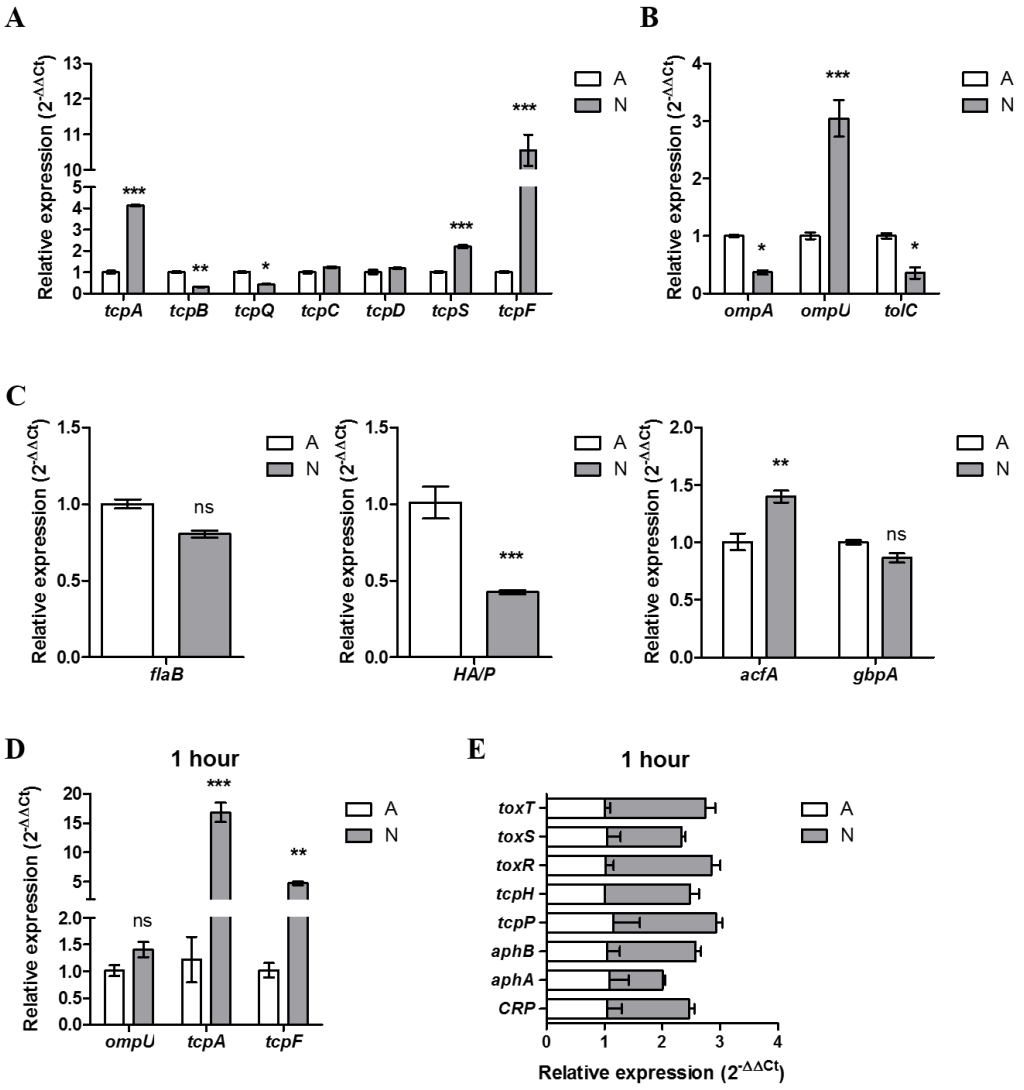


Figure 4. The expression of *tcpA, tcpF* and *ompU* in *V. cholerae* are increased in anaerobic conditions. (A~C) *V. cholerae* O1 El Tor Inaba strain T19479 was cultured in aerobic and anaerobic conditions until a mid-log phase. Bacterial RNA was extracted, and mRNA expressions of the cell adhesion-related gene were determined through real-time PCR. (D, E) Caco-2 cells were treated with *V. cholerae* at an MOI of 5 for 1 h.

Then the total RNA from the cell-adherent *V. cholerae* was extracted. The expressions of *ompU, tcpA, tcpF* and *toxR* regulon-related genes were analyzed through real-time PCR. Each gene expression was normalized against the endogeneous expression of *recA* of *V. cholerae*. The expression level of each gene in an aerobic condition was set to 1.

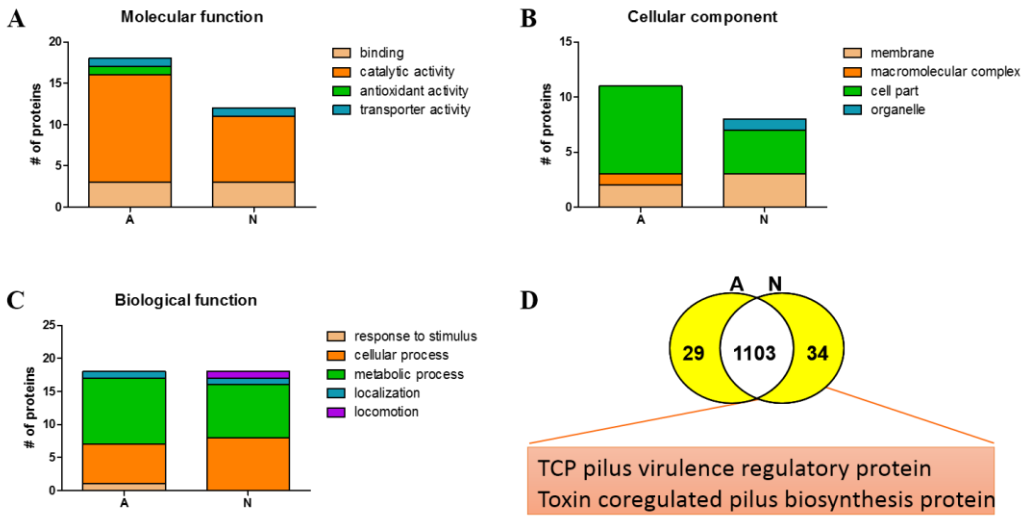


Figure 5. The expression of toxin coregulated pilus regulatory protein in *V. cholerae* are increased in anaerobic environment. *V. cholerae* at mid-log phase was harvested and sonicated for total protein extraction. Soluble proteins were analyzed using liquid chromatography-mass spectrometry. Obtained spectra was analyzed using Uniprot proteome database. Peptide result were analyzed with scaffold software by protein threshold: 95%, Minimum number of peptides: 3, peptide threshold: 95%, and about 1000 proteins were identified. To assign gene ontological categories, the identified proteins were classified according to (A) molecular function, (B) cellular component, and (C) biological function using the scaffold software. (D) In anaerobic culture conditions, the expression of 34 proteins was exclusively induced. And in those proteins, TCP related proteins were identified in an anaerobic condition. A, aerobic, N, anaerobic

3.4. An anaerobic culture rescues *V. cholerae* from hypo-adhesiveness to human epithelial cells.

V. cholerae deficient in any of adherence factors (*tcpA, tcpF* and *ompU*) exhibit a decreased colonization to epithelial cells in infant mice [76-78]. To verify adherence function of the three factors upregulated by anaerobiosis, the assessment of colonization efficiency of the mutant *V. cholerae* cultured in an anaerobic condition was made (Fig. 6A). Intriguingly, neither of the mutant stains showed the significant defect of colonization (Fig. 6A). Indeed, gene expression of other adherence factors was greater in each mutant strain than in the wild type counterparts under anaerobic environment (Fig. 6B). When the *ompU*-deficient strain was anaerobically cultured, the expression of *tcpA* and *tcpF* were increased about 10-fold and 2-fold, respectively. In the strains deficient of either *tcpA* or *tcpF, ompU* was higher about 3-fold, which seemingly suggested that *ompU* might complement both *tcpA* and *tcpF,* and vice versa. Meanwhile, *tcpA* expression was rather decreased in the *tcpF*-deficient strain, but *tcpF* expression was not changed in the *tcpA*-deficient strain; that is, *tcpA* and *tcpF* seem not to complement each other, unlike the relation of *ompU* to these two TCP genes. Therefore, the anaerobically cultured *V. cholerae* overcame a single loss of adherence factors by upregulating the others. Furthermore, these data suggested the feasible existence of a regulatory circuit which transcriptionally controls genes encoding adherence factors to maintain colonization in anaerobiosis.

It is worth noting that anaerobiosis maximized the adhesion of *V. cholerae* to human epithelial cells by upregulation of colonization factors. It seems that the adhesion of *V.*

cholerae Δ*tcpF* strain complemented with *tcpF* gene (Δ*tcpF*/cp) might reach apex in parallel with parental *V. cholerae* cultured in an anaerobic environment even though it expressed high levels of *tcpF* and *ompU* (Fig. 7A, B).

TcpA is known as major colonization factor, indeed *V. cholerae* A213 strain that is not expressing *tcpA* showed impaired adhesion to epithelial cells compared to *V. cholerae* A213/*tcpA*+ strain (Fig. 8A). Moreover, colonization factor TcpA was involved in protective effects on mice when it was tested through intranasal vaccination with heat-killed *V. cholerae* A213 (Fig. 8C).

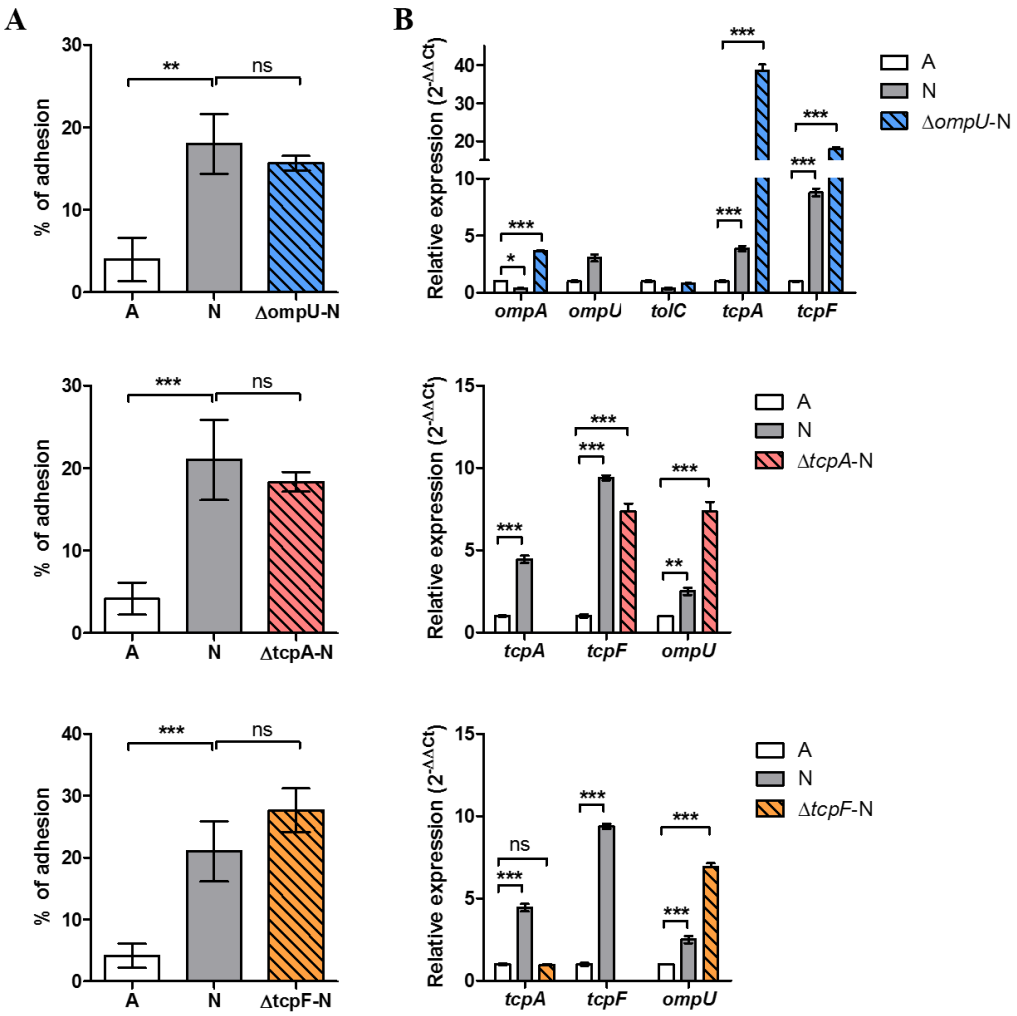


Figure 6. Anaerobic growth compensates the deficiency of *ompU, tcpA* and *tcpF* to retain the adhesion of *V. cholerae* to human epithelial cells. *V. cholerae* O1 El Tor Inaba strain T19479 was cultured in aerobic and anaerobic conditions. *V. cholerae O1 T19479 ompU-*, *tcpA-*, and *tcpF*-deficient strains were cultured in anaerobic environment until a mid-log phase. (A) Caco-2 cells were treated with *V. cholerae* at an MOI of 5 for 1 h. (B) Bacterial RNA was extracted and mRNA expressions of *ompU,*

tcpA and *tcpF* were determined through real-time PCR. Each gene expression was normalized against the endogenous expression of *recA* from *V. cholerae*.

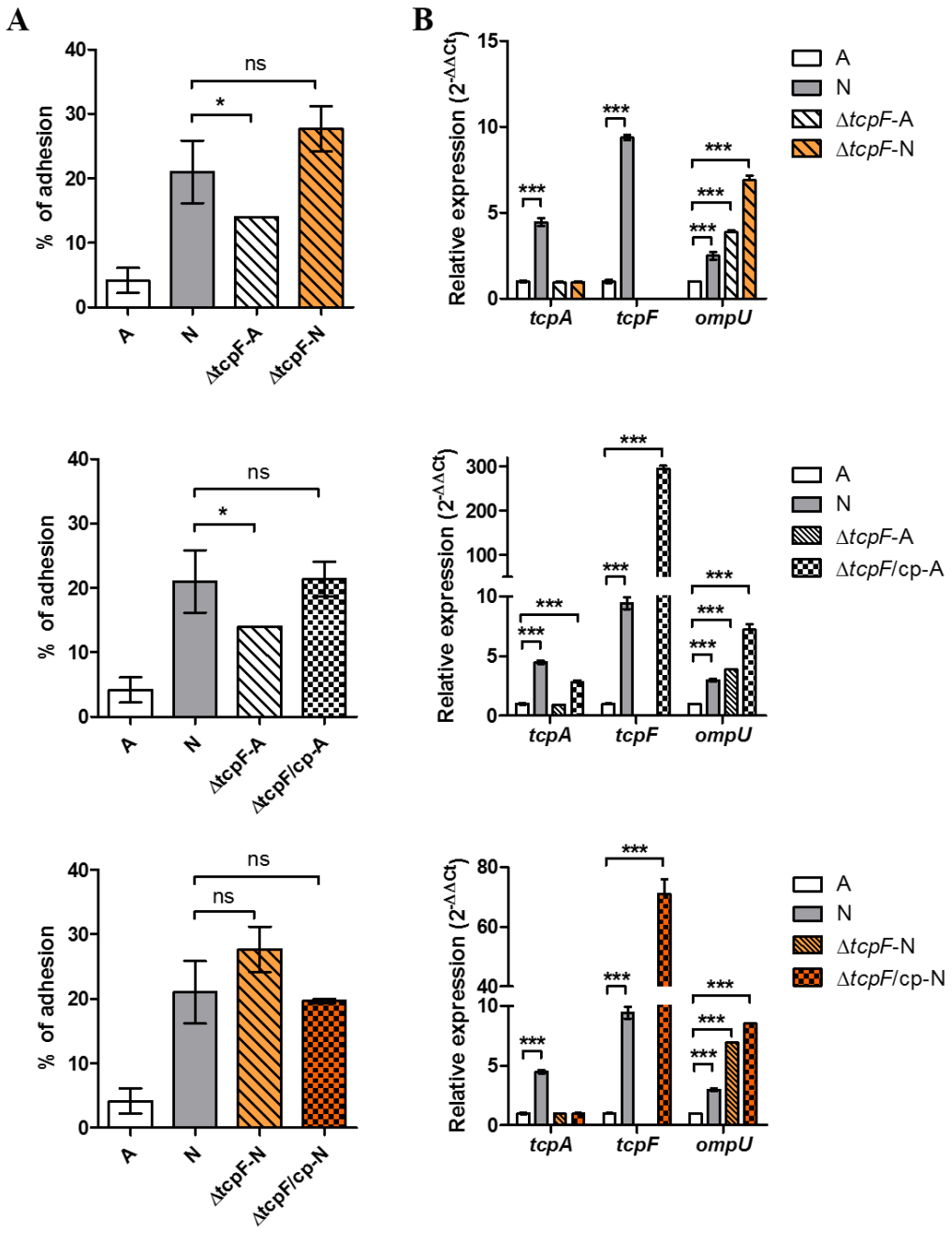


Figure 7. Anaerobiosis maximize the adhesion of *V. cholerae* to human epithelial cells. *V. cholerae* O1 El Tor Inaba strain T19479 wild type, *tcpF* deficient strain (Δ*tcpF*),

and Δ*tcpF* strain complemented with *tcpF* gene (Δ*tcpF*/cp) were cultured in aerobic (-A) and anaerobic (-N) conditions until a mid-log phase. (A) Caco-2 cells were treated with *V. cholerae* at an MOI of 5 for 1 h. (B) Bacterial RNA was extracted and mRNA expressions of *ompU, tcpA* and *tcpF* were determined through real-time PCR. Each gene expression was normalized against the endogenous expression of *recA* from *V. cholerae.*

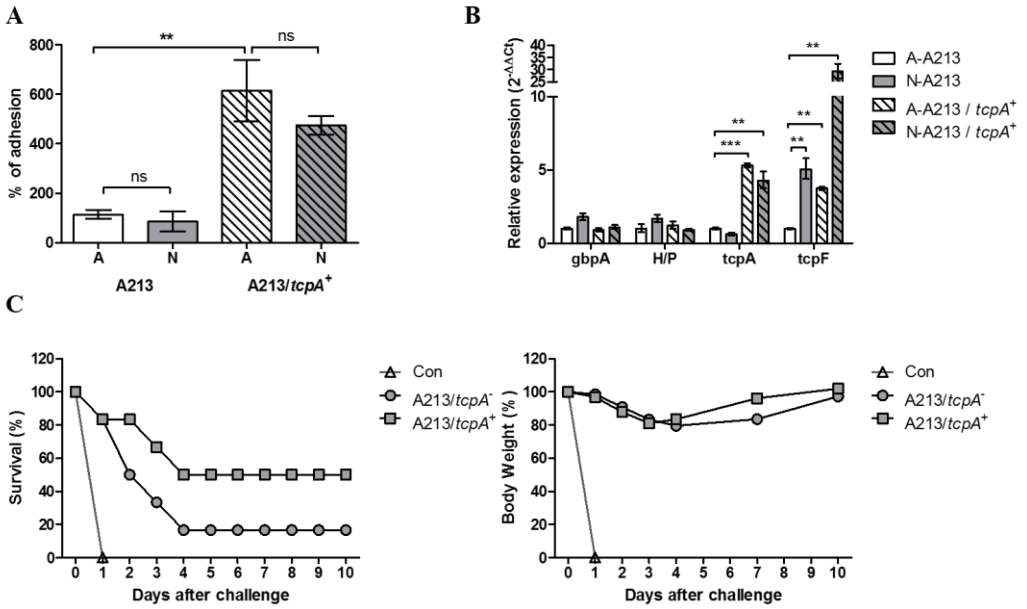


Figure 8. *V. cholerae* which is expressing TcpA highly adheres to epithelial cells and contributes to protect mice from cholera infection. *V. cholerae* A213 *tcpA* negative and positive strains were prepared in aerobic and anaerobic conditions. (A) Caco-2 cells were treated with *V. cholerae* A213 strains at an MOI of 5 for 1 h. (B) Bacterial RNA was extracted, and mRNA expressions of the cell adhesion-related gene were determined through real-time PCR. Mice were immunized twice at 2-week intervals with heat inactivated *V. cholerae* A213 prepared in aerobic and anaerobic conditions. Immunized groups were challenged with live *V. cholerae* O1 T9479 one week post last immunization. (C) Survival rates and (D) body weight changes after intranasal challenge were monitored for 10 days. (A-, aerobically cultured; N-, anaerobically cultured)

3.5. Outer membrane structure is involved in the effect of anaerobiosis on adherence of *V. cholerae*.

It was examined that whether the enhanced adhesion of the anaerobically cultured *V. cholerae* is conserved across strains. Like the result of T19479, hitherto used in this study, higher adherence to intestinal epithelial cells was also observed in anaerobically cultured N16961, another O1 strain, compared to aerobically cultured one; it was regardless to type of host epithelial cells (Fig. 9A). When this strain could further colonize and proliferate for additional 1.5 h, their final colonization was exponentially augmented in the anaerobically cultured one. However, 4260B, O139 serogroup, showed lesser adhesive capacity under an anaerobic condition (Fig. 9B). Outer membrane of 4260B strain contains well-exposed LPS O antigen and capsule [79], which lack in O1 serogroup. The adhesive capacity of CIRS134 strain, a capsule-deficient variant of O139 [80] was tested and observed that this mutant of O139 serogroup displayed enhanced adherence under anaerobic environment, analogous to O1 serogroup (Fig. 9C). This result is quite consistent with the previous report in that capsular polysaccharide of membrane could inhibit colonization as well as migration of *V. cholerae* [81]. Taken together, these results suggested that the enhancement in adherence of *V. cholerae* by anaerobic culture conditions might be associated with structural molecules of the outer membrane.

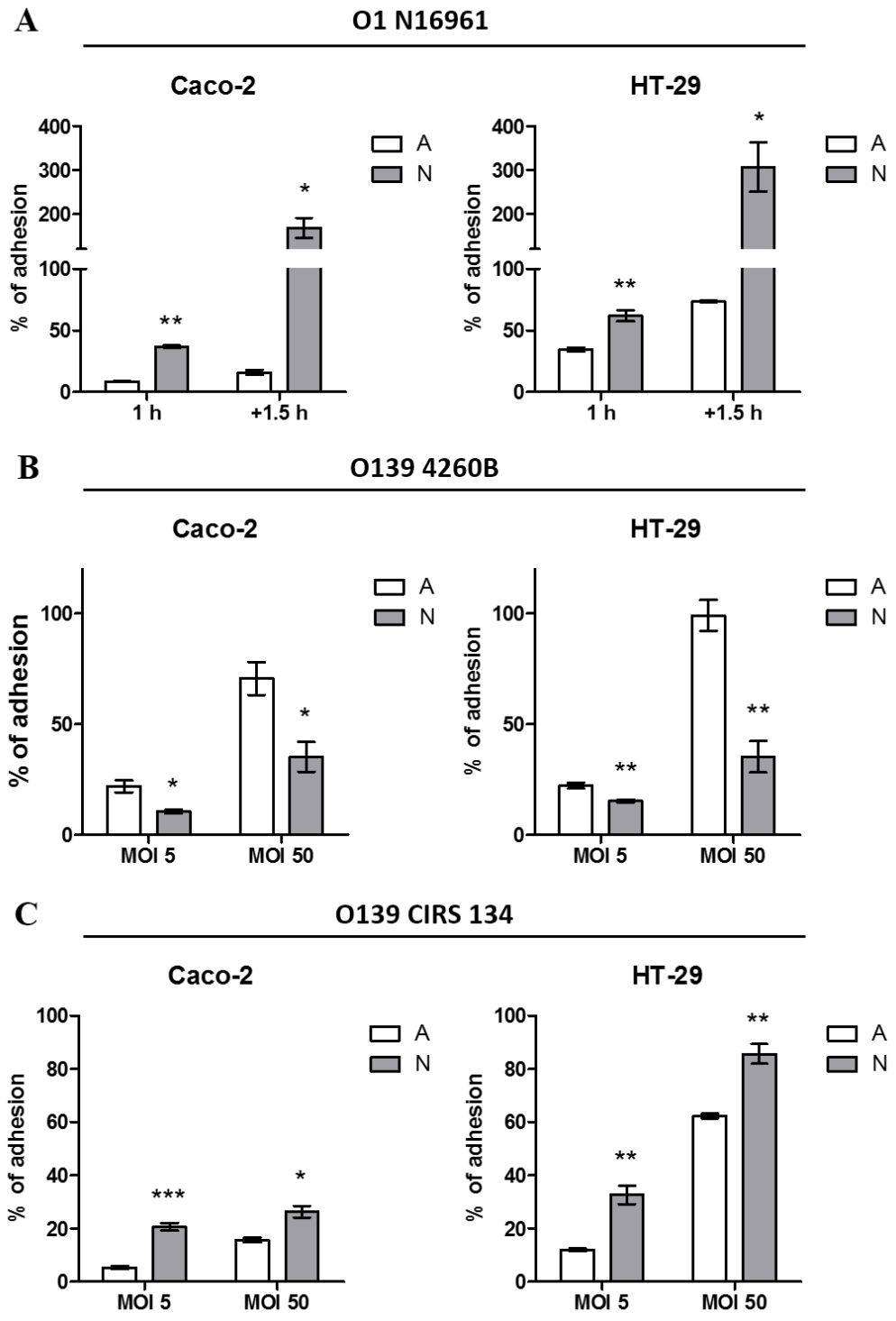

A
O1 N16961
Caco-2
HT-29
% of adhesion
400
300
200
100
50
0
A
N
**
*
1 h
+1.5 h
B
O139 4260B
Caco-2
HT-29
% of adhesion
100
50
0
A
N
*
**
MOI 5
MOI 50
C
O139 CIRS 134
Caco-2
HT-29
% of adhesion
100
80
60
40
20
0
A
N
***
*
**
MOI 5
MOI 50

Figure 9. Effect of anaerobiosis on the adhesion of *V. cholerae* O1 N16961, O139 CIRS134, and 4260B to human epithelial cells. Caco-2 and HT-29 cells were treated with *V. cholerae* grown in aerobic and anaerobic conditions. (A) Caco-2 and HT-29 cells were treated with *V. cholerae* O1 N16961 at an MOI of 5 for 1 h, and then washed and followed an additional incubation for 1.5 h**.** (B, C) Caco-2 and HT-29 cells were treated with *V. cholerae* O139 4260B and CIRS 134 at an MOI of 5 and 50 for 1 h. An asterisk indicates $P < 0.05$.

3.6. Growth phase is involved in the effect of anaerobiosis on adherence of *V. cholerae.*

Considering the difference of gene expression patterns of *V. cholerae* between mid-exponential and stationary phase [82], it was examined whether the growth phase might affect gene expression of adherence factors such as *tcpA, tcpF* and *ompU,* resulting in the changes on adhesion ability. Contrary to the mid-log phase when *tcpA*, *tcpF* and *ompU* were up-regulated in anaerobic condition (Fig. 4), early-stationary phase contained relatively lower levels of these genes in an anaerobic atmosphere (Fig. 10A). Most of all, at the early stationary phase, the adhesive capacity of *V. cholerae* anaerobically cultured was comparable with that of aerobically cultured ones (Fig. 10B). Therefore, these data indicate that anaerobic conditions regulate the adhesion of *V. cholerae* at the mid-log phase.

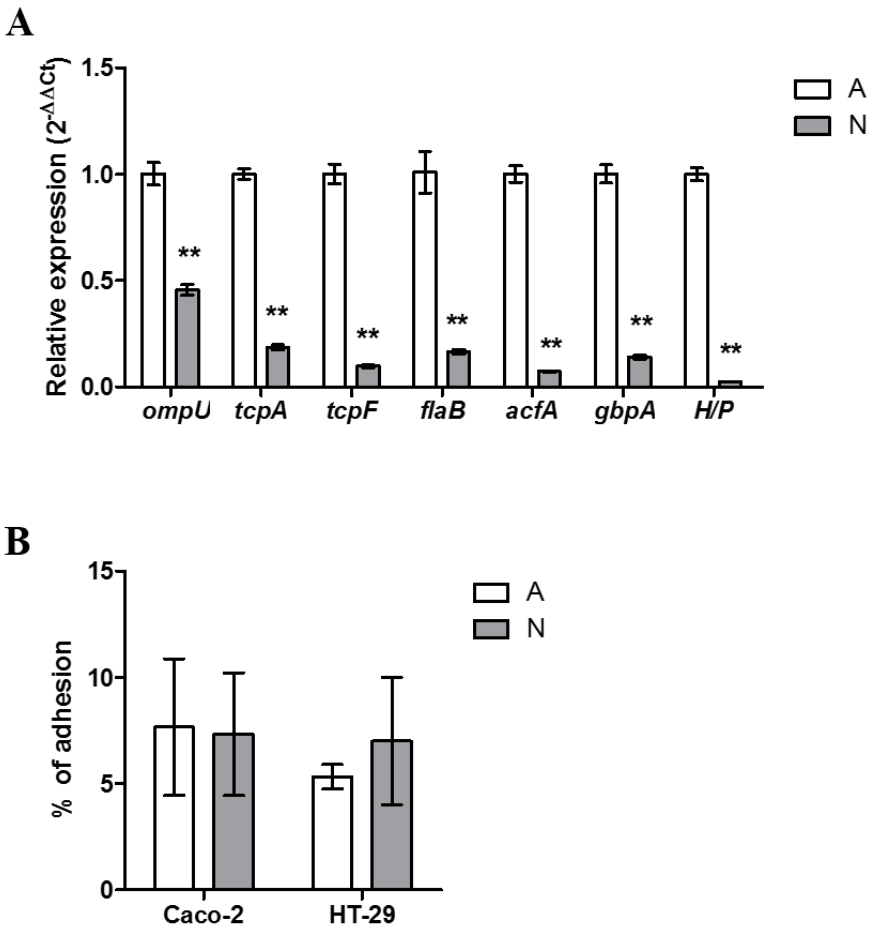


Figure 10. *V. cholerae* O1 T19479 grown until an early-stationary phase fails to demonstrate an increase of its adhesion ability. *V. cholerae* was grown in aerobic (A) and anaerobic (N) conditions until an early-stationary phase. (A) The quantitative expressions of several adhesion molecules of *V. cholerae* were analyzed through real-time PCR. Each gene expression was normalized against the endogenous expression of recA from *V. cholerae*. (B) Caco-2 and HT-29 cells were treated with *V. cholerae* at an MOI of 5 for 1 h.

3.7. Oxygen usage is involved in the effect of anaerobiosis on adherence of *V. cholerae.*

To further investigate the effect of oxygen level in culture environment, microaerophilic conditions were adopted in this study, as previously described [56]. *V. cholerae* was cultured under microaerophilic (static culture without shaking in an aerobic environment). Analogous to the anaerobic conditions, the microaerophilic conditions increased adherence of *V. cholerae* on Caco-2 and HT-29 cells compared to aerobic conditions (Fig. 11A). Indeed, the expressions of *tcpA, tcpF* and *ompU* were increased under the microaerophilic environment (Fig. 11B); *ompU* gene was dramatically induced in microaerophilic conditions. Therefore, the data confirmed that oxygen usage of *V. cholerae* is associated with their adherence to host intestinal epithelial cells.

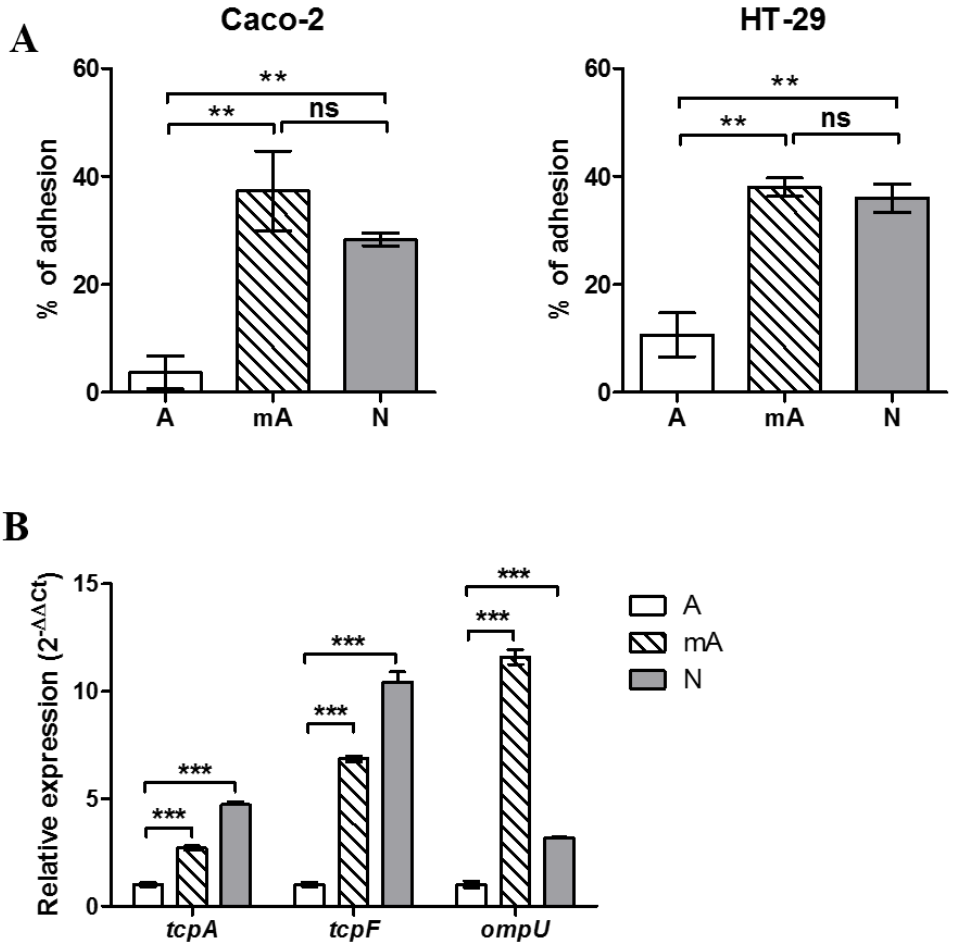


Figure 11. Oxygen limitation changes the adhesion of *V. cholerae* O1 T19479 against human epithelial cells. *V. cholerae* was grown in an aerobic (A), microaerophilic (mA) and anaerobic (N) conditions until the mid-log phase. (A) Caco-2 and HT-29 cells were treated with *V. cholerae* at an MOI of 5 for 1 h. (B) The quantitative expressions of *tcpA, tcpF* and *ompU* were analyzed from the cDNA of *V. cholerae* at mid-log phase. An asterisk indicates $^{**}P < 0.01$.

3.8. Anaerobically cultured *V. cholerae* O1 T19479 showed improved colonization to small intestine of mice *in vivo*.

To confirm the enhanced adherence of anaerobically cultured *V. cholerae* obtained from *in vitro* culture (Fig. 2), adult mice were instilled orally with *V. cholerae* to adopt *V. cholerae* colonization model under ketamine anesthesia [70]. As expected, significant difference of colonization between aerobically- and anaerobically-grown *V. cholerae* was observed, although the variation was quite large (Fig. 12). Therefore, anaerobically cultured *V. cholerae* showed enhanced colonization on the apical surface of host intestinal epithelial cells.

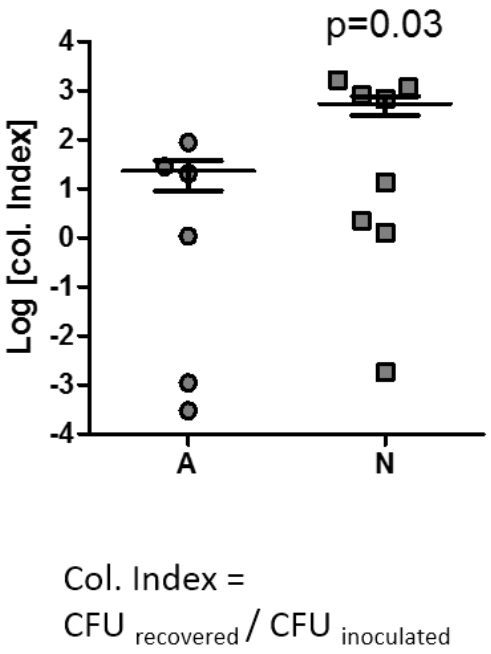


Figure 12. Anaerobic growth increases the colonization of *V. cholerae* O1 T19479 in mice intestines. Mice were orally administrated sodium bicarbonate and streptomycin resistant *V. cholerae* O1 T19479 (10^8 CFU per mouse) under anesthesia. The small intestines were harvested and homogenized for use in bacterial identification 20 h later. The amount of intestinal *V. cholerae* was calculated by the CFU of diluted intestinal contents on a streptomycin-containing agar plate. (Col. Index = CFU recovered/ CFU inoculated)

3.9. Infectivity of *V. cholerae* in streptomycin-treated mouse model

V. cholerae generally colonizes the small intestine and secretes cholera toxin to trigger the cholera disease. However, the association between colonization and virulence in cholera disease remains elusive. To get more insight about this, survival rate and the number of *V. cholerae* within fecal pellets were assessed from mice infected orally by *V. cholerae* O1 T9479 cultured in aerobic or anaerobic conditions. Here, streptomycin was treated to reduce colonization resistance of mice and make mice susceptible to orally administrated *V. cholerae* [65]. When 1.6 x 10^8 CFU of *V. cholerae* O1 T19479 were used, aerobically cultured *V. cholerae* clearly showed higher infectivity than anaerobically cultured one (Fig. 13A). 80% of mice survived when injected with anaerobically cultured *V. cholerae*, whereas all of the mice were dead at day 6 in the group administered with aerobically cultured *V. cholerae*. Indeed, CFU within fecal pellets lasted around 10^5 to 10^6 CFU / ml until 8 days in the aerobic group (Fig. 13B). However, the anaerobic group showed continuous decrease of CFU within the fecal pellets (Fig. 13B). Therefore, these findings demonstrated that an anaerobic culture environment makes *V. cholerae* less virulent with the enhanced adherence.

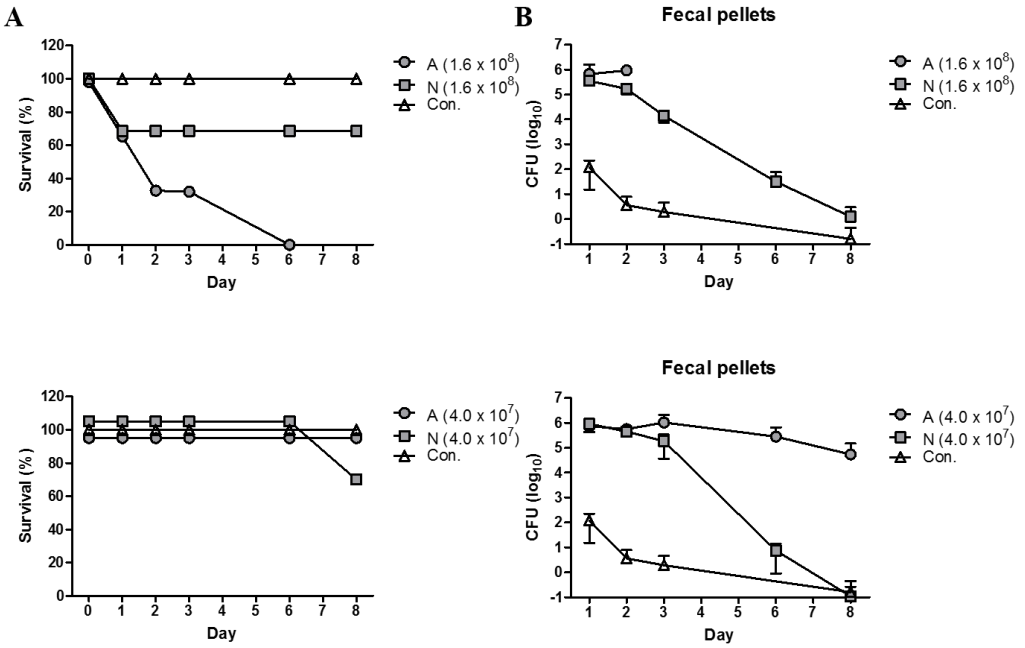


Figure 13. Oral administrations of *V. cholerae* O1 T19479 grown in an aerobic environment induces higher mortality in mice. Mice had ingested 5 mg/ml of streptomycin as drinking water for 24 h. Then mice were orally administered with 4 x 10^7 CFU (A) and 1.6 x 10^8 CFU (B) of streptomycin resistant *V. cholerae* cultured in aerobic and anaerobic conditions. After the administration, mice had ingested 0.2 mg/ml of streptomycin as drinking water for the experimental period. The control group was administered with 1.6 x 10^8 CFU of streptomycin resistant *V. cholerae* cultured in aerobic conditions without streptomycin treatment. Four fecal pellets (~0.07 g) were obtained every day after the administration. The number of *V. cholerae* that formed a colony on the agar plate was calculated from four fecal pellets /1ml PBS suspension. Survival curves were obtained from 3 BALB/c mice for 8 days.

3.10. Infectivity of *V. cholerae* in mouse pneumonia model

Consistent with high infectivity of *V. cholerae* cultured in aerobic environment in streptomycin-treated mice model (Fig. 13), the strong infectivity of aerobically cultured *V. cholerae* O1 T19479 in mouse cholera pneumonia model was also observed. The survival and body weight loss of mice infected intranasally by *V. cholerae* cultured in aerobic or anaerobic conditions were assessed based on the previously established pneumonia model of *V. cholerae* [67]. *V. cholerae* grown in an aerobic environment induced severe pneumonia resulting in death of 80% of mice and prolonged body weight loss (Fig. 14A). Whereas *V. cholerae* grown in an anaerobic environment failed to induce pneumonia symptoms. However, such a strong virulence of aerobically cultured *V. cholerae* was not observed when *V. cholerae* at early-stationary phase was used (Fig. 14B). Therefore, these results confirmed high infectivity of *V. cholerae* grown aerobic conditions.

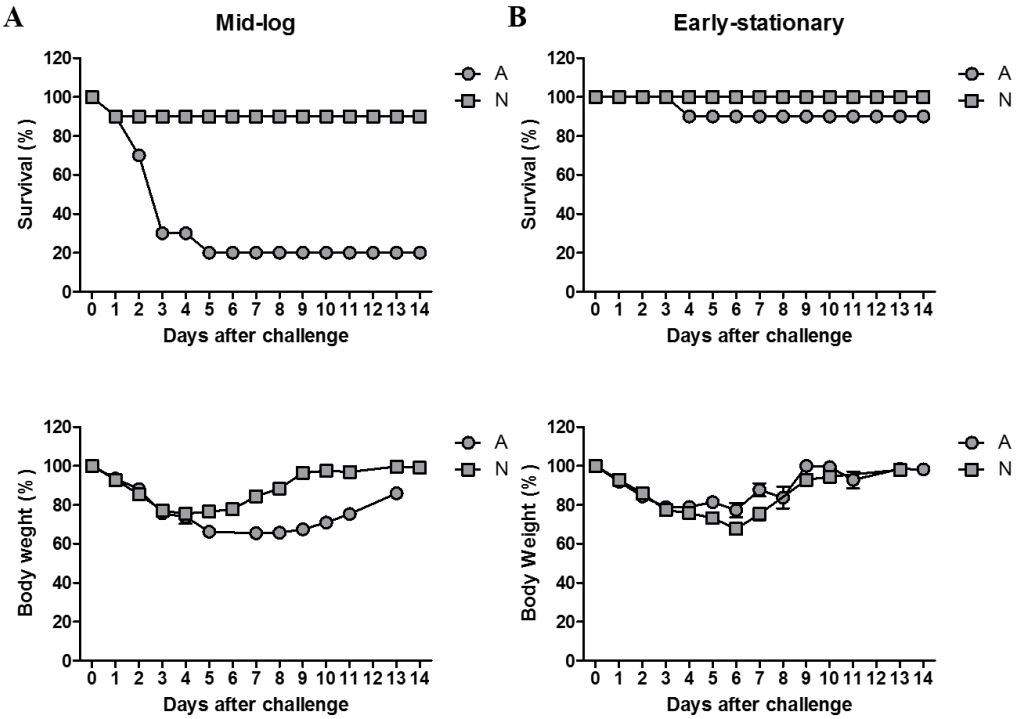


Figure 14. Intranasal administration of *V. cholerae* O1 T19479 grown in an aerobic environment causes pneumonia in mice. The mice were intranasally administered with 2.5 x 10^6 CFU of *V. cholerae* cultured in aerobic and anaerobic conditions. *V. cholerae* was prepared at mid-log phase (A) and early-stationary phase (B). Survival rate and body weight change were obtained from 10 BALB/c mice for 2 weeks. Values are expressed as mean ± SEM.

3.11. *V. cholerae* administered via nasal route resides in the lung in a few hours and disappears within 24 hours.

The high number of bacteria in a site of infection could produce lots of virulence factors and result in pathological changes in the host. In order to examine whether growth conditions make a difference in a bacterial burden at the site of infection, *V. cholerae* administered intranasally to mice and was traced (Fig. 15). The number of living *V. cholerae* in the lungs was measured after the challenge with 1 x 10^6 CFU/ mouse *V. cholerae*. At 6 h after the challenge, *V. cholerae* that colonized the lung, was approximately 1 x 10^4 CFU regardless of their growth conditions.

At 16 h, considerable amounts of anaerobically grown *V. cholerae* remained compared to aerobically grown ones, although the number of *V. cholerae* decreased rapidly overall. Moreover, they disappeared from the lungs within 24 h after administration. There was no spread of *V. cholerae* to the liver, spleen and blood. These results indicate that aerobiosis makes *V. cholerae* more infective than anaerobiosis, even though its existence in the site of infection is quite similar in terms of number and duration.

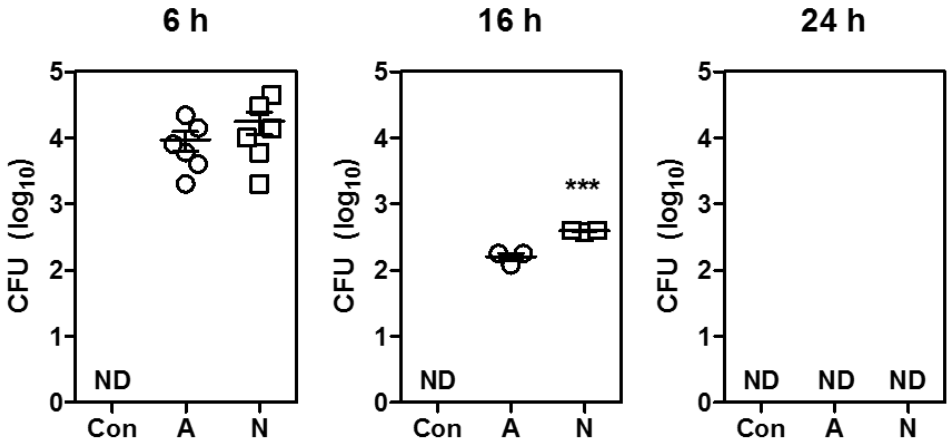


Figure 15. Bacterial load in the lung after intranasal challenge with *V. cholerae* grown in aerobic and anaerobic environments. The mice were administered with 1 x 10^6 CFU of streptomycin-resistant *V. cholerae* cultured under aerobic and anaerobic conditions. Lungs from mice were taken 6 h, 16 h and 24 h after challenge. Lungs were homogenized in 1ml of PBS and serial dilutions were made and plated on LB/agar containing streptomycin. Values are expressed as mean ± SEM. CFU, colony-forming unit. ***$P < 0.001$.

3.12. Protective effect of killed *V. cholerae* vaccine in mouse pneumonia model

Vaccination with inactivated whole bacteria could induce immunogenic responses depending on bacterial virulence factor. In order to examine vaccine effectiveness, two doses of heat-killed *V. cholerae* vaccines were instilled intranasally to mice. Survival and body weight of mice challenged with *V. cholerae* were recorded daily for 2 weeks. The vaccine prepared from *V. cholerae* cultured in an aerobic environment showed 80 % of protection against cholera infection while the vaccine from an anaerobic environment showed 20% of protection rate (Fig. 16). Significant body weight loss was not detected in both groups. These results indicate that an aerobic culture environment is important for preparation of cholera vaccines to elicit high protection against infection.

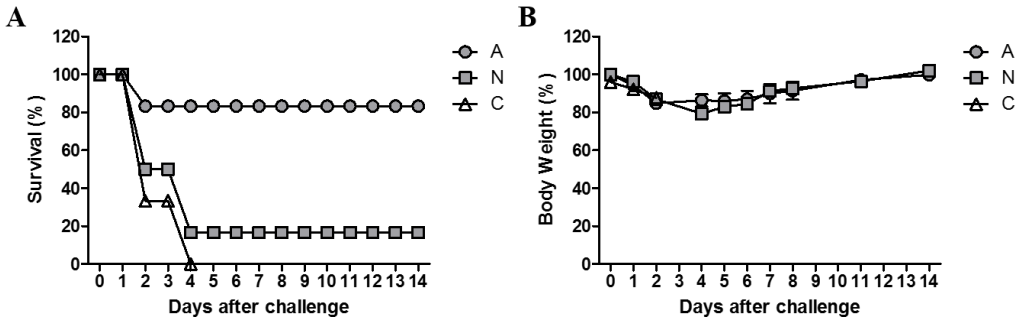


Figure 16. Vaccination with aerobically prepared vaccine induces high protection against lethal challenge with *V. cholerae*. Mice were intranasally immunized twice at 2-week intervals with heat inactivated *V. cholerae* O1 T19479 cultured in aerobic and anaerobic conditions. Immunized groups were challenged with live *V. cholerae* 1 week post last immunization. (A) Survival rates and (B) body weight changes after intranasal challenge were monitored for 14 days (6 mice/ group). Values are expressed as mean ± SEM.

3.13. Cholera vaccine prepared in aerobic conditions induces a higher production of anti-*V. cholerae* specific IgG in BAL fluids, lung extracts and sera.

Since cholera vaccines in this study is composed of inactivated bacterial whole cells, protection against cholera infection is considered to be mediated by anti-bacterial and anti- LPS antibodies. To get an understating about immunogenicity of cholera vaccines, the level of antibody in BAL fluids, lung extracts, and sera were analyzed from mice immunized with vaccines. Vaccines prepared in both aerobic and anaerobic conditions showed increased levels of anti-*V. cholerae* (Fig. 17) and anti-LPS specific (Fig. 18) antibodies compared to control groups. Of note, the vaccine prepared in an aerobic environment induced higher levels of anti-*V. cholerae* IgG but not IgM or IgA in BAL fluids, lung extracts, and sera compared to ones in an anaerobic environment (Fig. 17A, B, C). These results could elicit that the enhanced production of anti-bacterial IgG in the aerobically prepared vaccine group will take part in the protection of mice from cholera infections.

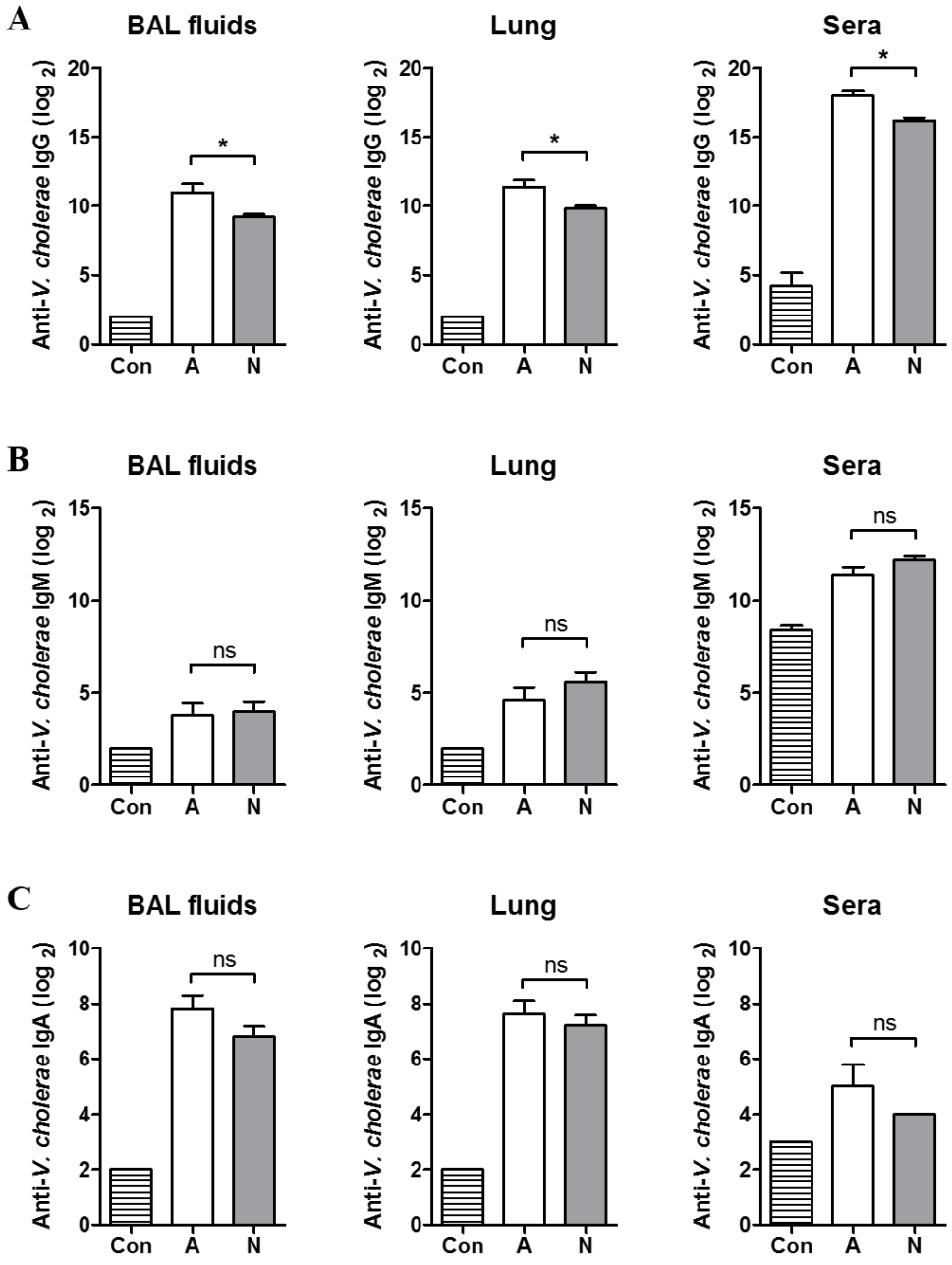


Figure 17. Intranasal immunization with heat-inactivated *V. cholerae* O1 T19479 grown in an aerobic environment induces higher *V. cholerae*-specific IgG than those

in an anaerobic environment in BAL fluids, lungs and sera. Mice were immunized twice at 2-week intervals with heat-inactivated *V. cholerae* cultured under aerobic and anaerobic conditions. The control group mice were immunized with PBS. BAL fluids, lungs, and sera were collected 1 week post last immunization. *V. cholera*-specific IgG (A), IgM (B), and IgA (C) were analyzed in BAL fluids, lungs and sera. Values are expressed as mean ± SEM. *$P < 0.05$. **$P < 0.01$

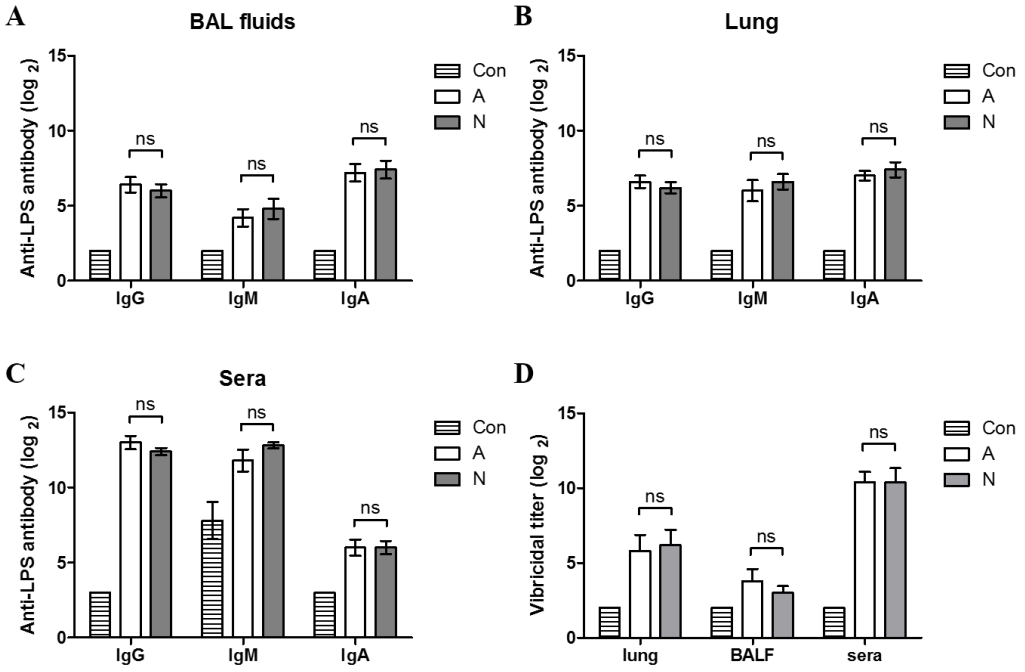
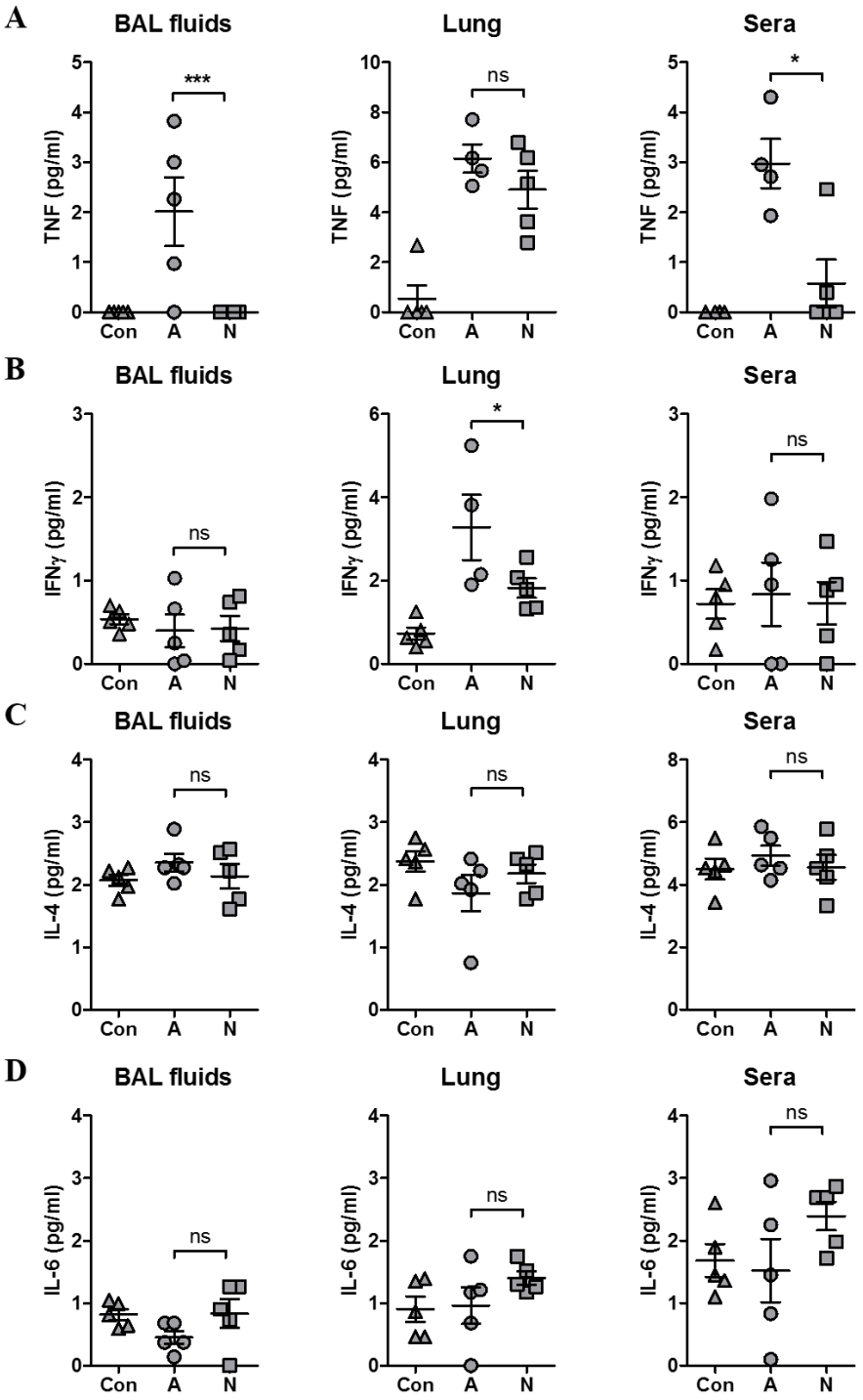


Figure 18. Intranasal immunization with heat-inactivated *V. cholerae* grown in an aerobic and anaerobic environment induces similar immunogenic properties. Mice were immunized twice at 2-week intervals with heat inactivated *V. cholerae* cultured under aerobic and anaerobic conditions. The control group mice were immunized with PBS. BAL fluids, lungs, and sera were collected 1 week post last immunization. *V. cholerae* LPS-specific IgG, IgM, and IgA were analyzed in (A) BAL fluids, (B) lung, and (C) sera. (D) The samples of BAL fluids, lung extract and sera were assayed for vibriocidal activity against *V. cholerae* O1 Inaba. Values are expressed as mean ± SEM.

3.14. Cholera vaccine prepared in the aerobic environment induces the production of TNF-α and IFN-γ.

Protective effects of vaccines come from not only humoral immune responses but also cellular immune responses. Although the vaccine prepared in the aerobic condition induced anti-*V. cholera*-specific IgG expressions more than ones in the anaerobic condition, it is inadequate to explain efficient protection against cholera infection. To get a better understanding about the immunogenicity of cholera vaccines, vaccine-induced expressions of Th1/Th2/Th17 cytokines from the BAL fluids, lung extracts and sera were investigated. The expressions of TNF-α, IFN-γ and IL-17A were enhanced by vaccination compared to the control group. However, IL-2, IL-4, IL-6 and IL-10 expressions were not changed after the vaccination. Notably, TNF-α is highly induced in BAL fluids and sera by vaccination with heat-killed *V. cholerae* grown in an aerobic environment and IFN-γ was increased in the lung extracts (Fig. 19A, B). The up-regulation of IL-17 was likewise detectable, although the existence was low-level and had quite large variations. These results suggest that TNF-α in BAL fluids and sera, and IFN-γ in the lung would be critical for inducing protection against *V. cholerae* induced pneumonia.


A
BAL fluids
Lung
Sera
TNF (pg/ml)
***
ns
*
B
IFNγ (pg/ml)
ns
*
ns
C
IL-4 (pg/ml)
ns
ns
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IL-6 (pg/ml)
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A
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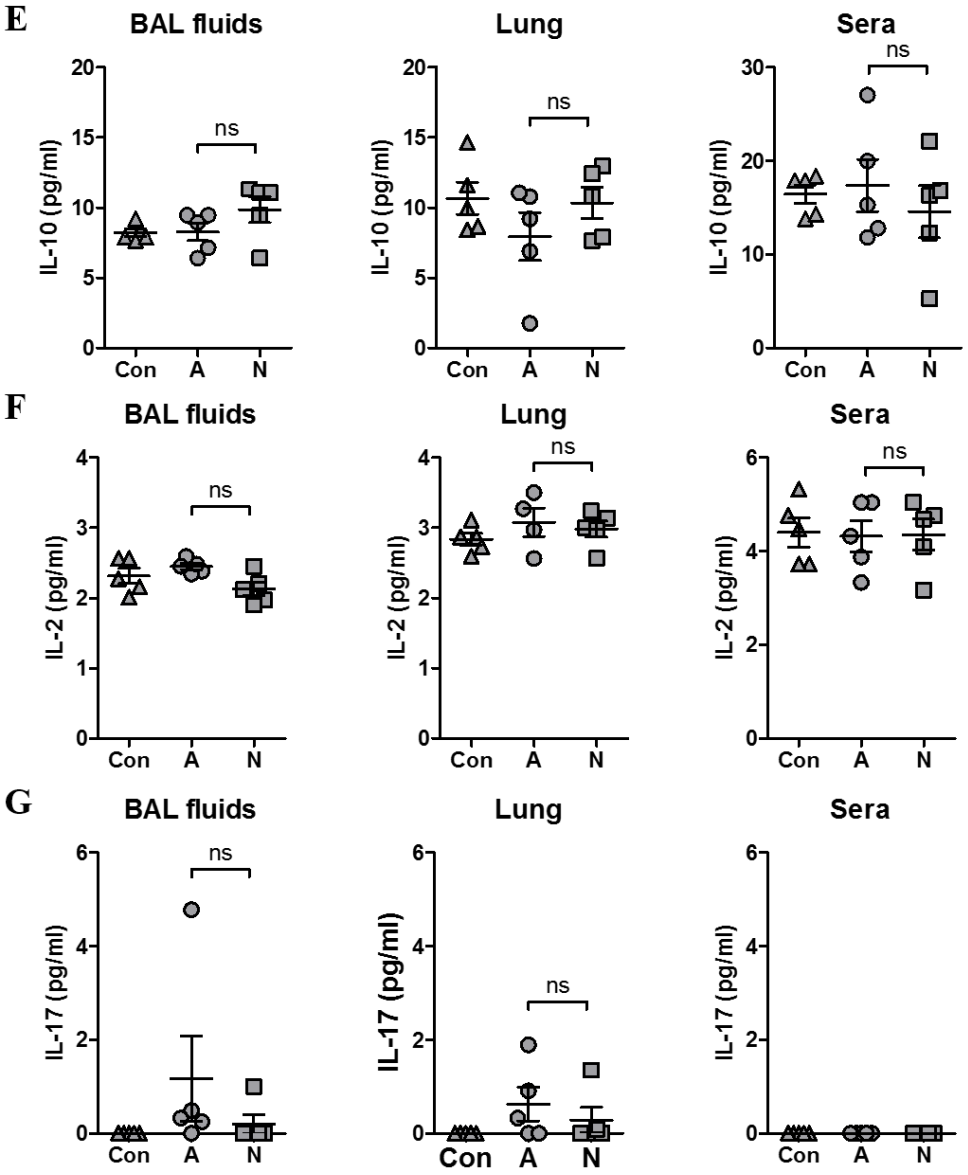

Figure 19. Intranasal immunization with heat-inactivated *V. cholerae* O1 T19479 grown under aerobic conditions induces TNF-α, IFN-γ and IL-17A. Mice were

immunized twice at 2-week intervals with heat inactivated *V. cholerae* prepared in aerobic (h-A) and anaerobic (h-N) conditions. The control group was immunized with PBS. BAL fluids, lungs and sera were collected 1 week post last immunization. The expression levels of TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-2 and IL-17 were analyzed through cytometry bead array. Values are expressed as mean ± SEM.

3.15. Cholera vaccines prepared in aerobic and anaerobic conditions elicit different immune responses in BMDCs.

To understand the role of antigen presenting cells mediating humoral or cellular immune responses, bone marrow-derived dendritic cells were treated with heat-killed vaccines prepared in aerobic and anaerobic conditions. Both heat-killed vaccines induced all cytokines and chemokines which are tested in the study in BMDCs. In addition, the vaccines generated immune responses discordantly (Fig. 20). BMDCs produced high levels of IL-6, IL-10, and IL-1β in response to vaccine prepared from aerobically-grown *V. cholerae* whereas the expression of pro-inflammatory cytokine TNF-α and IL-12 were reduced. It is worth noting that chemokines such as CCL-2, CXCL-10, CCL-12, and CXCL-1 were significantly increased in BMDCs activated with vaccine prepared from aerobically-grown *V. cholerae* compared to ones from anaerobic condition. It means vaccine prepared from aerobically-grown *V. cholerae* could activate the chemokine induced recruitment of immune cells such as monocyte/macrophage, neutrophil, T cell. In turn, this recruitment could act on the next step of responses to protect the host from cholera infection.

Moreover, BMDCs activated and matured in response to both vaccines. In consistent with cytokine expression, however, the cell surface molecules of BMDCs were differently regulated by two vaccines (Fig. 21). The high expression of CD14, TLR2 and MHC class II molecules of BMDCs was considered as ready-to-act state of DCs to respond and recognize next exposure of *V. cholerae*.

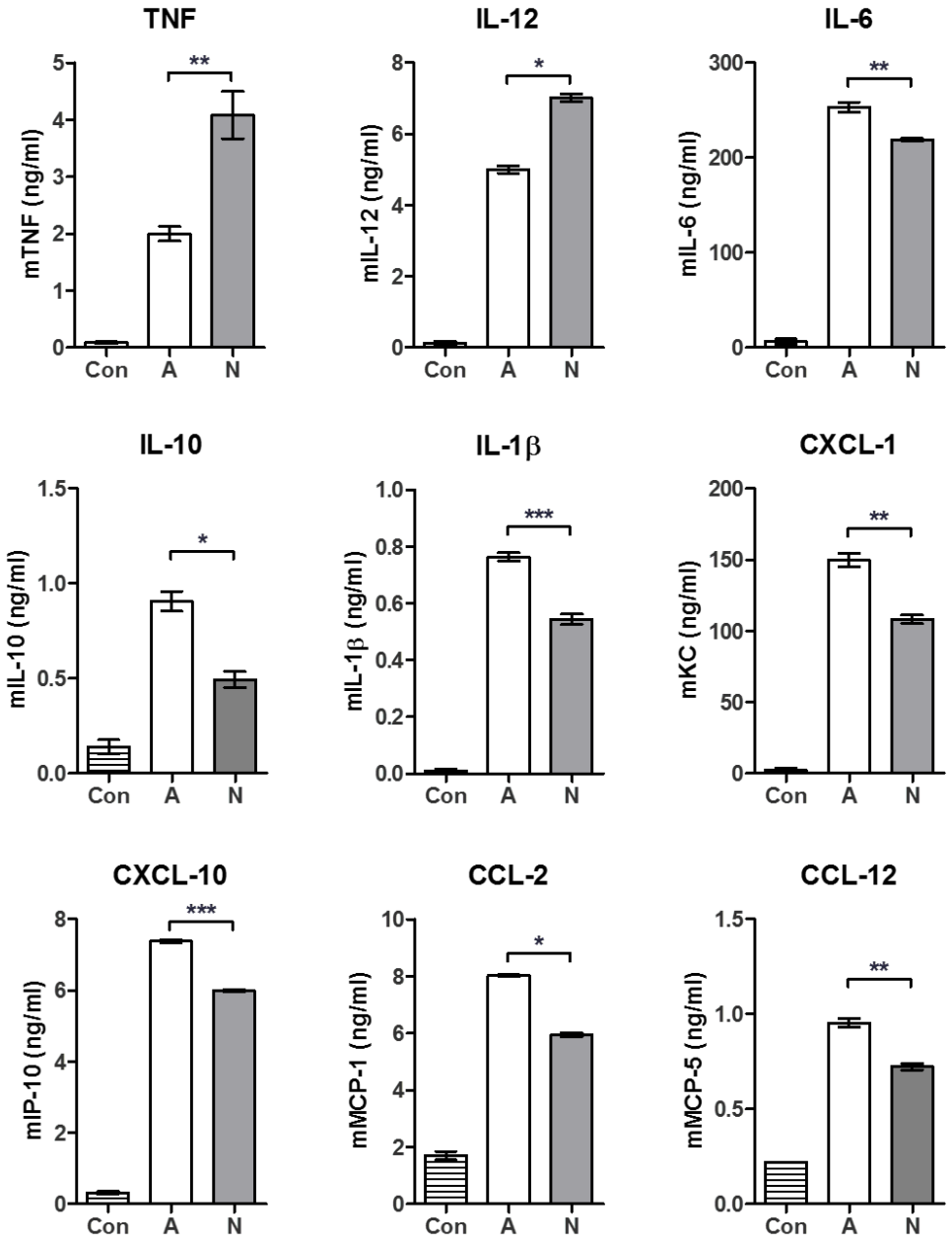

Figure 20. Effect of heat-killed *V. cholerae* on the secretion of cytokines and chemokines by BMDCs Mouse bone marrow derived dendritic cells were treated with

1 x 10^7 CFU heat-killed *V. cholerae* cultured in aerobic (A) and anaerobic (N) conditions for 24 h. Culture supernatant were used for analyzing the expression of TNF-α, IL-12, IL-6, IL-10, IL-1β, KC, IP-10, MCP-1, and MCP-5. The control group was treated with PBS for 24 h. Values are expressed as mean ± SD. An asterisk indicates $P < 0.05$.

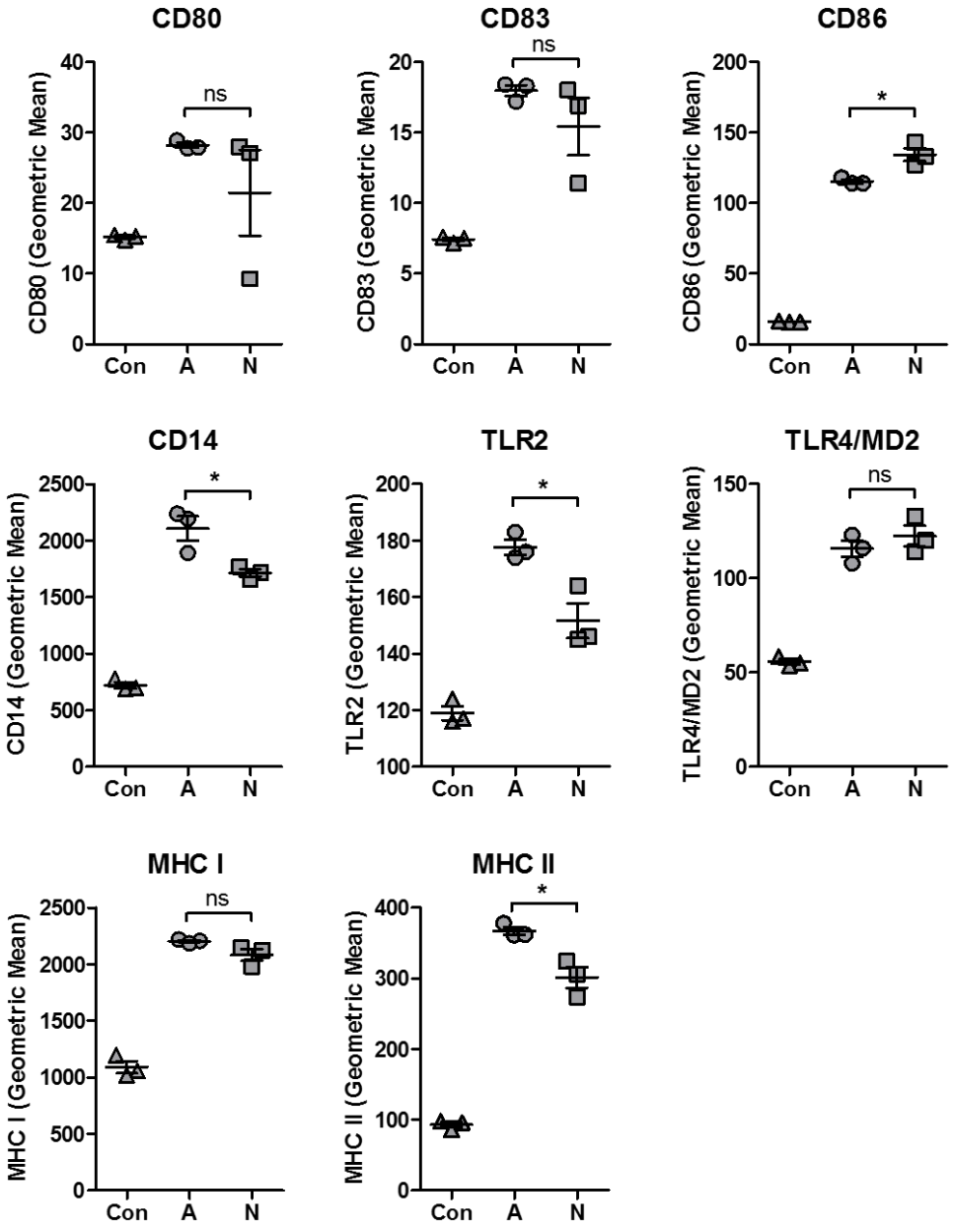

Figure 21. Effect of heat-killed *V. cholerae* on the expression of surface molecule on BMDCs Mouse bone marrow derived dendritic cells were treated with 1 x 10^7 CFU heat-killed *V. cholerae* cultured in aerobic (A) and anaerobic (N) conditions for 24 h.

The stimulated BMDCs were collected and stained with fluorescent dye-labeled CD80, CD83, CD86, CD14, TLR2, TLR4/MD2, MHC class I, and MHC class II antibodies. The fluorescence intensity was detected through flow cytometry and analyzed with FlowJo software. Values are expressed as mean ± SD. An asterisk indicates $P < 0.05$.

3.16. Protective effect of killed *V. cholerae* vaccine in mouse small intestinal colonization

Vaccination with inactivated whole bacteria could defend against bacterial colonization in the small intestine by producing antibodies against *V. cholerae* infection. In order to examine vaccine effectiveness, mice were administered orally with sodium bicarbonate buffer and then followed immediately with two doses of heat-killed *V. cholerae* vaccines under ketamine anesthesia. As expected, reduced colonization of *V. cholerae* in the small intestine of mice vaccinated with heat-killed *V. cholerae* was observed (Fig. 22). Of note, *V. cholerae* did not colonize well in mice vaccinated with heat-killed *V. cholerae* grown in an anaerobic environment compared to those grown in an aerobic environment although they had quite large variations. This may be caused by enhanced adhesion factors in *V. cholerae* cultured in anaerobic environments (Fig. 4A, B).

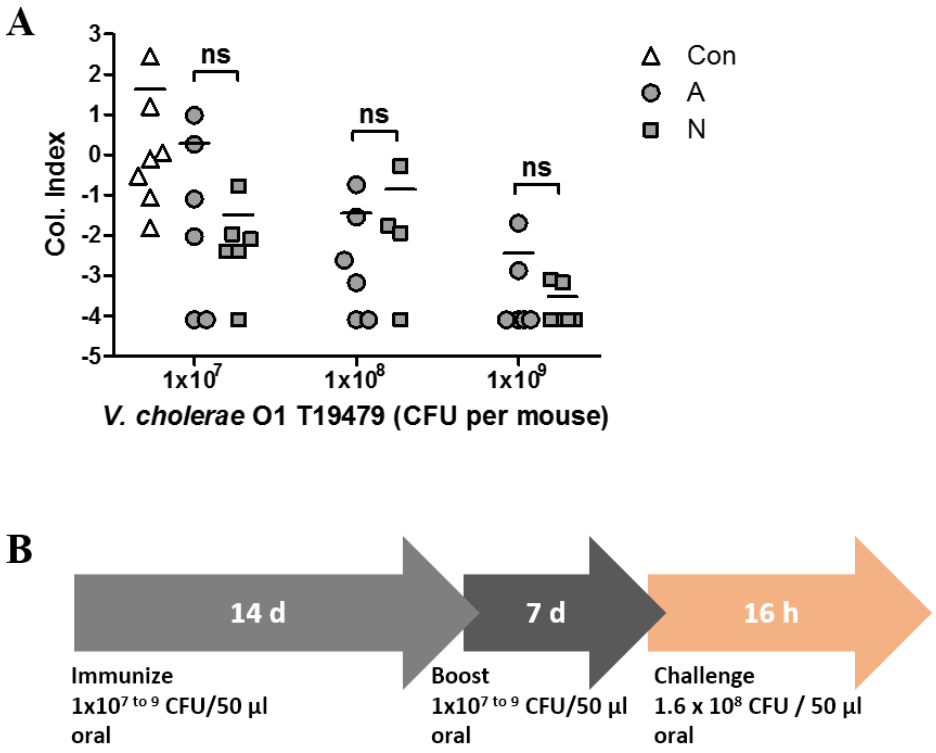

Figure 22. Oral vaccination with inactivated *V. cholerae* grown in an anaerobic environment contributes to diminishing colonization of *V. cholerae* in mice intestine. (A). Mice were orally immunized twice by 2 week-intervals with heat inactivated *V. cholerae* (as indicated CFU in x-axis) prepared in aerobic and anaerobic conditions. One week post last immunization, immunized groups were challenged with live *V. cholerae* (10^8 CFU per mouse) under anesthesia. The small intestines were harvested and homogenized for use in bacterial notification 20 h later. The amount of intestinal *V. cholerae* was calculated by the CFU of diluted intestinal contents on an agar plate. (Col. Index = CFU recovered / CFU inoculated) Values are expressed as mean ± SEM. **(**B) schematic diagram of experimental schedule.

Table 4. Primers used for the real-time PCR amplification

Gene	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)
aphA	aatgggggaacagggtttag	cgaccaatttacgcgattct
aphB	accaacagccaaggtgaaac	ggctggtgatctttgtggtt
ompU	catgctagccgcttcttacc	cgctgtttctgcattgttgt
toxT	tgggcagatatttgtggtga	gaaacgctagcaaacccaga
acfA	tcataccccattcatcagca	agtgcaccaaagcgattctt
flaB	aaatcgatggagcgtttgtc	gcgttccgaagaagagtttg
ctxA	ttgttaggcacgatgatgga	tggaatcccacctaaagcag
ctxB	atgcacatggaacacctcaa	gcaatcctcagggtatccttc
toxS	gtcgggtagacgtcacttcc	gctgcaattgctcgtcagta
toxR	ttaacccaagccatttcgac	gatgaaggcacactgcttga
tcpP	tgagtgggggaagataaacg	ttggattgttatccccggta
tcpH	gaccgatccacaaggtaacg	taagggaaggcgagaaaaca
ompA	gtgcagcaggcctagagttc	actggtgctggctcttcact
tolC	acatgatgcacaggcacaat	gctttttcgatcaacgcttc
CRP	cctcaaaccgatccaacact	tgtacgctcttggccttctt
tcpB	tacaagcgggaagctgaact	acctgctgtgaggcagtttt
tcpQ	gcacaaggagagatgcacaa	accgtgtaaatcagcccaag
tcpC	ctccgcctcagcaagattag	tcggcataaccatcatgcta
tcpD	actgggaggtggagcctatt	aaaaaccagttagcgcttgg
tcpS	tgcacccgttttatctcctc	attgcccttggaaaattgaa

Chapter IV. Discussion

The present study was intended to compare virulence and immunogenicity of *V. cholerae* grown between aerobic and anaerobic environments and demonstrated the following findings. 1) An anaerobic environment affected the growth of *V. cholerae*, but not morphology, cholera toxin secretion or biofilm formation. 2) Anaerobiosis improved the adhesive ability of *V. cholerae* on human epithelial cells through higher expressions of *tcpA, tcpF* and *ompU* genes. 3) Outer membrane structure, growth phase, and oxygen usage were involved in the effect of anaerobiosis on adherence of *V. cholerae.* 4) *V. cholerae* grown under the aerobic environment showed higher infectivity, causing mouse pneumonia than that under the anaerobic environment. 5) Cholera vaccine prepared in an aerobic environment induced a higher level of anti-*V. cholerae* IgG as well as of TNF-α and IFN-γ. 6) Vaccine derived from aerobic culture showed enhanced protection to cholera infection.

In this study, anaerobic environments conferred *V. cholerae* the ability to effectively adhere to human epithelial cells as well as the small intestines of mice. Bacterial attachment to apical surfaces of host epithelium could be a first step for forming a microcolony or biofilm, which eventually prevent bacterium from mechanical and chemical challenges such as fluid flow and antibiotics treatment [83, 84]; that is, *V. cholerae* commences to form biofilm in intestine *in vivo* after the initial colonization. However, in the study, an anaerobic environment did not enhance biofilm formation of *V.*

cholerae in vitro. Considering the fact that luminal side of human intestinal tracts are oxygen-limiting [85], *V. cholerae* under the anaerobic condition might go into inactive, latent state, after colonization without metabolic expenditure for growth. Then, successfully colonized *V. cholerae* might facilitate the expression of virulence factors when they were dispersed from epithelium to escape from the host body [86].

Increased adhesion of *V. cholerae* grown under anaerobic atmospheres seemed to be attributed to changes in expression level of colonization factors such as *tcpA*, *tcpF* and *ompU*. These facts are consistent with the previous reports which revealed increased expression of TcpA and TcpF and enhanced interaction of TcpP and ToxR to activate *toxT* under oxygen-limiting conditions [55-57]. Of note, upregulation of *ompU* in an anaerobic environment is firstly observed in this study. OmpU was known to play a role not only in IL-8 production but also adherence of *V. cholerae* in human IECs [72]. Meanwhile, the expression of *flaB* and *HA/P* were slightly reduced in an anaerobic environment than an aerobic one, which is consistent with the report in 2004, Kan *et. al*., in that flagellin B expression was decreased in anaerobic conditions [54]. The reduction of flagellin and HA/P in anaerobic environment might be advantageous to avoid immune recognition and clearance [87] beyond colonizing. However, the expression of flagellin and HA/P could be induced before *V. cholerae* reaches inner mucus of epithelium and when it escapes from the intestine [16, 88, 89]. Taken together, it is plausible that increased expression of flagellin and HA/P by aerobic atmospheres could guide *V. cholerae* to the inner region of the mucus layers in the intestinal epithelium. Then,

upregulated expression of TcpA, TcpF and OmpU could allow *V. cholerae* to attach on epithelial cells firmly.

Growth phase, outer membrane structure and oxygen usage of bacteria affected the adhesion ability of *V. cholerae*. The distinct effect of growth phase on adhesion and invasion was observed in *Eschelichia coli*, *Staphylococcus aureus*, and *Salmonella choleraesuis* [90-92], suggesting possible existence of conserved mechanism depend on growth phase on bacterial adherence. The expression of LPS and capsular polysaccharide of *V. cholerae* O139 was known to be correlated with colonization ability in the infant mouse small intestine [93], but the adhesion of *V. cholerae* O139 4260B was reduced in anaerobic environment. This might be partially caused by the structural discordance of co-existence of LPS and capsular polysaccharide and increased expression of colonization factors in anaerobic atmosphere. Further studies about the effect of anaerobiosis on bacterial surface structure (LPS and capsular polysaccharide) need to be investigated. In addition, finding the host receptors interacting with TcpA, TcpF and OmpU would give decisive explanation for the reduced adhesion ability of capsule containing *V. cholerae*, observed here. Ambient oxygen also regulates the adhesion of *V. cholerae* by regulating gene expression of colonization factors [55] either in the absence of oxygen (anaerobic (oxygen lacking)) or in microaerophilic (oxygen limiting) conditions, which suggest an importance of ambient oxygen levels for *V. cholerae* adherence.

In the present study, human colon epithelial cell lines were used to assess *in vitro* adhesion ability of *V. cholerae*, and the *in vitro* results was confirmed in mouse small intestinal colonization model *in vivo*. Although derived from a colon, the two human epithelial cells lines, HT-29 and Caco-2 cell showed fidelity of resembling *in vivo* human small intestinal cells [94, 95]. Moreover, differentiated and polarized HT-29 and Caco-2 cells resemble the enterocytes lining of the small intestine in their phenotype morphologically and functionally [96]. Considering the fact that the colonization data from *in vivo* mouse small intestine reflect adherence as well as growth of bacteria within intestinal tracts, tracing the colonization of fluorescently labeled *V. cholerae in vivo* or assaying adhesion in dissect small intestines *ex vivo* would provide alternative methods for further clarification.

V. cholerae cultured in aerobic atmosphere induced high mortality of mice compared to their anaerobic counterpart, consistent with the previous reports that planktonic bacteria had more invasive properties than those forming a biofilm [97, 98]. Unexpectedly, cholera toxin, known as a major effector virulence factor, was not likely to be associated with pathogenicity of the infected mice, since cholera toxin was not produced by *V. cholera* El Tor T19479 in both aerobic and anaerobic conditions. The aerobic culture environment let *V. cholerae* grow like as planktonic cells, whereas microcolony and biofilm formed in anaerobic culture environment as a survival strategy [99]. It is plausible that planktonic bacteria prefer to invade and kill host cells, whereas bacteria tend to form biofilm to employ defensive mechanisms for maintaining its niche

in the host. This study suggests that rather than anaerobic environment, the aerobic culture environment is better for induction of higher immunogenicity.

Although cholera toxin is the major pathogenic molecule of *V. cholerae*, there are several virulence factors which makes it as infectious. However, in-depth mechanism which virulence factors of *V. cholerae* grown in an aerobic environment are involved in the high mortality to mice was not investigated here. The previous studies, which comparing the expression of proteins from *V. cholerae* between the aerobic and anaerobic culture conditions, showed different levels of flagellin, outer membrane protein, colonization factor, and chemotaxis-related proteins [54, 55, 100]. However, these proteins seem to be more associated with bacterial colonization and mobility rather than the infectivity. Although there is no report yet about the expression of other cholera toxins such as accessory toxin, RTX (repeats-in-toxin) and Zot (zonula occludens toxin) under anaerobic condition, they could respond to oxygen limiting environment. Indeed, hemolysin and the RTX toxin could act as virulence factors during intestinal infection of mice [101] and take part in colonization [70]. Zot increased the permeability of the small intestine by affecting intercellular tight junctions [102]. However, many aspects of aerobiosis which regulates virulence of *V. cholerae* need to be clarified in the further.

It is worth noting that *V. cholerae* cultured in aerobic conditions was more virulent in spite of their low adhesion ability. Although colonization has considered as one of the major virulence factors, there might be more complicated modes of action in virulence

of *V. cholerae* beyond colonization. The colonization and forming microcolonies might be a defense mechanism of *V. cholerae* to protect itself from intestinal peristalsis and chemical destruction. Therefore, it is plausible that enhanced adhesion might give *V, cholerae* a chance to stay in the intestine, and *V, cholerae* detached and dispersed from intestine might act on the late stage of symptoms [86] not cause of acute diarrhea. The exact mechanism was not discovered yet, virulence factors besides colonization factors may play a role in the pathogenesis of *V. cholerae* grown in aerobic condition. Moreover, toxin expressions and corresponding antibodies at the site of infection need to be analyzed to get more understanding about virulence mechanism of *V. cholerae* in mouse models.

Here, an aerobic culture environment was important to elicit higher immunogenicity and good protective effect when it was used as killed whole-cell vaccine. The effect of bacterial culture condition on the immune responses has been less-explored field of research in vaccine development for enteric disease. Although, in vaccine development against *Streptococcus pneumoniae*, anaerobic conditions were adopted in the production processes for pneumococcal capsules to obtain high yields [103], most inactivated (killed) vaccines are prepared from bacteria which sufficiently grown in the bioreactor complemented with oxygen. It seemed obvious that enteric bacteria could induce different immune responses depend on their growing condition such as aerobic and anaerobic [104]. Although the higher immunogenicity which induced by vaccine prepared from aerobically-grown *V. cholerae* is still elusive, current oral cholera

vaccines prepared by aerobic-cultured bacteria may be better to induce immunogenicity of vaccine.

To evaluate immunogenicity of current cholera vaccines, vibriocidal antibody titers have long been measured. However, there is no difference of vibriocidal titer between vaccines from *V. cholerae* cultured in aerobic and anaerobic conditions. Cholera vaccine from *V. cholerae* cultured in aerobic environment seemed to elicit an increased expression of *V. cholerae*-specific IgG, TNF-α and IFN-γ in mice for protective effect against infection. The vaccine-induced substantial increase of IFN-γ and IL-17 is consistent with the previous report that elevations of IFN-γ and IL-17 in cholera patients and vaccinees [105]. Although TNF-α has understood as endotoxic molecule which is induced by outer membrane vesicle of O1 serogroup [106], TNF-α could lead to neutrophil influx following antigen challenge of immunized mice to clear the infectious agents [107]. The increased expression of IgG could activate phagocytic immune cells, classical pathway of complement system, neutralization of toxin and also play a role in antibody-dependent cell-mediated cytotoxicity systemically. It seems that vaccine prepared from aerobically-grown *V. cholerae* induces protective immunity through IgG induced neutralization and/or phagocytosis of *V. cholerae* and Th1 and Th17 biased immune responses.

In the study, both vaccines prepared from aerobically- and anaerobically-grown *V.*

cholerae induced a variety of cytokines, chemokines, co-stimulatory molecules, and pattern recognition receptors, however the expression levels were differently modulated depend on the growth conditions of *V. cholerae*. Vaccine prepared from aerobically-grown *V. cholerae* produced low levels of pro-inflammatory cytokines and higher levels of chemokines and TLR2 receptor compared to counterpart prepared from anaerobically-grown *V. cholerae*. The high expressions of TNF-α and IL-12 might be caused by increased colonization factors such as OmpU under anaerobic atmosphere [108, 109]. Up-regulation of TLR2 on BMDCs in response to vaccine prepared from aerobically-grown *V. cholera*e might activate TLR2 signal transduction and subsequent immune responses. The killed whole-cell vaccine-induced chemokine expression in BMDCs is consistent with the previous result that showed increased chemokine expression in human intestinal epithelial cells [110, 111], then, it could recruit immune cells such as monocyte/macrophage, neutrophil, T cell at the site of infection. Therefore, DCs may mediate T cell-mediated immune responses such as Th1 or Th17 responses depending on immunological properties of *V. cholerae*.

Collectively, this study describes that *V. cholerae* might adapt and protect itself in the intestine by means of colonization since anaerobiosis increased the colonization ability and the expression of colonization factors from *V. cholerae*. Moreover, aerobic condition could affect immunogenicity of *V. cholerae* critically when it adopted in live-attenuated vaccines because planktonic and dispersed *V. cholerae* from biofilm could be more immunogenic causing humoral and cellular immune responses in the host compare to

colonized *V. cholerae*. Finally, the enhanced immunogenic responses and protective immunity of vaccine prepared from aerobically-grown *V. cholerae* might give a consideration of how to prepare the whole cells before the physical inactivation for whole-cell killed vaccine development.

Chapter V. References

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국문초록

호기배양과 혐기배양 콜레라균의 병독성과 면역원성의 비교

장미선

협동과정 유전공학전공

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콜레라는 급성장내감염 질환으로 콜레라균 (*Vibrio cholerae*)에 의해서 발병된다. 콜레라균은 주로 식품위생시설이 부족하거나 불충분한 위생 기반 시설을 가진 저개발국가에서 풍토적으로 발생한다. 현재 WHO 의 승인을 받고 다양한 국가에서 사용 중인 백신은 Dukoral, Shanchol, Euvichol 세 개이다. 비록 콜레라균이 장내환경인 혐기 환경에서 병인기전을 보여주고 있지만 현재 사용 중인 경구투여백신 세 종류는 호기 환경에서 배양된 콜레라균을 이용하여 만들어졌다. 또한 혐기배양 환경이 유도하는 미생물의 유전자 조절 및 병 독성 조절은 콜레라균에서 잘 알려져 있지 않다.

본 연구에서는 산소가 부족한 혐기환경에서 배양한 콜레라균이 가지는 세포부착 및 집락 형성능 (colonization), 감염능 (infectivity)과 면역유도능

(immunogenicity)에 대해서 조사하였다. 혐기환경에서 배양된 콜레라균은 *tcpA, tcpF ompU* 의 발현 증가와 함께 증가된 상피세포 부착능을 보여주었다. 콜레라균의 세포부착능 증가는 캡슐이 없는 콜레라균 (O1 T19479, O1 N6961 과 O139 CIRS134) 에서만 확인되었고, 세포생장 중 대수기에 해당되는 콜레라균만이 이러한 현상을 보여주었다. 세포부착능은 콜레라균의 주요 병독 인자로 알려져 있지만, 혐기배양으로 인한 콜레라균 세포 부착능의 증가는 마우스를 이용한 콜레라 모델에서 감염성의 증가와 상관관계를 보여주지는 않았다. 열처리로 제작한 콜레라 사백신의 마우스 비강 접종 실험을 통해, 호기적 조건에서 배양한 콜레라균이 혐기적 조건에서 배양한 콜레라균보다 높은 항-콜레라균 면역글로불린 G 를 마우스 비강세포세척액, 폐조직액, 혈청에서 유도하는 것을 확인하였다. 이와 더불어 Th1 과 Th17 에 관여하는 사이토카인의 발현 역시 호기배양으로 만들어진 콜레라 백신에서 증가되었다. 또한 면역세포의 활성화와 이동에 관여하는 케모카인의 발현이 혐기배양백신보다 호기배양백신을 처리한 골수유래 수지상세포에서 더 많이 유도되는 것을 확인하였다. 이러한 호기배양 사백신의 면역원성 증가는 콜레라 감염 모델에서 높은 방어능으로 표현되었다.

결론적으로 본 연구는 혐기성 환경은 콜레라균의 장내 세포로의 부착능을 증가시킬 수 있지만, 동시에 콜레라균의 감염성과 면역원성을 약하게 할 수

있다는 사실을 보여준다. 이러한 결과는 장내감염균의 백신연구에 있어 의미있는 통찰을 제시해 줄 것으로 기대된다.

주요어: 콜레라균, 혐기배양, 백신, 부착능, 감염

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