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Pathophysiology of enteropathogenic *Escherichia coli* during a host infection

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea in developing countries. However, sporadic outbreaks caused by this microorganism in developed countries are frequently reported recently. As an important zoonotic pathogen, EPEC is being monitored annually in several countries. Hallmark of EPEC infection is formation of attaching and effacing (A/E) lesions on the small intestine. To establish A/E lesions during a gastrointestinal tract (GIT) infeciton, EPEC must thrive in diverse GIT environments. A variety of stress responses by EPEC have been reported. These responses play significant roles in helping E. *coli* pass through GIT environments and establishing *E. coli* infection. Stringent response is one of those responses. It is mediated by guanosine tetraphosphate. Interestingly, previous studies have demonstrated that stringent response is a universal virulence regulatory mechanism present in many bacterial pathogens including EPEC. However, biological signficance of a bacterial stringent response in both EPEC and its interaction with the host during a GIT infection is unclear. It needs to be elucidated to broaden our insight to EPEC pathogenesis. In this review, diverse responses, including stringent response, of EPEC during a GIT infection are discussed to provide a new insight into EPEC pathophysiology in the GIT.

Keywords: Enteropathogenic *Escherichia coli*; pathophysiology; gastrointestinal tract; Guanosine Tetraphosphate

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) has been a major cause of infant diarrhea in developing countries during the 20th century [1]. Remarkable advances in EPEC research in the 1980s and early 1990s have been achieved to understand how EPEC causes diarrhea, focusing on its mechanisms [2]. Although the number of EPEC outbeak cases was decreased by develping appropriate therapeutic interventions and improving sanitary conditions in the early 2000s [3], recent surveilance data on food-borne outbreaks imply a possible re-emergence of EPEC infections in humans [4,5]. Since EPEC is an enteric bacterial pathogen, it is improtant to understand its pathophysiology during a gastrointestinal track (GIT) infection as such understanding can provide a novel insight to intervention measures for controlling re-emerging diseases caused by EPEC. In this review, we especially focus on

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how EPEC interacts with diverse environmnets in the GIT during a host infection, including molecular pathogenesis [2,6].

RE-EMERGENCE OF EPEC

Bacterial pathogens have been serious concerns to global health under the concept of One Health [7]. Among them, *Escherichia coli* is an etiological agent that causes significant mortality of children and young animals in the world [1,8]. Zoonotic *E. coli* is often transmitted between humans and livestock animals. It is an important threat to public health and livestock industry. Transmission of *E. coli* occurs primarily via consumption of contaminated water and foods such as undercooked meat products and raw milk [9]. In recent years, many *E. coli* outbreaks are caused by consumption of agricultural produce such as romaine lettuce, which is likely to be contaminated by animal feces during cultivation or handling [10]. Direct contact with livestock, companion animals, and wild animals has also been suggested as a transmission route of *E. coli* infection [11].

E. coli is a Gram-negative, facultative, and rod-shaped bacterium that can colonize GITs of warm-blood animals [12]. Most *E. coli* strains are harmless. They are predominantly parts of normal GIT flora. However, some pathogenic *E. coli* have acquired specific virulence factors, which allow them to cause intestinal or extraintestinal diseases [13]. Based on their clinical manifestations, pathogenic *E. coli* can be devided into three pathovars: (i) diarrheagenic *E. coli*, (ii) uropathogenic *E. coli*, and (iii) sepsis and meningitis-associated *E. coli*. Diarrheagenic *E. coli* can be further classified into six pathotypes based on their different abilities to induce diarrheal diseases: (i) enteropathogenic *E. coli* (EPEC), (ii) enterohaemorrhagic *E. coli* (EHEC), (iii) enterotoxigenic *E. coli* (ETEC), (iv) enteroaggregative *E. coli* (EAEC), (v) enteroinvasive *E. coli* (EIEC), and (vi) diffusely adherent *E. coli* (DAEC) [14]. Among them, EPEC was the first recognized pathotype of diarrheagenic *E. coli* that could cause human diseases. This microorganism was first reported by John Bray in 1945 as a causative agent of infantile diarrhea in England [15].

Initially, EPEC caused frequent outbreaks of infantile diarrhea in United States and United Kingdom in 1940s and 1950s [16]. Although its occurence disappeared in developed countries after 1950s, EPEC became a major cause of infantile diarrhea in developing countries during the 20th century. EPEC infection was responsible for 5%–10% of infantile diarrhea in developing countries such as Brazil, Chile, Peru, and Iran [17]. However, recent studies suggest that EPEC seems to re-emerge in developed countries such as Northern Europe, Oceania, and East Asia. In Norway, for example, EPEC is one of the most common pathogens founded in stools of hospitalised patients with diarrhea. A total of 122 EPEC were isolated from diarrheal stools in a Norway university hospital during 2013–2015 [18]. In Finland, EPEC caused an outbreak of diarrhea in 237 human patients due to consumption of contaminated salads in 2016 [4]. In Australia, a total of 61 EPEC clinical isolates between 2008 and 2011 were analyzed [19]. In New Zealand, 21 EPEC were isolated from diarrheal stool samples from a public hospital during four months of 2014 [20]. High prevalence of EPEC from diarrheal patients has been also reported in East Asia. In South Korea, EPEC has caused a total of 26 diarrheal outbreaks during 2009-2010, affecting 1,791 human patients [21]. A high incidence of EPEC in diarrheal patients has been reported in Japan [22]. Collectively, these epidemiological reports strongly suggest that a great public health concern is necessary to control and prevent a zoonotic diarrheal EPEC infection locally, nationally, and globally.

EPEC can also induce diarrheal diseases in animals, causing huge economic losses of the livestock industry [23]. The prevalence of EPEC in calves and piglets with diarrheal diseases has been previously reported. In Belgium, a total of 104 EPEC strains were isolated from calves with diarrhea at 42 domestic farms during 2008–2015 [24]. Interstingly, 42 of these 104 EPEC isolates were serotyped as O80:H2 closely related to EHEC O80:H2 isolated from human patients during 2013–2016 [25]. In Spain, 156 EPEC strain were isolated from suckling or weaning piglets with diarrhea between 2006 and 2016 [26]. Among them, four colistin-resistant EPEC isolates harboring *mcr-1* gene were clustered into sequence type (ST) 10 clonal complex together with ETEC and EHEC porcine isolates carrying the *mcr-1* gene. These results suggest that EPEC isolates from animals have zoonotic potential. They are likely to spread antibiotic resistance genes in their clonal lineage. Moreover, EPEC is one of the most important enteropathogens in companion animals that can cause acute gastroenteritis, vomiting, diarrhea, and dehydration in infected dogs and cats [27,28]. Majority of EPEC isolates from diarrheal dogs and humans in Brazil were clustered together into ST 10 clonal lineage, implying their zoonotic risks [29].

MAJOR VIRULENCE FACTORS OF EPEC

EPEC produces a characteristic histopathological lesion in the GIT known as an attaching and effacing (A/E) lesion [30]. An A/E lesion occurs by serial events including (i) initial attachment of EPEC to small intestinal epithelial cells, (ii) formation of microcolony, (iii) effacement of brush border microvilli, (iv) intimate attachment to cell membrane, and (v) actin polymerization beneath the attached EPEC [6, 31]. EPEC has two major virulence factors responsible for the formation of an A/E lesion: type IV bundle forming pilus (BFP) and the locus of enterocyte effacement (LEE) (**Fig. 1**).

Type IV BFP

Type IV BFP is defined as a dynamic fibrillar organelle that can extend out and retract into bacterial surface [32]. BFP initiates a long-range, non-intimate attachment of EPEC to intestinal epithelial cells [33]. Subsequently, BFP recruits individual EPEC cells into aggregates, resulting in the formation of microcolony on host membrane. Such adherence pattern of EPEC is referred to as a localized adherence (LA) phenotype [34]. Fourteen genes for biogenesis of BFP are encoded in a ~80 kb plasmid (pEAF), which produces EPEC adherence factor (EAF) [35]. Therefore, a pEAF-cured strain could not form a typical LA phenotype [36]. Plasmid encoded regulator A (PerA) is known to activate the expression of BfpA, which is the major pilus subunit, so called pre-bundlin [37]. Processing of pre-bundlin to its mature form is mediated by prepilin peptidase, BfpP [38]. In addition, two nucleotide-binding proteins, BfpD and BfpF, mediate pilus extension and retraction, respectively. BfpD promotes the aggregation of EPEC, whereas BfpF separates EPEC cells from aggregates for the next step of the infectious process [39,40]. Dissociation of aggregates by BfpF permits the intimate attachment of individual EPEC cells on host membrane, allowing effective translocation of bacterial effector proteins via type III secretion system (T3SS) [41].

LEE

After dissociation of bacterial aggregates, EPEC expresses LEE for intimate attachment. LEE is a well-known pathogenicity island in genomes of bacteria including EPEC, EHEC, *Escherichia albertii*, and *Citrobacter rodentium* [42]. In EPEC, a 35,624-bp LEE pathogenicity island (LPI) contains 41 genes in five major polycistronic operons (LEE1 to LEE5) [43,44].

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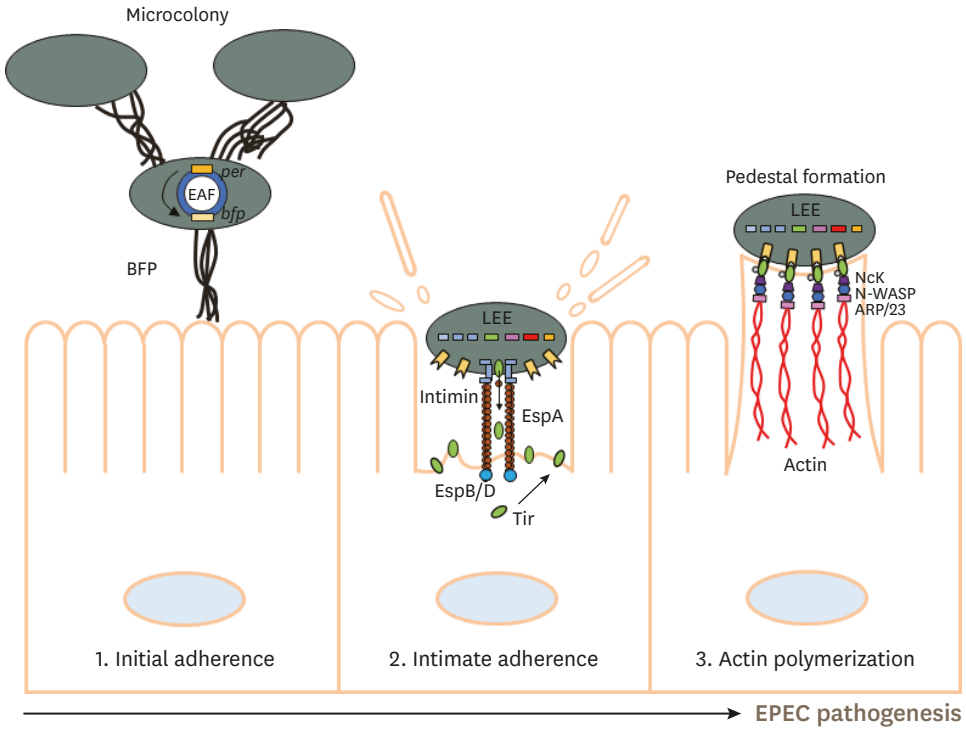

Note. Adapted from "Diarrheagenic *Escherichia coli*" by J. P. Nataro and J. B. Kaper, 1998, Clin Microbiol Rev, 11(1):142–201. Copyright 1998, American Society for Microbiology

Fig. 1. AE lesion formation by Type IV BFP and LEE of EPEC on the small intestine. (i) Type IV BFP and its activator Per mediate the initial adherence and microcolony formation of EPEC on epithelial cell layers. (ii) After dispersal of microcolonies, EPEC activates LEE operons to translocate T3SS effector proteins into epithelial cells via EspABD complex, causing effacement of microvilli. (iii) Adhesin intimin binds to Tir located at epithelial cell surface, allowing EPEC to attach intimately on cells. Phosphorylated Tir recruits host cellular proteins to induce actin polymerization beneath attached EPEC.
EAF, EPEC adherence factor; BFP, bundle forming pilus; LEE, locus of enterocyte effacement; Tir, translocated receptor; A/E, attaching and effacing; EPEC, Enteropathogenic *Escherichia coli*.

LEE1, LEE2, and LEE3 encode genes for synthesis and assembly of T3SS, a machinery that transfers bacterial effector proteins into host cells [45]. LEE-encoded T3SS structure is composed of three major components: (i) a needle complex in the outer membrane (EscC, EscD, EscF, EscI, and EscJ), (ii) an export apparatus in the inner membrane (EscRST, EscU, and EscV), and (iii) a cytoplasmic sorting platform (EscA, EscK, EscL, EscN, and EscQ) [46]. LEE4 encodes genes for extracellular proteins secreted by T3SS (EspA, EspB, and EspD) and forms a translocation apparatus [47]. Six LEE-encoded effectors (Tir, Map, EspF, EspG, EspZ, and EspH) are translocated into host cells via the EspABD translocon apparatus. These effectors can induce tight junction disruption, mitochondrial dysfunction, and membrane filopodia formation in host cells [48]. Furthermore, EspB itself is an effector of T3SS. It contributes to microvilli effacement [49]. LEE5 encodes genes for adhesin (Intimin) and its translocated receptor (Tir), which mediate intimate attachment of EPEC. Intimin is a 94 kDa outer membrane protein of EPEC and Tir is an effector protein translocated into the host cell membrane via T3SS [50]. Binding of intimin to Tir enables EPEC to attach intimately on cell membrane [51]. After intimate attachment, tyrosine residues in the cytoplasmic domain of Tir are phosphorylated by host cell kinases derived from Ab1/Arg, Src, and Tec families [52]. Phosphorylated Tir can bind two adaptor proteins, Nck1 and Nck2, to recruit actin nucleation-promoting factor, N-WASP [53]. N-WASP activates the Arp2/3 complex that assembles actin beneath EPEC [54]. These signaling events generate characteristic actin-rich pedestals on host cell membrane accompanied by inflammatory response and diarrhea [55].

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STRESS RESPONSES OF EPEC IN HOST ENVIRONMENTS

Acid resistance

EPEC encounters diverse environments during a GIT infection. Although EPEC posesses key virulence factors such as Type IV BFP and LEE, their enviroment-dependent and timely expression is very important for establishing bacterial infection in the GITs. It is well known that various environmental cues such as pH, immune response, nutrients limitation, and so on can induce bacterial stress responses, leading to the expression of virulence factors at the appropriate time and place [56]. For instance, the extremely low pH (1.5 to 3.0) in the stomach can trigger the acid resistance system in bacteria to survive for approximately 2 h [57]. Pathogenic *E. coli* including EPEC possess four acid resistance systems (AR1 to AR4) when exposed to enviroments with acidic pH. The AR1 is an oxidative acid resistance system repressed by glucose [58]. This system requires an alternative sigma factor, RpoS, which controls gene expression by interacting with RNA polymerase [59]. A previous study has demonstrated that the lack of RpoS strongly impairs the acid resistant phenotype of EPEC [60].

Molcular mechanism of AR1 has not been determined, although it is known that AR2 to AR4 require extracellular amino acids such as glutamate, arginine, and lysine. AR2 is known to be the most effective acid resistance system for protecting *E. coli* at an extremely low pH condition [61]. It contains three components: glutamate decarboxylases alpha, beta (GadA, GadB), and a glutamate/gamma-aminobutyric acid antiporter (GadC) [62]. Under acidic conditions, GadC can exchange extracellular glutamate and intracellular gamma-aminobutyric acid (GABA). Subsequently, GadAB can convert externally-derived glutamate to GABA, expelling intracellular protons to the extracellular space via GadC [63], resulting in maintaining the internal pH of *E. coli*. Similar to AR2, AR3 contains an arginine decarboxylase alpha (AdiA) and a arginine/agmatine antiporter (AdiC), while AR4 has a lysine decarboxylase alpha (CadA) and a cadaverine/lysine antiporter (CadB) [64,65]. All these four acid resistance systems help EPEC pass through a gastric stomach and reach the small intestine.

Inhibition of phagocytosis

In the small intestine, EPEC can adhere to not only absorptive cells, but also microfold cells (M cells) of peyer's patches [66,67]. Since peyer's patches are surrounded by host immune cells (B cells, T cells, dendritic cells, and macrophages), EPEC is known to induce acute immune responses as early as 12 h post infection [68]. M cells can facilitate the uptake of EPEC by endocytosis and deliver them to resident macrophages in subepithelial space [69]. Macrophages are crucial phagocytes and sentinel cells in body's first line of defense [70]. Phagocytosis proceed through serial steps including (i) bacterial binding to receptors on macrophages, (ii) activation of intracellular signal pathways, (iii) pseudopod extension by actin rearrangement and membrane expension, and (v) bacterial internalization into phagosome [71]. Interestingly, EPEC can inhibit phagocytosis to evade *E. coli* death in macrophages. Previous studies have demonstrated that EPEC can secrete T3SS effector proteins to impair phagocytosis. For example, EspB can interfere with pseudopod extension and phagosome closure of macrophages [49]. EspF can inhibit phosphoinositide 3-kinase (PI3K) dependent F-actin rearrangement [72]. EspJ can prevent opsonization with immunoglobulin G (IgG) and a complement component iC3b [73]. EspH can repress the activation of Rho guanine nucleotide exchange factors (RhoGEF), which regulates actin rearrangement [74]. These T3SS effectors allow EPEC to bypass host immune responses and enable successful colonization in the small intestine.

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Modulation of inflammatory signaling pathways

In addition to phagocytosis, macrophages can recognize pathogen associated molecular patterns (PAMPs) to activate inflammatory responses for recruiting immune cells to pathogens [75]. EPEC possesses many PAMPs such as lipopolysaccharides (LPS), flagellin, and outer membrane vesicles (OMVs) that can promote inflammatory signaling pathways in host cells [76]. For example, flagellin of EPEC can activate MAPK signaling pathways and induce IL-8 secretion [77]. On the other hand, EPEC can also secrete T3SS effector proteins to subvert PAMPs-induced inflammatory responses in host cells (**Fig. 2**) [78,79]. Previous studies have demonstrated that Tir and non-LEE encoded effectors such as NleB and NleE can inhibit NF-κB signaling pathways [80-82]. Other effectors (NleC, NleH) can directly target the NF-κB complex to prevent its nuclear translocation [83,84]. Since NF-κB is a prerequisite for the activation of NLRP3 inflammasome, all effector proteins targeting NF-κB can suppress NLRP3 activity [85]. EPEC can also secrete NleA and NleF, which directly target the NLRP3 complex to block caspases activation [86,87]. In contrast, some T3SS effector protiens such as EspB are known to trigger inflammatory signaling pathways for recruiting neutrophils to EPEC [88]. A recent study has shown that EPEC can activate NLRP3 inflammasome signaling pathways by T3SS effector proteins, distinct from those by LPS transfection or non-pathogenic *E. coli* infection [89]. Taken together, these findings suggest that there EPEC might have certain sophisticated mechanisms to modulate host inflammatory responses by T3SS effector proteins.

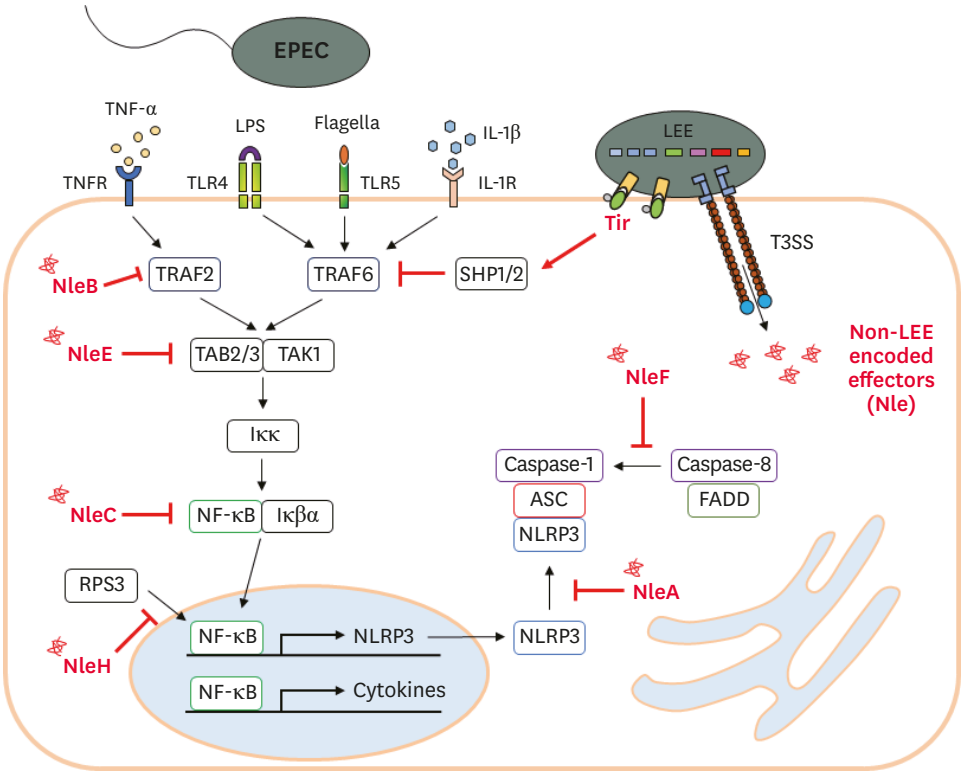

Fig. 2. Inhibitory effects of T3SS effector proteins of EPEC on inflammatory signaling pathways in host cells. T3SS effector proteins inhibit the activation of NF-κB and NLRP3 signaling pathways in host cells. Tir and NleB inhibit TRAF signaling. Tir recruits SHP1/2 to inhibit TRAF6 and NleB glycosylates GAPDH to suppress TRAF2. NleE inactivates TAB2/3, a downstream signal molecue of TRAF. NleC and NleH target the NF-κB complex. Protease NleC cleaves p65 subunit of NF-κB complex and NleH prevents nuclear translocation of RPS3 subunit of NF-κB complex. NleA and NleF target NLRP3 inflammasome complex. NleA blocks the assembly of NLRP3/ASC/Caspase-1 complex and NleF inhibits Caspase-8 activation.
EPEC, Enteropathogenic *Escherichia coli*; TNF-α, tumor necrosis factor alpha; LPS, lipopolysaccharides; IL, interleukin; LEE, locus of enterocyte effacement; Tir, translocated receptor; NF, nuclear factor.

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Nutrient acquisition from host

To limit bacterial pathogenicity during a host infection, host cells should sequester nutrients such as carbon and iron from EPEC [90,91]. Thus, EPEC must compete with host cells for nutrients, which are essential for bacterial growth and virulence. A previous study has demonstrated that EPEC can produce LEE-encoded T3SS injectisome components, so called "CORE," in order to extract nutrients from host cells [92]. CORE can mediate the formation of another protuding membranous nanotube, allowing EPEC to directly extract nutrients from host cell cytoplasm. It has been shown that all EPEC clinical isolates can execute a CORE-dependent nutrient acquisition, whereas a non-pathogenic *E. coli* K12 strain fails to do so. In addition, it has been reported that EPEC can inhibit the uptake of vitamin B1 (thiamin) in host intestinal epithelial cells by T3SS effector proteins EspF and EspH [93].

STRINGENT RESPONSE OF EPEC

Stringent response and guanosine tetraphosphate

In addition to struggling for nutrients, EPEC also needs to re-allocate its cellular resources and save energy by repressing biosynthesis of DNA, stable RNA (ribosomal RNA and transfer RNA), and ribosomal proteins [94]. This so-called "hunger response" occuring in bacteria has been referred to as a stringent response. It is known that a stringent response is mediated by a guanosine tetraphosphate (ppGpp), a nucleotide-based signaling molecule [95]. When *E. coli* faces depletion of nutrients, both RelA (a monofunctional ppGpp synthetase) and SpoT (a bifunctional ppGpp synthetase and hydrolase) rapidly increase intracellular concentration of ppGpp [96]. ppGpp controls the expression of numerous stringent response genes in cells by binding to RNA polymerase (RNAP). Three mechanisms have been proposed to explain how ppGpp binding alters the transcriptional activity of RNAP: (i) ppGpp can bind to the secondary channel of β'-subunit and cause an allosteric signal transduction to the RNAP active site that contains catalytic Mg^{2+}, hence regulating the catalytic activity of RNAP [97]; (ii) ppGpp can bind to mobile modules (shelf and core domains) and form a shelf-core ratcheting, resulting in conformational changes of RNAP, through which ppGpp can regulate the stability of RNAP-promoter complexes [98]; and (iii) ppGpp can reduce the affinity of housekeeping σ-factor (σ^{70}) to core RNAP, thus promoting the binding of alternative σ-factors (σ^{*}) to core RNAP. This σ-factor competition can direct RNAP to transcribe a set of stringent response genes [99]. Thus, *E. coli* can alter various physiological and cellular processes such as growth and morphology during a stringent response [100,101].

Environmental cues for stringent response in EPEC

In 1969, ppGpp was first discovered by Michael Cashel and collegues who identified two unusual spots from amino acid-starved *E. coli* cells using thin layer chromatography [95]. These 'magic spots' were generated by addition of a pyrophosphate (PPi) to the 3' carbon of guanosine diphosphate (GDP) and guanosine triphosphate (GTP), collectively referred to as ppGpp [102]. Various environmental cues are known to induce ppGpp synthesis in EPEC (**Fig. 3**). Deficiency of amino acid can result in the accumulation of uncharged transfer RNA (tRNA), which binds to ribosomal A site. A ribosome-associated enzyme, RelA, can sense the presence of uncharged tRNA in A site and synthesize ppGpp to initiate a stringent response [103]. Another enzyme, SpoT, can mediate the stringent response when various nutrients including phosphate, carbon, iron, and unacylated fatty acids are limited [104]. For instance, depletion of fatty acids can lead to accumulation of unacylated acyl carrier proteins (ACP) that bind to threonly-tRNA synthetase, GTPase, and SpoT (TGS) domain of SpoT

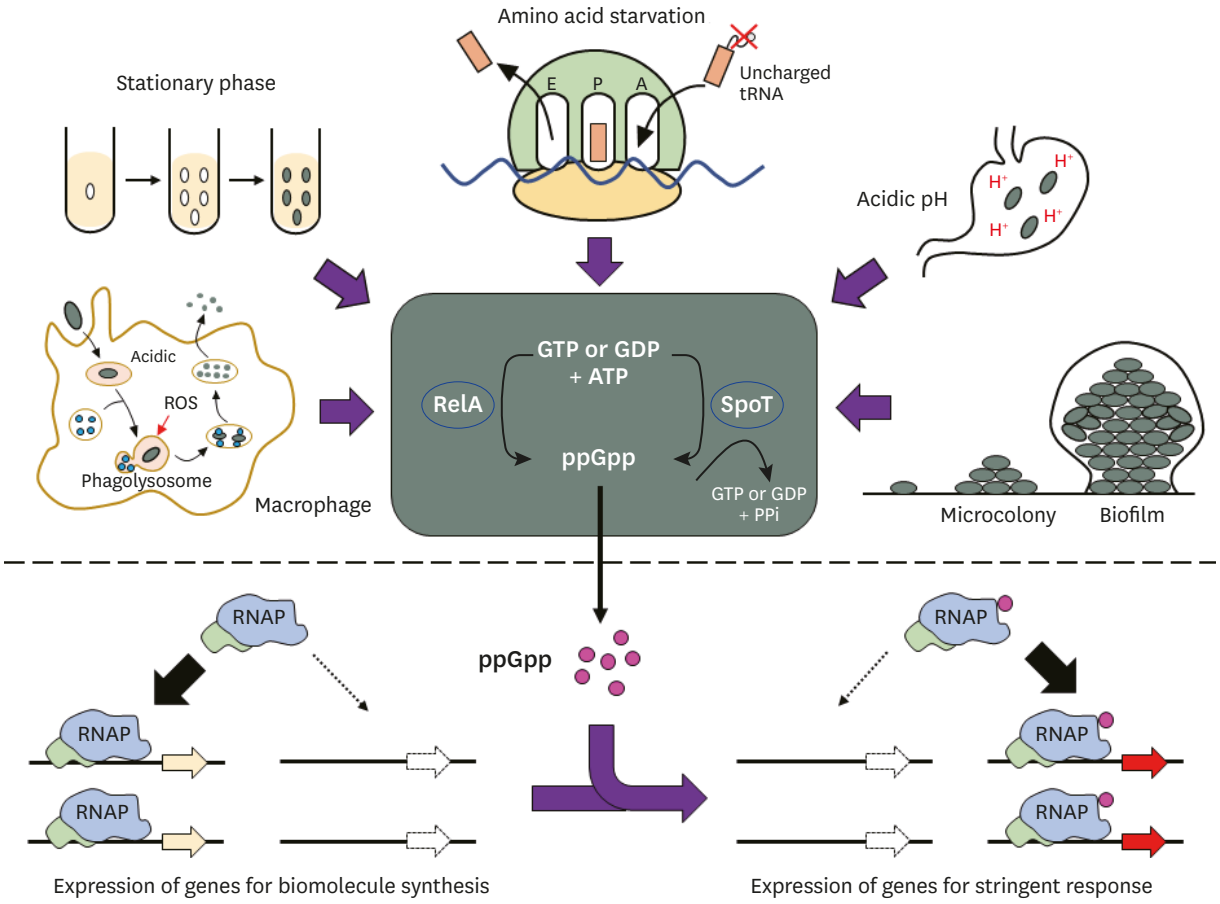

Fig. 3. Various environmental cues that induce stringent response in EPEC. Bacterial stringent response is induced by various environmental cues such as amino acid starvation, acidic pH, localized famine in cell aggregates, stationary growth phase, and host immune responses. In response to signals, RelA and SpoT enzymes synthesize ppGpp by adding PPi to the 3'carbon of GTP/GDP. To balance intracellular ppGpp levels, SpoT hydrolyzes ppGpp to GTP/GDP and PPi. As a stringent response mediator, ppGpp alters the affinity of RNAP toward promoters. Transcription levels of biomolecule synthetic genes for replication are decreased, whereas those of genes for stringent response are increased.
GTP, guanosine triphosphate; GDP, guanosine diphosphate; ppGpp, guanosine tetraphosphate; PPi, pyrophosphate; RNAP, RNA polymerase.

[105]. Such binding influences the bifunctional activity of SpoT, with its synthetic acitity being increased while its hydrolytic activity being decreased. Other environmental cues such as high osmolarity, oxidative burst, and extreme pH can also trigger a stringent response [106-108]. To survive in the stomach, enteropathogens can utilize ppGpp in response to acidic stress. For example, ppGpp can bind to lysine decarboxylase and regulate its activity in *E. coli*, confering an acid resistant phenotype [108]. In *Helicobacter pylori*, ppGpp is rapidly synthesized in response to pH downshift [109]. Besides, host immune responses to bacteria can trigger a stringent response. For example, harsh environments in macrophage such as iron sequestration, oxidative burst, and acidic pH in phagosome can trigger bacterial stringent response [110-112]. To survive in macrophages, intracellular bacterial pathogens can synthesize ppGpp to induce their specialized virulence determinants [94]. Furthermore, heat shock, high density population in stationary phase, and biofilm formation can induce a stringent response [113-115]. Taken together, various environmental cues can trigger bacterial stringent response, implying that this adaptive response is closely related to EPEC pathogenesis during a host infection.

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Stringent response and virulence factors of EPEC

Bacterial stringent response is important for the regulation of its virulence, invasion, and persistence [116]. In case of EPEC, a previous study has constructed a single Δ*relA* mutant of LRT9 strain (EPEC O111:abH2) to examine its expression level of adhesins and adherent efficiency compared to those of a wildtype strain [117]. Expression levels of two adhesins (BFP and Intimin) and their regulator Per were decreased in this single Δ*relA* mutant, which adherent rate was 75% lower than that of the wildtype strain [117]. This result suggested that ppGpp levels reduced by RelA inactivation diminished the adherence of EPEC. Interestingly, ppGpp is known to increase the expression of LEE-encoded genes in EHEC [118]. Compared to EPEC, EHEC has a 43,359-bp LPI containing additional 13 ORFs of a cryptic prophage at the 5' end. However, nucleotide sequences of major regions (LEE1 to LEE5) of EHEC share 93.9% similariteis with those of EPEC [119]. ppGpp can activate both LEE-encoded regulator (Ler) and Pch to increase the expression of LEE genes [118,120]. Pch is a non-LEE encoded transcription regulator of the LEE operon in EHEC [118,121]. Since EPEC has Per, a Pch homologous protein encoded in pEAF, further studies about EPEC are needed to unveil the relationship between ppGpp and LEE expression. In addition, it has been reported that ppGpp can regulate the biosynthesis of lipid A, a highly conserved structure of Gram-negative bacteria [122,123]. Lipid A is an innermost component of LPS (also called endotoxin) causing host cell damages [124]. In *E. coli*, ppGpp controls the degradation of enzyme LpxC that catalyzes the deacetylation at C-2 position (UDP-3-O-acyl-GlcNAc) of lipid A [123]. According to cellular needs for lipid A biosynthesis, LpxC is degraded in slow-growing cells, but stabilized during fast growth. However, it should be noted that the lack of ppGpp deregulates the LpxC degradation. Further studies are needed to elucidate the role of ppGpp-mediated LpxC degradation in EPEC pathogenesis.

Stringent response and host immunity

Based on the fact that EPEC can modulate host immune responses, its full virulence depends on host immunity. However, it is unclear if a stringent response can modulate host immune responses to EPEC. A few studies have addressed the role of stringent response in modulating host immune responses to other bacteria. For example, intracellular bacterial pathogens require ppGpp to replicate in phagocytes. In *Salmonella* Typhimurium, ppGpp can activate SlyA to transcribe antimicrobial peptides resistance genes [125]. In *Francisella tularensis*, ppGpp can activate PigR, which interacts with MglA-SspA complex to express genes for phagosome escape [126]. In *Legionella pneumophila*, ppGpp can activate two non-coding regulatory RNAs (RsmY and RsmZ) to sequester carbon storage regulator (CsrA) from target mRNA [127]. CsrA is a transcriptional repressor that can bind to mRNAs of genes for bacterial transmission in phagocytes. Therefore, inhibiting CsrA by ppGpp can promote the transmission of *L. pneumophila* for evading phagosome. Another study has examined host inflammatory responses to ppGpp-defective bacterial pathogens. Guinea pigs infected with ppGpp-defective *Mycobacterium tuberculosis* show lower transcription levels of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) in lungs than those infected with its wildtype strain [128]. In *Salmonella* spp, a ppGpp-defective mutant strain has been used to develop a live attenuated vaccine. Interestingly, immunization with a ppGpp-defective *S.* Typhimurium can elicit significant IgG and IgA antibodies in BALB/c mice and confer protective immunity against the wildtype strain [129]. Likewise, immunization with a ppGpp-defective *S.* Gallinarum can elicit both IgG and IgA in chicken, allowing protective immune responses to the wildtype strain [130]. These results demonstrate significant increases in the proliferation of T cells as well as the expression of both IFN-γ and TGF-β4 in chicken. Taken together, these results imply that EPEC can also induce stringent response to modulate host immune responses like other bacterial pathogens.

Anti-infective strategy targeting stringent response

Stringent response-mediated regulation of virulence factors and host immunity can affect the degree of bacterial virulence *in vivo*. Previous studies have analyzed the virulence of ppGpp-defective mutants of bacterial pathogens using animal infection models. In general, their virulence are attenuated, implying that stringent response contributes to bacterial full virulence [128,131-133]. Therefore, inhibiting stringent response has been suggested to be a novel antibacterial strategy to weaken bacterial pathogens. Anti-infective molecules targeting stringent response could substitute for conventional antbiotics in order to fight antimicrobial resistant bacteria. For example, Relacin is a synthetic ppGpp analogue that can inhibit RelA and SpoT homologue family of Bacillus species [134]. This molecule can block ppGpp synthesis and disrupt essential phenotypes such as biofilm formation and sporulation of *B. subtilis*. Relacin and its derivatives can also inhibit RelA enyzmes of Gram-negative bacteria, showing a broad-spectrum activity [135]. Other synthetic ppGpp analogues can prevent ppGpp accumulation by specific binding to Rel enzyme in *Mycobacterium smegmatis* [136]. Those molecules can inhibit Rel-mediated ppGpp synthesis in a dose-dependent manner and prevent biofilm formation and long-term persistence of *M. smegmatis*. In addition to nucleotide inhibitors, a synthetic peptide 1018 can degrade intracellular ppGpp and interfere with biofilm formation of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Syaphylococcus aureus*, *Salmonella* Typhimurium, and *Burkholderia cenocepacia* [137]. In a murine cutaneous abscess model, treatment of *P. aeruginosa* with synthetic peptides (1018 and DJK-5) can suppress *spoT* promoter activity during abscess formation and result in virulence attenuation, leading to decreased abscess size and reduced bacterial colony forming unit recovered from the absecess [138]. These phenotypes are similar to those of a ppGpp-defective *P. aeruginosa* mutant infection. Collectively, all proposed molecules targeting stringent response could be applied as novel anti-infective agents against EPEC.

CONCLUSION

EPEC is a major zoonotic pathogen causing diarrhea in both developing and developed countries. This microorganism can produce Type IV BFP and LEE necessary for the formation of a characteristic intestinal histopathology called AE lesion. Although molecular machanisms of individual virulence factors of EPEC are clearly defined, the pathogenesis of EPEC remains unclear because it can trigger diverse stress responses to environmental cues during a host infection. Numerous viruelnce genes of EPEC are under the control of stress responses to environmental cues including acidic pH, phagocytosis, inflammatory response, and nutrient limitation. Sophisticated regulation of virulence factors by stress responses allows EPEC to establish a successful infection. One of these stress responses under nutrient limitation is stringent response, which is mediated by ppGpp. Stringent response can also be induced by other stressful conditions in host gut environments. Its biological significance on pathogenesis has been confirmed in other bacteria. However, very few studies have documented the role of stringent response in both EPEC and its host immune response. Elucidation of the role of stringent response in EPEC will provide a deeper understanding on EPEC pathogenesis.

Since ppGpp is a master regulator of stringent response, it has become a novel target for attenuating the virulence of bacteria. High-throughput screening assay for the identification of new compounds that can inhibit ppGpp synthesis is strongly recommended for molecular targeted therapy. In addition, further studies aiming to understand immunomodulatory effects of a ppGpp-defective EPEC could offer great opportunitis to develop a new vaccine against EPEC infection.

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