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Escherichia coli O157:H7 senses microbiota-produced riboflavin to increase its virulence in the gut

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Riboflavin is produced by most commensal bacteria in the human colon, where enterohemorrhagic *Escherichia coli* (EHEC) colonizes and causes diseases. Sensing environmental signals to site-specifically express the type-III secretion system (T3SS), which injects effectors into host cells leading to intestinal colonization and disease, is key to the pathogenesis of EHEC. Here, we reveal that EHEC O157:H7, a dominant EHEC serotype frequently associated with severe diseases, acquired a previously uncharacterized two-component regulatory system *rbfSR*, which senses microbiota-produced riboflavin to directly activate the expression of LEE genes encoding the T3SS in the colon. *rbfSR* is present in O157:H7 and O145:H28 but absent from other EHEC serotypes. The binding site of RbfR through which it regulates LEE gene expression was identified and is conserved in all EHEC serotypes and *Citrobacter rodentium*, a surrogate for EHEC in mice. Introducing *rbfSR* into *C. rodentium* enabled bacteria to sense microbiota-produced riboflavin in the mouse colon to increase the expression of LEE genes, causing increased disease severity in mice. Phylogenic analysis showed that the O55:H7 ancestor of O157:H7 obtained *rbfSR* which has been kept in O157:H7 since then. Thus, acquiring *rbfSR* represents an essential step in the evolution of the highly pathogenic O157:H7. The expression of LEE genes and cell attachment ability of other EHEC serotypes in the presence of riboflavin significantly increased when *rbfSR* was introduced into them, indicating that those serotypes are ready to use RbfSR to increase their pathogenicity. This may present a potential public health issue as horizontal gene transfer is frequent in enteric bacteria.

enterohaemorrhagic *Escherichia coli* | virulence regulation | evolution | two-component regulatory system | gut microbiota

Significance

EHEC colonizes the human colon, and site-specific expression of T3SS which injects effectors into colonocytes inducing diseases is essential for its pathogenicity. We found that the highly pathogenic EHEC O157:H7 acquired a TCS (RbfSR) enabling it to sense microbiota-produced riboflavin to increase T3SS expression and virulence in the colon. Introducing *rbfSR* into *C. rodentium*, a surrogate for EHEC in mice, increased bacterial pathogenicity responding to commensal bacteria-produced riboflavin in mice. Among nine prevalent EHEC serotypes, *rbfSR* is present in O157:H7 and O145:H28. Introducing *rbfSR* into other EHEC serotypes increased their virulence. Thus, obtaining *rbfSR* represents an important step in the evolution of O157:H7, and there is a risk that *rbfSR* may increase the pathogenicity of other EHEC serotypes in future evolution.

Enterohemorrhagic *Escherichia coli* (EHEC) is an important human pathogen that specifically colonizes the large intestine, resulting in various diseases including bloody diarrhea, hemorrhagic colitis, and life-threatening systemic hemolytic uraemic syndrome (1). It has a very low-infection dose, which is estimated to be 50 colony-forming units (CFUs) (1). EHEC O157:H7, one of the most dominant and important EHEC serotypes in several countries, is frequently associated with severe diseases and outbreaks (2, 3). EHEC senses multiple environment signals as spatial landmarks in the large intestine to precisely induce the expression of the Type-III secretion system (T3SS), one of the major virulence determinants of EHEC (1). This is a key prerequisite for the establishment of infection and essential for the survival of EHEC, as expression of virulence factors, including T3SS, constitutes a considerable metabolic burden for pathogens, and their dysregulated expression always leads to decreased bacterial growth and fitness (4) (5).

T3SS encoded by locus of enterocyte effacement (LEE) pathogenicity island is a molecular syringe that EHEC uses to inject multiple effectors into the host epithelial cells, leading to the formation of attaching and effacing (A/E) lesions on intestinal epithelial cells, which is a hallmark of EHEC pathogenesis (6, 7). The A/E lesion is characterized by the destruction of epithelial microvilli and the rearrangement of the cytoskeleton of epithelial cells to form a pedestal-like structure that cups the bacterium in the infection site, thereby allowing intimate attachment of EHEC to host intestinal epithelial cells (1). Transcription of all LEE genes is under the control of a master regulator, Ler, encoded within the LEE island (4, 8).

Riboflavin produced by microbiota in the large intestine directly promotes the maturation of the immune system in infants, and depletion of riboflavin-producing commensal bacteria in early life permanently impairs the development of the immune system and ultimately affects tissue homeostasis (9, 10). Additionally, riboflavin is a cofactor for enzymatic reactions and involved in several essential cellular processes (11). As mammalian hosts cannot produce riboflavin, it has been suggested that the supply of riboflavin from

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microbiota in the large intestine is essential for maintaining host riboflavin homeostasis (12). As a result of coevolution of humans and microbiota, approximately 60% of commensal bacterial species in the large intestine of humans, such as *Lactobacillus* and *Bifidobacterium* species, can produce riboflavin (13, 14).

As one of the basic stimulus-response-coupling mechanisms, the two-component regulatory systems (TCSs) enable bacteria to adapt their cellular process and virulence to a wide variety of environmental signals (5, 15). TCSs consist of a histidine sensor kinase (HK) that senses a signal input and a response regulator (RR) that mediates the output (16, 17). In response to certain environmental signals, HKs undergo autophosphorylation and then transfer their phosphate to RRs. Subsequently, RRs activated by phosphorylation regulate the expression of different sets of genes, thereby coordinating the response of bacteria to different environmental cues.

In this study, we found that EHEC O157:H7 obtained a previously uncharacterized TCS, RbfSR, for sensing microbiota-produced riboflavin in the large intestine of the host to increase LEE gene expression, leading to significantly increased bacterial pathogenicity. We revealed the molecular mechanism underlying this riboflavin-mediated regulation: RbfSR is activated by riboflavin, leading to specific binding of phosphorylated RbfR to the *ler* promoter, which results in increased LEE gene expression. Introduction of *rbfSR* into *Citrobacter rodentium*, which is a murine pathogen extensively employed as a surrogate animal model for EHEC, enabled bacteria to increase LEE gene expression in response to riboflavin with a concentration range close to that in the host large intestine. This led to increased bacterial virulence in the large intestine of specific pathogen-free (SPF) mice but not in microbiota-depleted mice. Comparative genomics analysis showed that *rbfSR* is only present in EHEC O157:H7 and O145:H28 but absent from other EHEC serotypes. We also showed that the O157:H7 lineage obtained this TCS before it diverged from *E. coli* O55:H7 and maintained it since then, indicating that acquisition of *rbfSR* contributed to the evolution of the highly pathogenic EHEC O157:H7. Introduction of *rbfSR* into EHEC serotypes without native *rbfSR* increased bacterial attachment ability to HeLa cells, indicating that the lateral transfer of *rbfSR* has the potential to increase the virulence of other EHEC serotypes.

Results

Riboflavin Promotes LEE Gene Expression. B vitamins are persistently present in the large intestine of hosts and indispensable for human health (18). Among the eight B vitamins, vitamin B12 and biotin have been reported to be used by EHEC O157:H7 as the signal to regulate the expression of its LEE genes in the host large intestine (19, 20). We speculated that some other members of the B vitamins may also serve as spatial landmark signals for the regulation of EHEC O157:H7 virulence. Thus, we systematically investigated whether six other B vitamins (folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin, and thiamine) can regulate the expression of LEE genes of EHEC O157:H7. The expressions of six representative LEE genes, including *ler*, *escC*, *escN*, *eae*, *tir*, and *espB*, were tested in EHEC O157:H7 wild-type strain EDL933 (WT) grown in SCEM medium (which mimics the colonic environment) supplemented with each of these six B vitamins (10 μM), respectively. The growth of EDL933 was unaffected by these B vitamins (*SI Appendix*, Fig. S1*A*). qRT-PCR assays showed that LEE genes were upregulated in the presence of thiamine, riboflavin, and nicotinic acid (*SI Appendix*, Fig. S1 *B–D*), while riboflavin exhibited the most significant regulatory effect (2.3–3.3 fold increase) (*SI Appendix*, Fig. S1*C*).

We then analyzed the concentration of riboflavin in the intestine of C3H mice by HPLC assays. The results showed that the concentration of riboflavin in the colonic content and ileal content of mice was approximately 22.8 μM and 0.2 μM (Fig. 1*A*), respectively. In addition, HPLC assays showed that there is no riboflavin present in the colonic content of antibiotics-treated mice (abx-treated mice) devoid of microbiota (Fig. 1*A*). These data are consistent with that riboflavin in the large intestine is produced by local microbiota(12).

Next, we investigated whether the riboflavin concentration in the colon and ileum can induce the expression of LEE genes in EDL933. qRT-PCR assays showed that the expression of LEE genes was significantly upregulated (3.6–4.7 fold) in the presence of 20 μM riboflavin but exhibited no changes in the presence of 0.2 μM riboflavin (Fig. 1*B*). It indicates that the riboflavin concentration in the colon, but not in the ileum, is sufficient to induce LEE gene expression of EHEC O157:H7.

Riboflavin Increases LEE Gene Expression Through a Putative TCS. To determine the factors through which EHEC O157:H7 senses riboflavin to induce LEE gene expression, transcriptome analysis was performed on EDL933 grown in SECM supplemented with 20 μM riboflavin, while EDL933 grown in SECM without riboflavin was used as a control. The transcriptome results showed that 148 genes and 87 genes were upregulated and downregulated, respectively, in the presence of riboflavin (Dataset S1). In addition to LEE genes, the expression of *z5692* and *z5684* within a pathogenicity island (OI-167) exhibited a significant increase

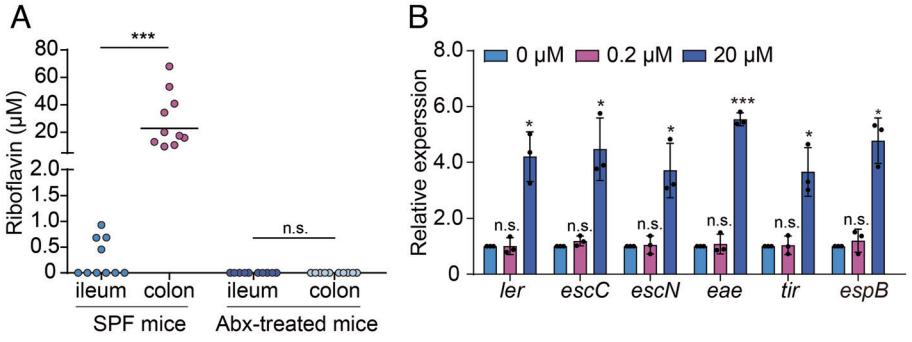

Fig. 1. Riboflavin increases LEE gene expression in vitro. (*A*) HPLC measurement of riboflavin concentration in the ileum and colon contents of SPF or abx-treated mice (n = 10 mice per group). (*B*) qRT-PCR analysis of LEE gene expression in EDL933 grown in SCEM medium containing 0.2 or 20 μM riboflavin. Data are represented the mean ± SD (n = 3). Significant differences were assessed by an unpaired *t* test. Error bars represent SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; n.s. no significant difference.

(3.0fold and 9.1fold, respectively) in response to riboflavin. The upregulation of *z5692* and *z5684* in riboflavin-treated EHEC O157:H7 was confirmed by qRT-PCR assays (Fig. 2*A*).

Z5692 shares 43% similarity to TorS at the amino acid level, which is a HK in *E. coli* (21) and contains a transmembrane sensor domain, an autophosphorylation domain (PF00512) and an ATP binding domain (PF02518) (*SI Appendix*, Fig. S2*A*). Z5684 shares 56% similarity to TorR at the amino acid level, which is a RR in *E. coli* (21), and contains a receiver domain (PF00072) and a DNA-binding domain (PF00486) (*SI Appendix*, Fig. S2*A*). These data indicate that *z5692* and *z5684* encode a putative TCS.

To investigate whether Z5692/Z5684 influences the LEE gene expression in response to riboflavin, mutants of *z5692* (Δ*z5692*) and *z5684* (Δ*z5684*) and the double mutant Δz5692Δz5684 were constructed. qRT-PCR assays showed that the LEE gene expression was not induced in Δ*z5692*, Δ*z5684*, and Δ*z5692*Δ*z5684* after treatment by riboflavin (Fig. 2*B*). The expression of LEE genes in Δ*z5692* and Δ*z5684* in response to riboflavin was restored to WT level when complemented with *z5692* or *z5684* (Fig. 2*B*). The growth of EDL933 in the presence of riboflavin was unaffected by the deletion of *z5692* or *z5684* or both (*SI Appendix*, Fig. S2*B*), indicating that the reduced LEE gene expression in these mutants was not due to different growth rates. Collectively, these data indicate that riboflavin activates a putative TCS (Z5692/Z5684) in EHEC O157:H7, which leads to the increase in LEE gene expression.

Functional Verification of RbfSR as a TCS. To biochemically verify whether Z5692/Z5684 forms a TCS activated by riboflavin, we first showed that purified Z5692 exhibited the autophosphorylation activity in the presence of ATP by in vitro phosphorylation assays (Fig. 2*C*). In addition, incubation with 20 μM riboflavin significantly enhanced the autophosphorylation level of Z5692 (Fig.2*C*). Next, we showed that incubation of purified Z5684 with the autophosphorylated Z5692 resulted in phosphorylation of Z5684 and a decreased autophosphorylation level of Z5692, indicating that Z5692 can transfer its phosphate to Z5684 in vitro (Fig.2*D*). These results indicate that Z5684/Z5692 constitute a functional TCS, which can be activated by riboflavin. Thus, we renamed Z5692 as RbfS for riboflavin-sensing histidine kinase and Z5684 as RbfR for the riboflavin-sensing RR, respectively.

Next, we investigated the range of riboflavin concentrations that can induce the expression of LEE genes through RbfSR. qRT-PCR assays on EDL933 grown in SCEM supplemented with different concentrations of riboflavin (0–200 μM) showed that the expression of *rbfSR* and LEE genes became induced when the riboflavin concentration exceeded 10 μM, reaching the highest level and plateauing in SCEM with 20–35 μM riboflavin, then decreasing when the riboflavin concentration exceeded 50 μM (*SI Appendix*, Fig. S2*C*). In vitro phosphorylation assays showed that RbfS was autophosphorylated to a similar level when incubated with 10–200 μM riboflavin (*SI Appendix*, Fig. S2*D*). Thus, the induction of *rbfSR* expression by riboflavin, which in turn induces the expression of LEE genes, was enhanced along with the increased concentration of riboflavin in the range 10–20 μM.

The effects of riboflavin on the expression of *rbfSR* and LEE genes began to diminish when its concentration reached 50 μM and completely disappeared when its concentration exceeded 100 μM (*SI Appendix*, Fig. S2*C*). This is due to the known antimicrobial

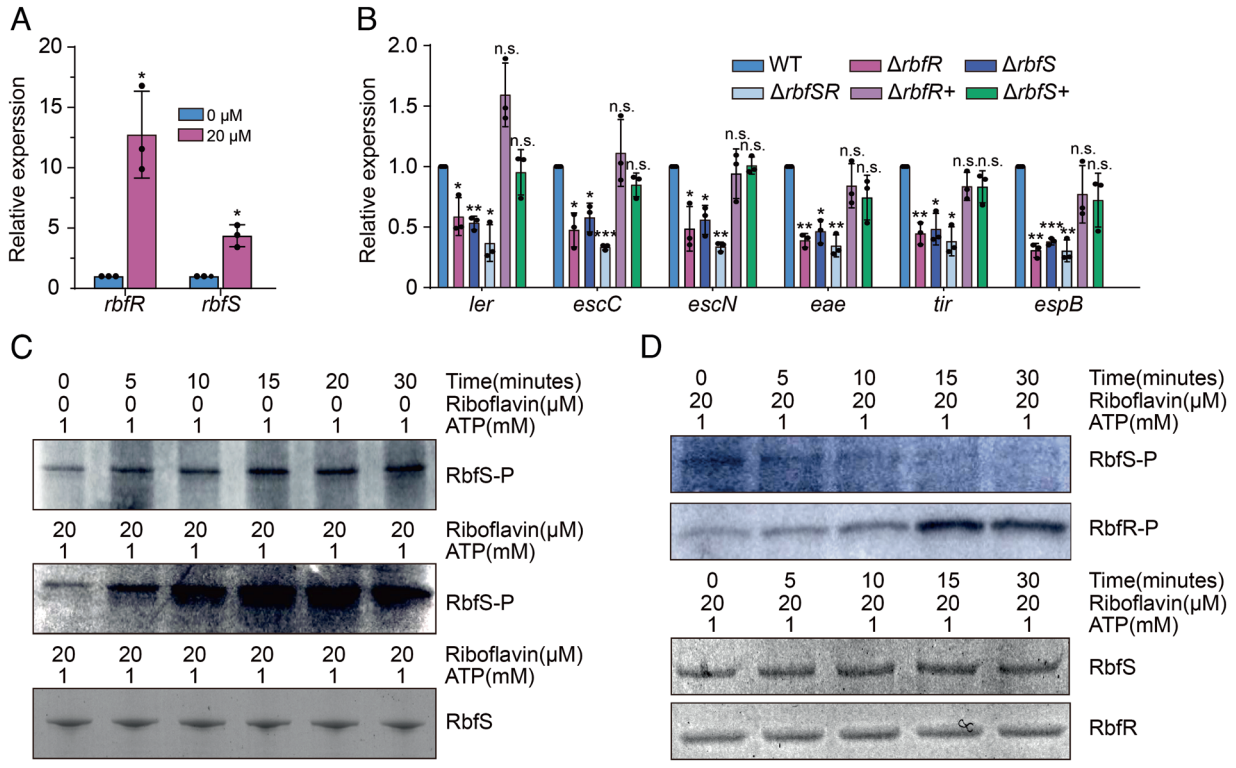

Fig. 2. Riboflavin increases LEE gene expression through RbfSR(Z5692/Z5684). (*A*) qRT-PCR analysis of *rbfR*(*z5684*) and *rbfS*(*z5692*) expression in WT grown in SCEM medium containing 0 or 20 μM riboflavin. Data are presented as the mean ± SD (n = 3). (*B*) qRT-PCR analysis of LEE gene expression in WT, Δ*rbfR*(Δ*z5684*) Δ*rbfS*(Δ*z5692*)Δ*rbfSR* (Δ*z5684*Δ*z5692*)Δ*rbfR*+(Δ*z5684*+), and Δ*rbfS*+(Δ*z5692*+) grown in SCEM medium containing 20 μM riboflavin. Data are presented as the mean ± SD (n = 3). Significant differences were assessed by an unpaired t test. Error bars represent SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; n.s. no significant difference. (*C*) Autophosphorylation of RbfS(Z5692). Coomassie gel of RbfS(Z5692) served as loading control. (*D*) Phosphotransfer from RbfS(Z5692) to RbfR(Z5684) (HK: RR=1:10). Coomassie gel of RbfS(Z5692) and RbfR(Z5684) served as loading control.

activity of riboflavin (22). Growth curve analysis showed that when the riboflavin concentration exceeded 50 μM, EDL933 began to exhibit a decreased growth rate in the exponential phase (*SI Appendix*, Fig. S2*E*). When the riboflavin concentration exceeded 100 μM, the growth of EDL933 was significantly inhibited (*SI Appendix*, Fig. S2*E*). This indicates that EDL933 exhibited a growth defect when the concentration of riboflavin exceeded 50 μM, which may impair the expression of *rbfSR* and LEE genes.

RbfR Specifically Binds to the Promoter of *ler*. To investigate the mechanism by which RbfR regulates LEE gene expression, the potential binding sites of RbfR on the genome of EHEC O157:H7 were screened via ChIP-Seq analysis. Then, 167 potential binding sites of RbfR were detected, including a site in the promoter region of *ler* (Dataset S2). Next, we analyzed the enrichment of the *ler* promoter in RbfR-ChIP samples compared with mock-ChIP control DNA using ChIP-qPCR assays. The results showed that the *ler* promoter was significantly enriched in DNA binding to RbfR compared to control DNA (8.9fold) (Fig. 3*A*). Electrophoretic mobility shift assays (EMSA) were performed to confirm that the phosphorylated RbfR specifically binds to the promoter region of *ler* in a concentration-dependent manner in vitro, whereas the binding capacity of nonphosphorylated RbfR was significantly reduced (Fig. 3*B* and *SI Appendix*, Fig. S6). Meanwhile, phosphorylated RbfR did not bind to the negative control (*rpoS*) under the same experimental condition (Fig. 3*C*). Using a dye-based DNase I foot-printing assay, we revealed a specific RbfR-bound sequence containing a 21-base pair motif (5- TCTCACATAATTTATATCATT-3) in the promoter region of *ler* (Fig. 3*D*), which is located -94bp to -73bp from the proximal transcriptional start site. Deletion of this identified that binding site completely abolished the binding of phosphorylated RbfR to the *ler* promoter (Fig. 3*E*). These data indicate that RbfR directly regulates the expression of *ler* by binding to its promoter region.

We next confirmed that RbfR regulates LEE gene expression via Ler in the presence of riboflavin. As expected, expression of the LEE genes was significantly reduced in Δ*ler* as revealed by qRT-PCR assays (Fig. 3*F*), while there was no further decrease in the LEE gene expression level in Δ*ler*Δ*rbfR* compared to that in Δ*ler* in the presence of riboflavin (Fig. 3*E*).

RbfSR Promotes EHEC O157:H7 Attachment to Epithelial Cells In Vitro. To investigate the effects of RbfSR on bacterial adherence in vitro, HeLa cells were infected with the WT, Δ*rbfS*, Δ*rbfR*, Δ*rbfS*Δ*rbfR* and complementary strains (Δ*rbfS*+ and Δ*rbfR*+), respectively, in the presence of riboflavin. The results showed that Δ*rbfS*, Δ*rbfR*, and Δ*rbfS*Δ*rbfR* exhibited 3.1–5.3-fold decrease in cell adherence ability compared to WT (Fig. 4*A*). The defects of these mutants in cell adherence ability were restored to the WT level by complementation with the corresponding genes (Fig. 4*A*). Furthermore, immunofluorescence microscopy analysis revealed that the numbers of HeLa-attached bacteria and formed actin-pedestals per infected cell for Δ*rbfS*, Δ*rbfR*, and Δ*rbfS*Δ*rbfR* were much lower than those for WT and complemented strains (Fig. 4 *B* and *C*). These data indicate that RbfSR promotes the adherence ability of EHEC O157:H7 when riboflavin is present in vitro.

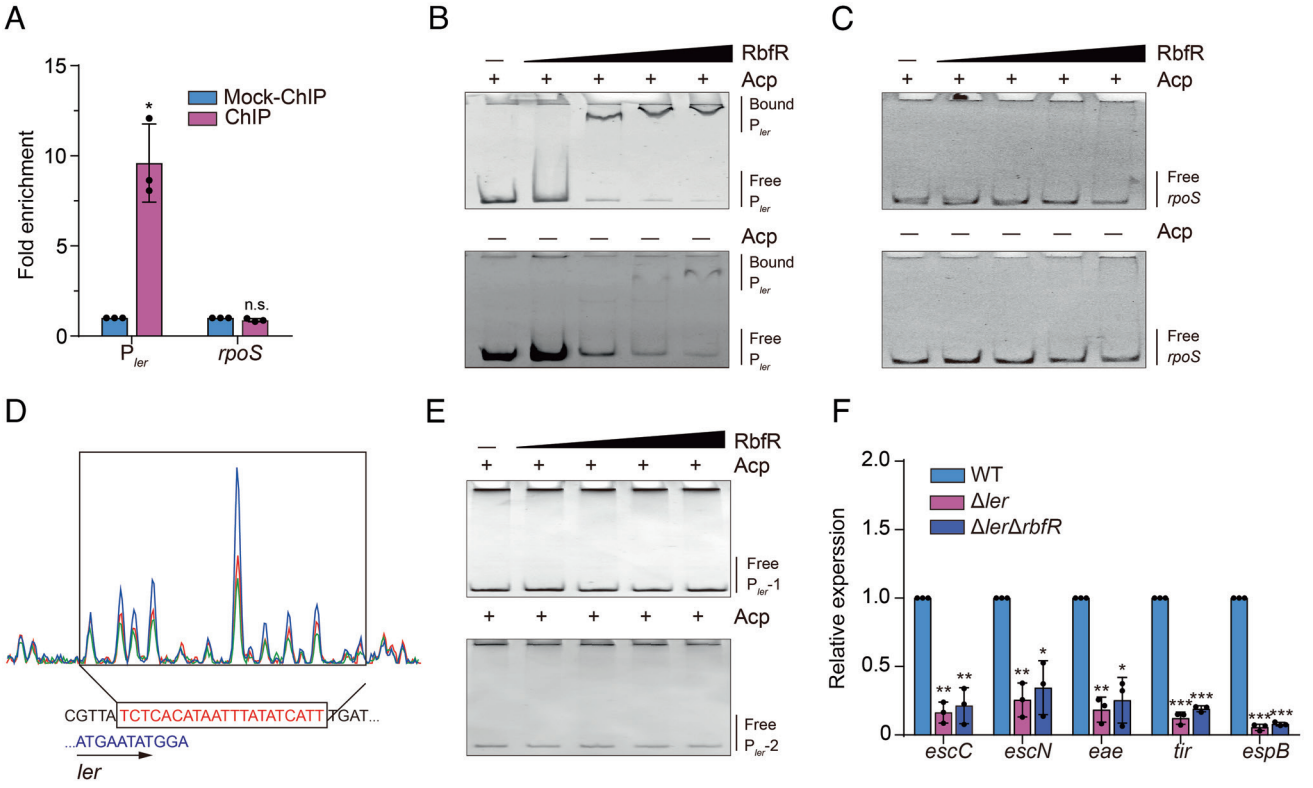

Fig. 3. RbfR specifically binds to the promoter of *ler*. (*A*) Fold enrichment of the promoter region of *ler* (P_{ler}) and coding region of *rpoS* in RbfR-ChIP samples, as measured by qRT-PCR. *rpoS* served as negative control. Data are presented as the mean ± SD (n = 3). (*B* and *C*) EMSA of the binding of purified RbfR to P_{ler}(b) and *rpoS*(c) with or without acetyl phosphate (Acp). (*D*) RbfR binds to a motif in P_{ler}. The protected region shows a significantly reduced peak intensities (green) pattern compared with those of the control (blue and red). The identified RbfR-binding motif is shown in a box at the *Bottom* of the figure. (*E*) EMSA of the binding of purified RbfR to P_{ler}-1 (the binding motif was deleted) and P_{ler}-2(the binding motif was mutated to 5- CTCTGTGCGGCCCGCGCTGCC-3) of EDL933 with or without Acp. (*F*) qRT-PCR analysis of LEE gene expression in WT, Δ*ler*, and Δ*ler*Δ*rbfR* grown in SCEM medium containing 20 μM riboflavin. Data are presented as the mean ± SD (n = 3). Significant differences were assessed by an unpaired *t* test. Error bars represent SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; n.s. no significant difference.

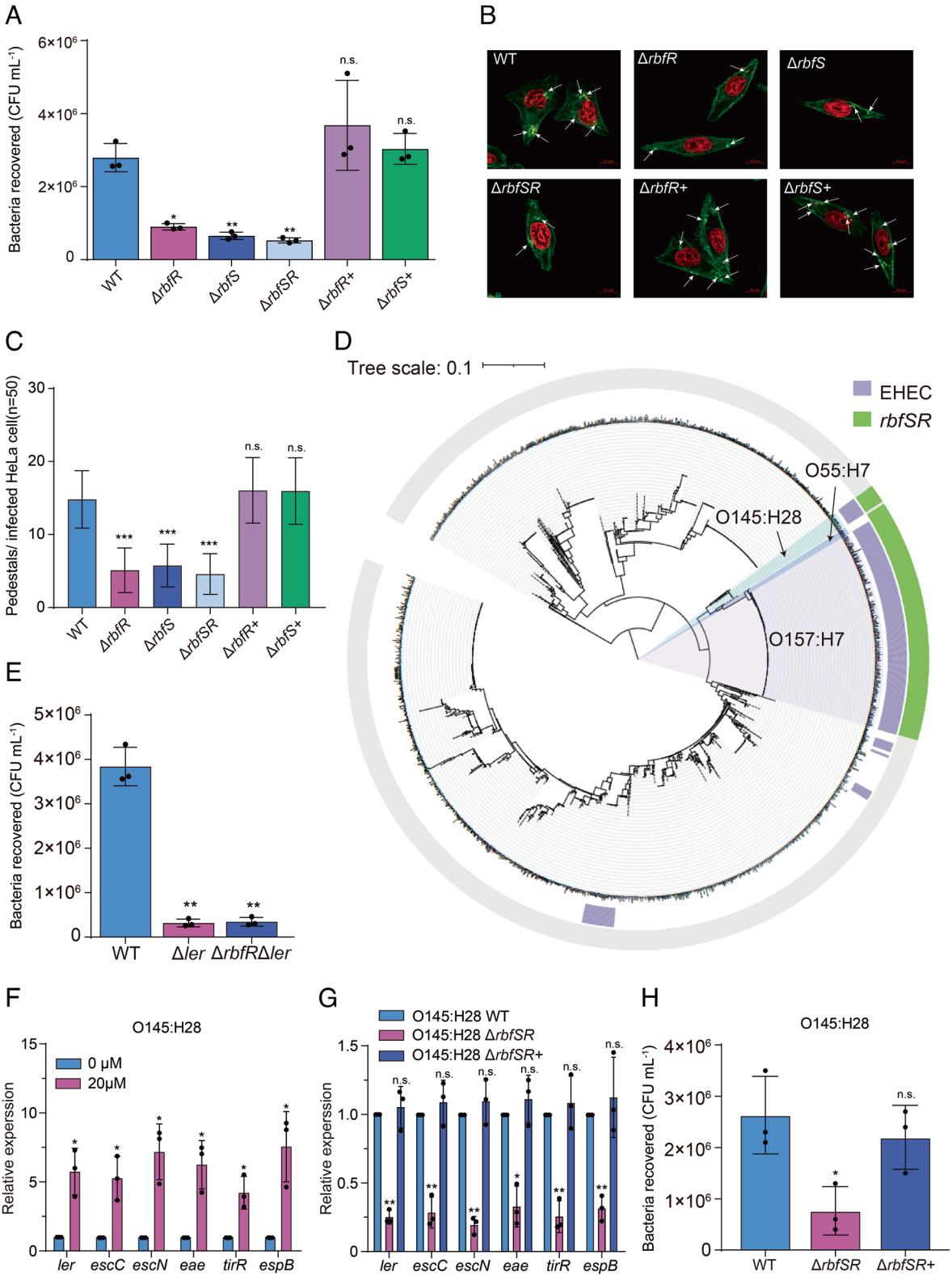

Fig. 4. Expression of *rbfSR* increases the cell attachment ability of EHEC. (*A*) Adherence of WT, Δ*rbfR*, Δ*rbfS*, Δ*rbfR*Δ*rbfS*, Δ*rbfR*+, and Δ*rbfS*+ to HeLa cells. Data are presented as the mean ± SD (n = 3). (*B*) Detection of AE lesion formation by WT, Δ*rbfR*, Δ*rbfS*, Δ*rbfR*Δ*rbfS*, Δ*rbfR*+, and Δ*rbfS*+ by FAS in HeLa cells at 3 h post infection. The HeLa cell actin cytoskeleton (green) and nuclei of bacterial and HeLa cells (red) are shown. (*C*) FAS assay quantification of the number of pedestals per infected cell (n = 50). (*D*) Phylogenetic analysis of 2,134 publicly available *E. coli* complete genomes. Purple semicircle on the inner ring indicates the EHEC strains. The presence of *rbfSR* is labeled with a green semicircle on the outer ring. (*E*) Adherence of WT, Δ*ler*, and Δ*rbfR*Δ*ler* to HeLa cells. Data are presented as the mean ± SD (n = 3). (*F*) qRT-PCR analysis of LEE gene expression in EHEC O145:H28 grown in SCEM medium containing 0–20 μM riboflavin. Data are presented as the mean ± SD (n = 3). (*G*) qRT-PCR analysis of LEE gene expression in O145:H28 WT, O145:H28 Δ*rbfSR*, and O145:H28 Δ*rbfSR*+ grown in an SCEM medium containing 20 μM riboflavin. Data are presented as the mean ± SD (n = 3). (*H*) Adherence of O145:H28 WT, O145:H28 Δ*rbfSR*, and O145:H28 Δ*rbfSR*+ to HeLa cells. Data are presented as the mean ± SD (n = 3). Significant differences were assessed by an unpaired *t* test. Error bars represent SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; n.s. no significant difference.

We then showed that Δ*ler* exhibited a significantly decreased bacterial adherence ability compared to WT (Fig. 4*E*), while deletion of *rbfR* in Δ*ler* did not result in a further decrease of bacterial adherence ability (Fig. 4*E*). Thus, RbfSR promotes EHEC O157:H7 attachment ability via Ler.

rbfSR Is Present in EHEC O157:H7 and O145:H28 and Expression of RbfSR in Other EHEC Serotypes Increased Their Cell Attachment Ability. To investigate the distribution of *rbfSR* in *E. coli*, a comparative genomics analysis was performed using all 2,134 publicly available *E. coli* complete genomes. The results showed that *rbfSR* is only present in EHEC strains and *E. coli* O55:H7 that is closely related to EHEC O157:H7 (Fig. 4*D*). Next, we performed a detailed analysis on the distribution of *rbfSR* and the RbfR binding site in EHEC. Among the nine prevalent EHEC serotypes (23, 24), *rbfSR* is only present in O157:H7 and O145:H28 but is absent from all other seven serotypes (Fig. 4*D*). The identical RbfR binding site is present in the *ler* promoter of all nine serotypes (*SI Appendix*, Fig. S3*A*). Phylogenetic analysis showed that EHEC O157:H7 and O145:H28 are separated by serotypes without *rbfSR* in the tree, indicating that these two serotypes independently acquired *rbfSR* via lateral gene transfer events (Fig. 4*D*).

We next investigated whether *rbfSR* also regulates the virulence of EHEC O145:H28. qRT-PCR assays showed that the expression of LEE genes in EHEC O145:H28 was increased in response to riboflavin (Fig. 4*F*). Deletion of *rbfSR* in EHEC O145:H28 led to a decrease of LEE gene expression and reduced bacterial attachment ability to HeLa cells in the presence of riboflavin (Fig. 4 *G* and *H*), and these defects were restored to the WT level by complementation with corresponding genes (Fig. 4 *G* and *H*). This indicates that *rbfSR* also promotes the cell attachment ability of bacteria via enhancing LEE gene expression in EHEC O145:H28 when riboflavin is present.

As the other seven EHEC serotypes all have RbfR binding sites in their *ler* promoters, it is likely that they are ready to use *rbfSR* to increase virulence if they can obtain this TCS. To test this hypothesis, a plasmid (p-*rbfSR*) containing *rbfSR* with a native promoter was introduced into three representative EHEC serotypes: O26:H11, O111:H8, and O103:H2. qRT-PCR assays showed that the expression of LEE genes in these serotypes harboring p-*rbfSR* exhibited a 2.1–5.2-fold increase in response to riboflavin in vitro (*SI Appendix*, Fig. S3 *B–D*), while riboflavin did not influence the expression of LEE genes in the control strains (*SI Appendix*, Fig. S3 *E–G*). Bacterial adherence assays showed that the cell attachment ability of these serotypes harboring p-*rbfSR* exhibited a 2.1–2.7-fold increase compared to those harboring a blank plasmid in the presence of riboflavin (*SI Appendix*, Fig. S3 *H–J*). These data indicate that introduction of *rbfSR* into other EHEC serotypes can lead to increased LEE gene expression and cell attachment ability of bacteria.

***C. rodentium* That Does Not Carry a Native RbfSR Acquires the Ability to Sense Riboflavin to Increase LEE Gene Expression When RbfSR of EHEC O157:H7 Was Introduced.** EHEC does not cause enteric disease in rodents (25). The natural mouse pathogen *C. rodentium*, which employs a LEE-encoded T3SS similar to EHEC for intestinal colonization, has been used extensively as a model for EHEC infection (26). Thus, we employed the *C. rodentium* mouse infection model to investigate whether RbfSR increases bacterial virulence in vivo.

Analysis of the genome sequence showed that *C. rodentium* strain DBS100, a strain widely used in the mouse infection model, does not contain *rbfSR*. However, there is a potential RbfR binding site in the *ler* promoter of DBS100, which is homologous to the RbfR binding site identified in the *ler* promoter of EHEC O157:H7 with two base differences (*SI Appendix*, Fig. S4*A*). EMSA assays showed that phosphorylated RbfR of EHEC O157:H7 specifically binds to the promoter regions of *ler* in DBS100, while the binding capacity of nonphosphorylated RbfR was significantly reduced (Fig. 5*A* and *SI Appendix*, Fig. S6). Furthermore, deletion or mutation of this specific binding site completely abolished the binding of RbfR to the *ler* promoter of DBS100 (Fig. 5*B*). These data indicate that RbfR of EHEC O157:H7 can specifically bind to the *ler* promoter of DBS100.

We next investigated whether RbfSR regulates the expression of LEE genes in DBS100. We constructed strain DBS100-*rbfSR* by introducing p-*rbfSR* into DBS100. qRT-PCR assays showed that the expression of LEE genes in DBS100-*rbfSR* exhibited a 4.1–14.5-fold increase in response to riboflavin in vitro (Fig. 5*C*), while riboflavin has no effect on the expression of LEE genes in DBS100-p (DBS100 with an introduced blank plasmid) (Fig. 5*D*). We further analyzed whether RbfSR regulates the expression of LEE genes in DBS100-*rbfSR* in vivo. At 3 d post infection (d.p.i.), qRT-PCR assays showed that the LEE gene expression of DBS100-*rbfSR* in the colon of mice was 17.5–29.7 fold higher than that of DBS100-p (Fig. 5*E*). These data indicate that *rbfSR* directly increases LEE gene expression in *C. rodentium* in response to the presence of riboflavin in vitro and in vivo.

RbfSR Increases *C. rodentium* Virulence During Murine Infection. We next performed competitive infection assays in mice to determine whether the introduction of *rbfSR* into strain DBS100, which increases LEE gene expression, influences *C. rodentium* colonization in the colon of mice (Fig. 6*A*), which is strictly dependent on the LEE-encoded T3SS (27). A 1:1 mixture of DBS100-*rbfSR* and DBS100-p was administered to mice, and at 3 d.p.i. the competitive index (CI) value of DBS100-*rbfSR* versus DBS100-p was 11.5 in the colonic epithelium of mice (Fig. 6*B*). This indicates that RbfSR increases the colonization of DBS100 in the colon of mice.

Intestinal colonization of *C. rodentium* leads to the induction of gut inflammation and lethality, which are key features of diseases caused by *C. rodentium* and EHEC in hosts (28, 29). We next investigated the influence of RbfSR on the virulence of *C. rodentium* by monitoring the survival and histological damage of infected mice. Survival curves of infected mice showed that DBS100-*rbfSR* presented accelerated mortality compared with DBS100-p (Fig. 6 *A–C*). Histological damage in the colon of mice infected by DBS100-*rbfSR* or DBS100-p was also evaluated by analyzing the presence of mononuclear and polymorphonuclear infiltrates in the lamina propria, changes in the architecture of the mucosa, villus height, goblet cell depletion, and attached bacteria. Histopathology scores in DBS100-*rbfSR*-infected mice were much higher than those of DBS100-p-infected mice (Fig. 6 *D* and *E* and *SI Appendix*, Fig. S4*B*), indicating that the severity of disease caused by DBS100-*rbfSR* infection in mice was increased compared to that caused by DBS100-p infection. Collectively, these data indicate that the introduction of *rbfSR* can increase the pathogenesis of *C. rodentium* during murine infection.

RbfSR Senses Microbiota-produced Riboflavin to Increase *C. rodentium* Virulence in the Mouse Colon. The above results showed that *C. rodentium* does not carry a native *rbfSR* and that the introduction of *rbfSR* into *C. rodentium* increases bacterial intestinal colonization causing increased disease severity in mice. We next investigated whether this effect of RbfSR requires the presence of microbiota which produce riboflavin in the mouse

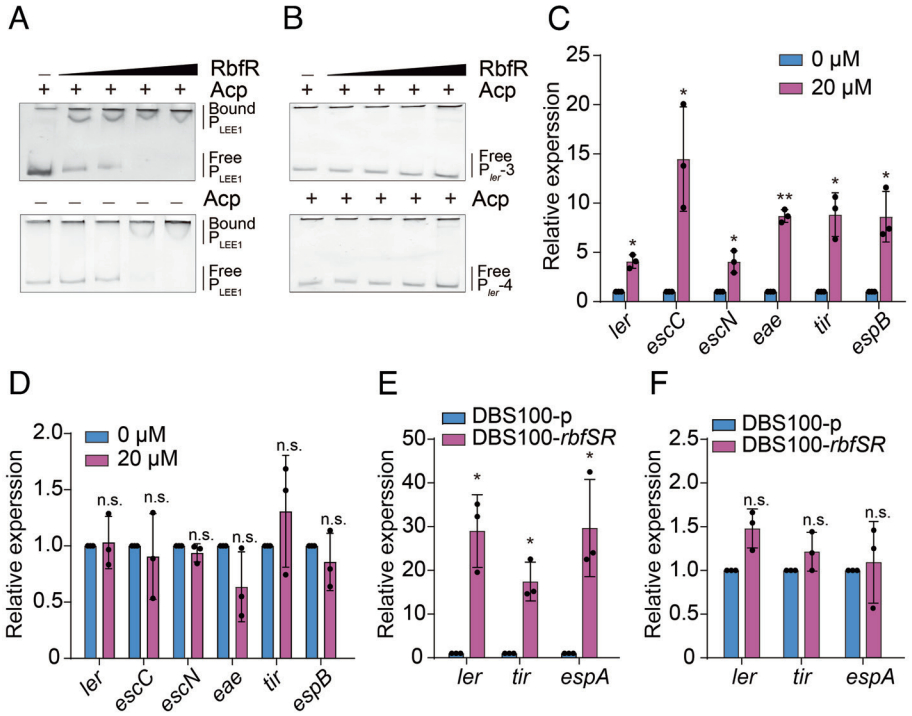

Fig. 5. Expression of *rbfSR* in DBS100 increases LEE gene expression in vitro and in vivo. (*A* and *B*) EMSA of the binding of purified RbfR to P_{ler} (a), P_{ler}-3 (b, the binding motif was deleted), and P_{ler}-4(b, the binding motif was mutated to 5-CTCCGTGCGGCCCGCGGTGCC-3) of DBS100 with or without Acp. (*C* and *D*) qRT-PCR analysis of LEE gene expression in DBS100-*rbfSR*(c) and DBS100-p(d) grown in SCEM medium containing 20 μM riboflavin. Data are presented as the mean ± SD (n = 3). (*E* and *F*) qRT-PCR analysis of LEE gene expression in DBS100-p and DBS100-*rbfSR* in SPF mice(*E*) and abx-treated mice(*F*). Data are presented as the mean ± SD (n = 3). Significant differences were assessed by an unpaired *t* test. Error bars represent SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; n.s. no significant difference.

colon. We first compared the expression of LEE genes and intestinal colonization ability of DBS100-*rbfSR* and DBS100-p in abx-treated mice devoid of microbiota. qRT-PCR and competitive infection assays showed that the LEE gene expression and bacterial colonization in the colon of mice exhibited no significant difference between DBS100-*rbfSR* and DBS100-p in abx-treated mice (Figs. 5*F* and 6*B*). Then, we used abx-treated mice fed with riboflavin, in which 16.2 μM riboflavin (Fig. 6*F*) was present in the colon, to perform the competitive infection assays. The results showed that DBS100-*rbfSR* exhibited a significant colonization advantage over DBS100-p (Fig. 6*G*). This is different from the situation in the colon of abx-treated mice fed with PBS, where no riboflavin is present and the two strains (DBS100-*rbfSR* and DBS100-p) compete evenly (Fig. 6*G*). These data indicate that the influence of RbfSR on the LEE gene expression and intestinal colonization of *C. rodentium* in the mouse colon depends on the riboflavin produced by microbiota.

To further confirm that the effect of RbfSR on the *C. rodentium* virulence in mice depends on microbiota-produced riboflavin, we used abx-treated mice monocolonized with riboflavin-producing commensal bacteria to perform the competitive infection assays. Among the members of the gut microbiota, *Lactobacillus* species synthesize considerable amounts of riboflavin (14). We administered abx-treated mice with *L. plantarum* WCFS1 harboring a mutated riboflavin biosynthesis gene cluster (30), and riboflavin-producing strain *L. plantarum* ATCC8014 (31), respectively. After 3 d of administration, similar amounts of WCFS1 and ATCC8014 were recovered from the colon of mice, indicating that both strains colonized in the colon with a similar efficiency (*SI Appendix*, Fig. S4*C*). HPLC assays showed that 17.9 μM riboflavin was detected in the colonic content of mice administered with ATCC8014 (Fig. 6*F*), while no riboflavin was detected in the colonic content of mice administered with WCFS1 (Fig. 6*F*). Next, a 1:1 mixture of DBS100-*rbfSR* and DBS100-p was administered to abx-treated mice fed with WCFS1 or ATCC8014, respectively. At 3 d.p.i., DBS100-*rbfSR* exhibited a significant colonization advantage over DBS100-p in the colon of abx-treated mice administered with ATCC8014 but not in abx-treated mice administered with WCFS1 (Fig. 6*H*). It further confirms that the increase of *C. rodentium* virulence in vivo resulting from the introduction of *rbfSR* requires riboflavin produced by commensal bacteria in the mouse colon.

Overall, these data indicate that C. rodentium does not have a native rbfSR and that the bacteria obtain the ability to sense riboflavin produced by microbiota to increase virulence in mice when the rbfSR of EHEC O157:H7 was introduced. As the C. rodentium murine infection model is suitable for studying EHEC pathogenesis in vivo (26), these data confirm that RbfSR activated by microbiota-produced riboflavin plays an important role in the virulence of EHEC O157:H7 by increasing LEE gene expression within the host. The results also indicate that acquisition of *rbfSR* by other EHEC serotypes that do not carry native *rbfSR* might enable bacteria to sense riboflavin to increase bacterial colonization in the large intestine, causing more severe disease in humans.

Discussion

EHEC has evolved to sense multiple signals in the colon to exquisitely control the expression of T3SS. Those intricate regulatory systems are essential for the pathogen to outcompete commensal bacteria and colonize the densely populated colon with a very small infection dose (50 CFUs) (1). In this study, we showed that the highly pathogenic EHEC O157:H7 utilizes a TCS to sense microbiota-produced riboflavin to increase the expression of T3SS in the colon. Introduction of this TCS into other EHEC serotypes and *C. rodentium* significantly increased bacterial virulence in vitro

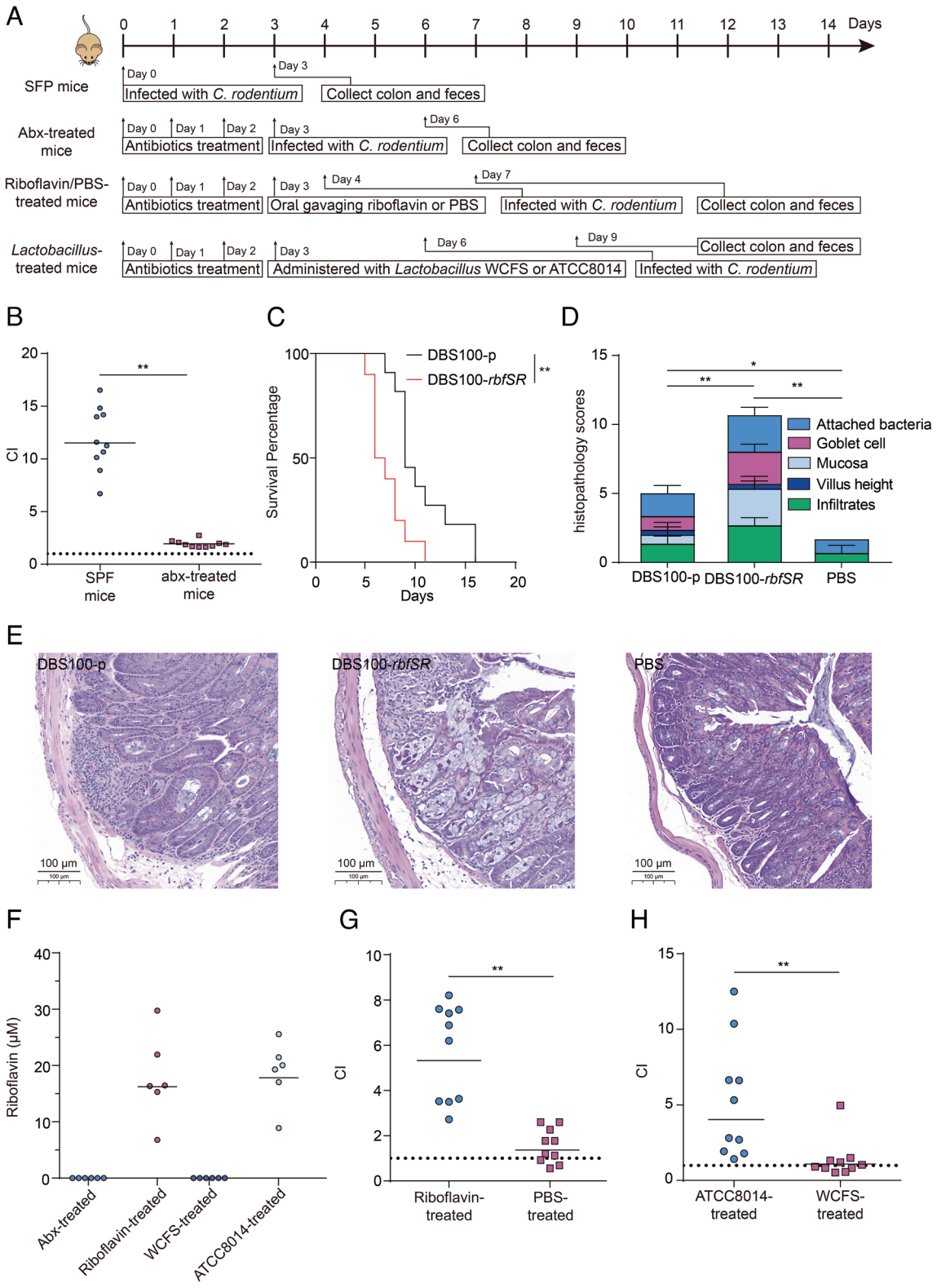

Fig. 6. Riboflavin produced by microbiota increases *C. rodentium* intestinal colonization through RbfSR. (*A*) Schematic of *C. rodentium* colonization in different infection models. (*B*) Competition assay comparing the intestinal colonization ability of DBS100-p and DBS100-*rbfSR* in SPF mice and abx-treated mice (n = 10 mice per group). (*C*) Survival curve of mice infected with DBS100-p or DBS100-*rbfSR* (n = 10 mice per group). (*D* and *E*) Histological score (*D*) and representation (*E*) of colon 3 d after DBS100-p and DBS100-*rbfSR* infection (n = 3). (*F*) HPLC measurement of riboflavin concentrations in the colon contents of abx-treated miceabx-treated mice fed with 20 ug riboflavin (Riboflavin-treated), and abx-treated mice administered with WCFS (WCFS-treated) or ATCC8014 (ATCC8014-treated) (n = 6 mice per group). (*G*) Competition assay comparing the intestinal colonization ability of DBS100-p and DBS100-*rbfSR* in abx-treated mice fed with 20 ug riboflavin (Riboflavin-treated) or PBS(PBS-treated) (n = 10 mice per group). (*H*) Competition assay comparing the intestinal colonization ability of DBS100-p and DBS100-*rbfSR* in abx-treated mice administered with WCFS (WCFS-treated) or ATCC8014 (ATCC8014-treated mice) (n = 10 mice per group). Significant differences were assessed by log-rank (Mantel-Cox) test (*C*), unpaired *t* test (*D*), and Mann–Whitney U test (*B*, *G*, and *H*). Each symbol represents an individual mouse. Error bars represent SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; n.s. no significant difference.

or in a mouse infection model. Evolutionary analysis showed that O157:H7 obtained this TCS before it diverged from the ancestral O55:H7. Thus, it is highly likely that acquiring this TCS played an essential role in the generation of the highly pathogenic EHEC O157:H7 and that other EHEC serotypes may obtain this TCS to increase their pathogenicity during their future evolution, which represents a potential risk for public health.

Intestinal microbiotas provide a barrier known as "colonization resistance" to bacterial pathogens, which limits pathogen expansion and transmission via different mechanisms, such as production of antagonistic molecules, competition for niches and nutrients, and contact-dependent killing (32). T3SS of EHEC injects bacterial effectors into colonic epithelial cells to induce the A/E lesion formation, which triggers the intimate attachment of EHEC to epithelial cells (6, 7). This enables EHEC to colonize the surface of the colonic epithelium, a unique niche inaccessible to the microbiota, and thus confers a competitive advantage to EHEC (4, 33). We found in this study that EHEC O157:H7 gained the ability to use the microbiota-produced riboflavin as a signal to further increase T3SS expression, which results in the significantly increased ability of O157:H7 to attach to epithelial cells and colonize the colon, leading to a higher disease severity. We expect that acquiring this signal-sensing ability may have further decreased the infection dose of O157:H7. The fact that riboflavin is necessary to the host body and is produced by about 60% of commensal bacterial species in the large intestine ensured that this is a reliable signal. In addition to riboflavin, this study also showed that the other two microbiota-produced B vitamins, thiamine and nicotinic acid, also influence the expression of LEE genes of O157:H7 in vitro. The underlying mechanisms whereby EHEC O157:H7 senses thiamine and nicotinic acid to regulate virulence gene expression and the contribution of these mechanisms to bacterial pathogenicity in vivo will be the subject of future studies.

In order to precisely recognize the large intestine and site-specifically induce LEE gene expression, EHEC and *C. rodentium*, which share similar pathogenic mechanisms and identical LEE genes, exquisitely regulate the transcription of LEE genes via multiple signaling transduction pathways in response to signals in the large intestine, such as succinate, galacturonic acid, and L-arginine (28, 34, 35). In this study, in addition to known signal-sensing mechanisms shared by those two pathogens, we found that EHEC O157:H7 uses RbfSR to sense riboflavin with a concentration range close to that in the host large intestine to increase the expression of LEE genes. RbfR can bind to the same region of the *ler* promoters in both EHEC O157:H7 and *C. rodentium* (Figs. 3*B* and 5*A*). Expression of *rbfSR* in *C. rodentium* promotes its intestinal colonization by increasing bacterial attachment ability to epithelial cells, thus enhancing the bacterial virulence in mice as shown by increased disease severity and accelerated mortality (Fig. 6 *C*–*E*). In addition, we also showed that the introduction of *rbfSR* into other EHEC serotypes significantly increased the LEE gene expression and cell attachment ability of bacteria in the presence of riboflavin (*SI Appendix*, Fig. S3 *B*–*D*, *H*, and *I*). Thus, obtaining RbfSR increased the pathogenicity of EHEC O157:H7 over other EHEC serotypes.

E. coli is a highly clonal species that is classified into five phylogenetic groups (36, 37). It is known that EHEC has independently evolved from nonpathogenic *E. coli* ancestors six times in two phylogenetic groups, generating six distinct EHEC lineages (24, 38). To investigate the evolutionary history of *rbfSR*, we first constructed an updated genome-wide phylogenetic tree using 2,134 currently publicly available *E. coli* complete genomes, including 379 EHEC genomes. The tree showed that all nine prevalent EHEC serotypes are distributed among six lineages (Fig. 4*D*). *rbfSR* is only present in two EHEC serotypes, O157:H7 and O145:H28, which belong to two different EHEC lineages. Separation of these two lineages by strains without *rbfSR* indicates that they independently acquired *rbfSR* via distinct horizon gene transfer events (Fig. 4*D*). In non-EHEC *E. coli*, *rbfSR* is only found in serotype O55:H7. It is known that EHEC O157:H7 and *E. coli* O55:H7 originated from an ancestral O55:H7 clone (39). The presence of *rbfSR* in both O157:H7 and O55:H7 (Fig. 4*D*) indicates that *rbfSR* was introduced into their common ancestor prior to the divergence of O157:H7 from O55:H7. Consistent with this, phylogenetic analysis showed that *rbfSR* of O157:H7 is more closely related to that of O55:H7 than to that of O145:H28 (*SI Appendix*, Fig. S5 *A* and *B*). These data further confirm that O157:H7 acquired *rbfSR* via a genetic event distinct from the event that introduced *rbfSR* into O145:H28. EHEC O157:H7 is an important human pathogen that is more often associated with severe disease and death than other EHEC serotypes (40, 41). Taking into consideration the ability of RbfSR to increase bacterial virulence, the fact that the lineage obtained *rbfSR* during the early stage of evolution and has retained it ever since indicates that acquisition of this TCS was a critical step in the generation of the highly pathogenic EHEC O157:H7. In addition, the observation that two distinct EHEC lineages separately acquired *rbfSR* during evolution further suggests that this TCS might be important for the pathogenicity of EHEC.

The evolutionary path of different EHEC lineages is repeatedly obtaining a core set of virulence factors via horizontal gene transfer to generate the same pathogenic features, chief of which is the independent acquisition of the LEE pathogenicity island encoding T3SS (38). The fact that RbfSR binding sites in the promoter of ler are conserved in all EHEC serotypes indicates that the evolutionary precursor of the EHEC LEE pathogenicity island contained the RbfR binding site and was under regulation by RbfSR. The LEE pathogenicity island and rbfSR were transferred into O157:H7 or O145:H28 by separate events, and in the cases of other EHEC serotypes, only the LEE pathogenicity island was transferred. Healthy ruminant animals, particularly cattle, are the primary reservoir of EHEC (42). In cattle, EHEC is distributed along the entire gut, including the rumen, small intestine, and large intestine, without causing diseases (43, 44), and LEE genes have been suggested to contribute to the bovine gut colonization of EHEC (45). This differs from the situation in humans and mice, where EHEC and *C. rodentium* specifically colonize the large intestines of their respective hosts and cause diseases. We found in this study that EHEC and *C. rodentium* sense the large intestinal microbiota-produced riboflavin via RbfSR to increase LEE gene expression, leading to enhanced bacterial colonization ability and virulence. Dietary riboflavin is mainly absorbed in the small intestine of mammalian hosts (12), and we showed that the riboflavin concentration in the small intestine of mice is too low to activate RbfSR (Fig. 1*A* and *SI Appendix*, Fig. S2 *C* and *D*). It is likely that at gut sites of cattle where little or no riboflavin is present, RbfSR would have no influence on the LEE gene expression and colonization of EHEC. Thus, we speculate that the selection pressure that promotes EHEC to acquire *rbfSR* is lower than that which promotes EHEC to obtain LEE genes at gut sites other than the large intestine in cattle. This may be the primary reason why all EHEC serotypes harbor LEE genes, but only two of them harbor *rbfSR*. We showed that when *rbfSR* was introduced into those other EHEC serotypes, the expression of LEE genes and the bacterial attachment ability of these serotypes to HeLa cells was significantly increased. Owing to the high prevalence of EHEC in cattle and extensive lateral gene transfer among enteric bacteria (46–48), it

is highly likely that other EHEC serotypes may eventually obtain *rbfSR* from O157:H7, O145:H28, or other commensal bacteria harboring this TCS during their future evolution, under selection pressure in the large intestine of cattle where RbfSR may benefit the colonization of EHEC. Obtaining this TCS will make those EHEC serotypes more competitive in colonizing the human colon and able to cause more severe disease. This risk requires attention in the prevention and control of EHEC infection.

Materials and Methods

Bacterial Strains, Plasmids and Cell Culture. The bacterial strains and plasmids used in this study are listed in *SI Appendix*, Table S1. Mutant strains of EHEC O157:H7 EDL933 were generated using the λ-Red recombination system. Complementary strains were established by cloning *rbfS* and *rbfR* and native promoters into the pACYC-184 plasmid and transformed into EHEC O157:H7 EDL933 and *C. rodentium* DBS100. The strain for RbfR and RbfS purification was generated by cloning *rbfR* and *rbfS* into the pET28a plasmid. Bacterial strains were grown in Luria-Bertani (LB) broth (Oxoid; LP0021 and LP0042), Dulbecco's modified Eagle medium (DMEM, Gibco; C11995500BT), or simulated colonic environment medium (SCEM, 6.25 g/L tryptone, 2.6 g/L D-glucose, 0.88 g/L NaCl, 2.7 g/L $KHCO_3$, 0.43 g/L KH_2PO_4, 1.7 g/L $NaHCO_3$ and 4.0 g/L bile salts) (49) with different concentrations of riboflavin (Sigma; R9504). Bacteria were inoculated into a fresh medium and grown at 37°C under shaking. When needed, isopropyl β-D-thiogalactoside (IPTG) and antibiotics were added to the medium at the following final concentrations: 0.1 mM IPTG, 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 10 μg/ml tetracycline. The primers used for all manipulations are listed in *SI Appendix*, Table S1.

Riboflavin Concentration Detection. Riboflavin was quantified using HPLC as described previously (50). Ileum (0.1 g) and colonic contents were weighed and vortexed for 5 min in 1 ml PBS buffer. The samples were centrifuged at 13,000 rpm for 10 min, and the supernatants were collected. The supernatants were then filtered through a 0.22-μm filter membrane. The filtered supernatants were transferred to an HPLC vial and analyzed by HPLC. For the riboflavin standard curve, 0–100 μM riboflavin was prepared in PBS and analyzed by HPLC. HPLC was performed using a reverse-phase C18 column (Agilent, 4.6×250 mm, 5 μm) at room temperature on a Surveyor Plus HPLC System (Thermo Finnigan) by isocratic elution at a flow rate of 1 mL/min using a mobile phase of 1% acetic acid:methanol (70:30, v/v). The sample injection volume was 20 μL. The detection wavelength was 282 nm. The peak area of the analytes in question was measured and used for quantification.

Detailed experimental protocols and materials and methods are described in *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. Relevant data are given within the manuscript and/or *SI Appendix*. RNA-seq data have been deposited in the NCBI SRA database under the accession codes PRJNA839165 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA839165). ChIP-seq data have been deposited in the NCBI SRA database under the accession codes PRJNA839186 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA839186).

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