Maturation of functional type III secretion machinery by activation of anaerobic respiration in enterohaemorrhagic *Escherichia coli*

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Enterohaemorrhagic *Escherichia coli* (EHEC) is a gastrointestinal pathogen that causes diarrhoea and more severe diseases in humans. A key feature of EHEC is the type III secretion system (TTSS), which translocates virulence factors (effectors) directly into host cells. In this study, the expression and secretion of effectors in EHEC grown under anaerobic conditions were examined. The secretion of effectors was greatly enhanced, without an increase in their expression levels, when EHEC was grown in the presence of specific electron acceptors, such as trimethylamine *N*-oxide (TMAO) and nitrate, for anaerobic respiration. The activation of the TTSS was dependent on the activity of respiratory systems, including electron-acceptor-specific signalling systems and reductases. Although *de novo* protein synthesis was not required for TTSS activation, the inhibition of respiratory activity abolished secretion. EHEC grown with either TMAO or nitrate possessed a more intact type III secretion (TTS) apparatus, including the needle protein EscF and the translocator protein EspA, than EHEC grown without an electron acceptor. These observations suggest that activation of either the TMAO- or the nitrate-specific respiratory system accelerates the maturation of functional TTS apparatus under anaerobic growth conditions.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) is a life-threatening human pathogen that causes haemorrhagic colitis, bloody diarrhoea, and haemolytic uraemic syndrome (Nataro & Kaper, 1998). EHEC is a member of the attaching and effacing (A/E) pathogens (Nataro & Kaper, 1998; Tzipori *et al.*, 1995), which is a group that includes enteropathogenic *E. coli* (EPEC) (Moon *et al.*, 1983) and the mouse pathogen *Citrobacter rodentium* (Schauer & Falkow, 1993). A/E lesions are characterized by the localized destruction of brush border microvilli, intimate attachment of the organism to the host cell membrane, and the formation of an actin-rich underlying structure in the host cell (Frankel *et al.*, 1998). The genes essential for causing the A/E lesions are encoded in a pathogenicity island designated the locus of enterocyte effacement (LEE). The LEE consists of more than 40 genes that encode the components of the type III secretion system (TTSS), including secreted proteins, chaperones, and adhesin and transcription regulators (Garmendia *et al.*, 2005).

The TTSS is associated with many Gram-negative pathogens that cause disease in humans, animals, insects and plants (Galan & Collmer, 1999). Features of the TTSS include: (i) the formation of a macromolecular complex that spans both membranes, and extends a needle-like projection out of the cell; and (ii) the delivery of virulence factors directly into the host cell (O'Connell *et al.*, 2004). Once injected into the host cell, the virulence factors, referred to as effectors, co-opt the signalling pathways of the cell to promote responses beneficial to the pathogen (Galan & Collmer, 1999). The TTSS of EPEC and EHEC is unique in that it attaches a filamentous extension to the needle complex (Sekiya *et al.*, 2001; Wilson *et al.*, 2001). The filament is composed of EspA, and appears to function as the conduit for the translocation of effector proteins. The effectors are delivered into the host cell through a translocation pore formed in the plasma membrane of the host cell by the translocator proteins EspB and EspD (Hartland *et al.*, 2000; Ide *et al.*, 2001).

Expression and secretion of the virulence factors are tightly regulated, and respond to environmental stimuli, such as temperature, pH and nutrient availability (Beltrametti *et al.*, 1999; Kenny *et al.*, 1997). Such regulation permits the coordinated and timely expression of the proteins required for virulence expression under changing environmental conditions. Clarification of the environmental factors and signal transduction systems that control virulence

Abbreviations: A/E, attaching and effacing; DIC, differential interference contrast; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *E. coli*; LEE, locus of enterocyte effacement; TMAO, trimethylamine *N*-oxide; TTS, type III secretion; TTSS, type III secretion system.

expression is important to improve our understanding of pathogenesis, and to identify drug targets. Most of the studies on the expression of virulence genes in EHEC have been carried out with bacteria grown under aerobic conditions, although the intestinal tract, which is the target site of EHEC infection, is anaerobic. Like other *E. coli* strains, EHEC is a facultative anaerobe, and it possesses a large number of systems for regulating anaerobic respiration in response to whatever terminal electron acceptor is available. These regulatory systems channel electrons from the donor to terminal acceptors, such that the overall difference in potential is maximized for any given growth condition (Shalel-Levanon *et al.*, 2005). The terminal electron acceptors used in anaerobic respiration are usually low-molecular-weight oxygen-containing molecules, such as trimethylamine *N*-oxide (TMAO), DMSO, nitrate and fumarate, which are abundant in the environment.

In this study, we performed what we believe to be the first investigation of the expression and type III secretion (TTS) of virulence factors occurring in response to electron acceptors under anaerobic conditions. Our results suggest that specific electron acceptors, TMAO and nitrate, enhance TTS by accelerating the formation of the fully assembled functional TTS apparatus.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise specified, the bacteria were pre-cultured in 2 ml Luria-Bertani (LB) medium for 14 h at 30 °C, with shaking, and

then 100 µl of the culture was used to inoculate 10 ml fresh LB medium, both with and without an electron acceptor (10 mM), in a 15 ml tube. The bacteria were further cultured for 3 h at 37 °C in an anaerobic jar, and for 3 h at 37 °C without shaking (standing conditions). To inhibit protein synthesis and respiration, excess amounts of chloramphenicol (100 µg ml⁻¹) or amyltal (5 mM) were added to the bacterial culture grown without an electron acceptor. After 30 min incubation at 37 °C, an electron acceptor was added, and the bacteria were incubated for an additional 1 h at 37 °C. The electron acceptors used were TMAO (Sigma), DMSO (Wako), nitrate (Sigma), and fumarate (Wako).

Construction of deletion mutants. To construct deletion mutants, we employed a one-step inactivation method (Datsenko & Wanner, 2000). Briefly, PCR products containing the chloramphenicol-resistance cassette flanked by 40 bp of sequence corresponding to the 5' and 3' ends of each gene were introduced by electroporation into the EHEC Sakai strain harbouring pKD46, which is a helper plasmid encoding the λ -Red, Gam and Bet proteins (Datsenko & Wanner, 2000). The chloramphenicol-resistant colonies were examined for the correct chromosomal structures by PCR tests using locus-specific primers. The DNA sequences of the primers are indicated in Table 2.

Adherence assay. Bacterial cultures grown as described above were added directly to tissue culture wells that contained a confluent monolayer of Caco-2 cells growing on glass coverslips (13 mm diameter). After 2 h incubation at 37 °C, the cells were washed five times with PBS to remove non-adherent bacteria. The cells were then incubated in fresh medium for an additional 1.5 h at 37 °C. After washing with PBS, the cells were fixed with 100% methanol for 10 min at room temperature, and stained with Giemsa staining solution. The adherence efficiency was determined by counting the number of bacteria adhering to the Caco-2 cells.

Electrophoresis and immunoblot analysis. SDS-PAGE (8–13.5%) or Tricine-SDS-PAGE (16%) was used throughout this

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Sakai (RIMD 0509952)	WT EHEC O157:H7	Hayashi <i>et al.</i> (2001)
SKI0073	Sakai $\Delta(\text{torSTR})::\text{cat}$	This study
SKI0156	Sakai $\Delta\text{narQ}::\text{cat}$	This study
SKI0171	Sakai $\Delta(\text{narXL})::\text{cat}$	This study
SKI0194	Sakai $\Delta\text{narP}::\text{cat}$	This study
SKI1188	Sakai $\Delta(\text{narVWYZ})::\text{cat}$	This study
SKI1189	Sakai $\Delta(\text{narGHJI})::\text{cat}$	This study
SKI1191	Sakai $\Delta(\text{torCAD})::\text{cat}$	This study
SKI1193	Sakai $\Delta\text{purK}::\text{cat}$	This study
SKI1195	Sakai $\Delta\text{escD}::\text{cat}$	This study
SKI1197	Sakai $\Delta\text{ubiE}::\text{cat}$	This study
SKI1201	Sakai $\Delta\text{menA}::\text{cat}$	This study
Plasmids		
pKD3	Template plasmid for λ -Red recombination system, Ap ^r	Datsenko & Wanner (2000)
pKD46	Red recombinase expression plasmid, Ap ^r	Datsenko & Wanner (2000)
pGEM-T-easy	TA cloning vector, Ap ^r	Promega
pGEM-grlA	pGEM-T-easy-grlA	This study

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5'→3')
grlA-U	GAATATGAAAAATGGAATCT
grlA-D	CCGAGAAAAAGGCTTACCCTGG
torSTR-H1	GAATATGAACAGATATGAACAGAATGAGTAAACCCCTCTGGTGTAGGCTGGAGCTGCTTC
torSTR-H3	ATGCCACTATTCTAGTTATTCTTAAGTGGCTGATATTGATATATGAATATCCTCCTTAGT
torCAD-H1	TCATTAAGATCGCTTCACTAAACCATAATTTTACAGGGGCGTGATAGGCTGGAGCTGCTTC
torCAD-H2	ATGCGACGCTAAACGCGTCTTATCAGGCTAACAAACGACGATATGAATATCCTCCTTAGT
NarP-H1	CTCAAACATAATGATTCTAATAAAACCTCAGGAGACTACTGTGTAGGCTGGAGCTGCTTC
NarP-H2	AAATAAAAAAGAGGCCCGATGGTGATGCCATCGGGCTATTTATATGAATATCCTCCTTAGT
NarQ-H1	CGAACTGGAACATTAATGATTTTTTGTGGAGAAAGACGCGTGTGTAGGCTGGAGCTGCTTC
NarQ-H2	ACCTTAAGTGCAAGTATTCTTTGGTCAGTAGGAGGCATTAATATGAATATCCTCCTTAGT
NarXL-H1	AAGGTTATTGCTCATTTAAAGCCTGAAGGAAGAGGTTTACGTGTAGGCTGGAGCTGCTTC
NarXL-H2	TGATGCACCCATACCGCTGCTTCCACGCGAGACTTGAGCTATATGAATATCCTCCTTAGT
narVWYZ-H5	CGTAGTCTAGGGTTAAGTAAGGACCTCGTCCTCAGTACAGGTGTAGGCTGGAGCTGCTTC
narVWYZ-H6	CCTCTTGATAGTATCGGACGTTCCACGGCCCTCTCGCTTCCCATATGAATATCCTCCTTA
narGHJI-H1	ATATGTTACCCATGGGGAATACTCCTTAATACCCATTTGCGTGTAGGCTGGAGCTGCTTC
narGHJI-H2	TGTAGGTCGGATAAGCGAAGCGCATCCGACAACCTACCCCATATGAATATCCTCCTTAGT
escD-H1	TTTCTCATTGTTCAACCACCAGGATGAATAAAATTTAAAGTGTAGGCTGGAGCTGCTTC
escD-H2	CAATAAAAAACCCCTCCGAAGAGGGGGAAGAGGATAAGAAAAATGAATATCCTCCTTAGT
ubiE-H1	GGGCATCGACGCCCAATCTGTACACTTCTGGAACAATTTGTGTAGGCTGGAGCTGCTTC
ubiE-H2	CTGCCGTCACATAAAGGTTTAAAGGCATTTCCGGTCTCCTCATATGAATATCCTCCTTA
menA-H1	CAAACCTACCCGACTTTCCGGGGTAAAAATAACCGCGCATGTGTAGGCTGGAGCTGCTTC
menA-H2	CAGACAACCGTTTTAGTAGTTAACAATTAACATAAACAGCATATGAATATCCTCCTTA
purK-H1	CGGACCCGCGAGGTGCGGCATGAAACAGGTTTGCGTCCTCGTGTAGGCTGGAGCTGCTTC
purK-H2	CGGATAAGGCGTTACGCGCATCCGGCAATCAAATGAGCATATGAATATCCTCCTTA

study (Laemmli, 1970; Schagger & von Jagow, 1987). For immunoblot analysis, proteins separated by SDS-PAGE or Tricine-SDS-PAGE were transferred to an Immobilon-P transfer membrane (Millipore) or an Immuno-Blot PVDF membrane (Bio-Rad). The proteins on the membranes were detected using primary antibodies specific for EspA, EspB, Tir (Tatsuno *et al.*, 2000), EscF (rabbit antibody raised against the MBP-EscF fusion protein), EscC (Sekiya *et al.*, 2001), DnaK (mAb; Calbiochem) and FLAG (mAb; Sigma). Proteins were visualized with a horseradish-peroxidase-conjugated secondary antibody using an ECL detection kit (Amersham Biosciences).

Analysis of proteins in culture supernatant and whole-cell extract. Bacteria grown as described above were harvested from 1 ml culture by centrifugation. The bacterial pellet was dissolved in SDS-sample buffer (100 µl per OD₆₀₀ unit of original culture). To prepare the proteins from the culture supernatant, 10 ml culture was spun, and the supernatant was passed through a filter (0.22 µm pore size; Millipore). Proteins in the filtered supernatant were precipitated by 6% (final) trichloroacetic acid, and dissolved in SDS sample buffer. The proteins were separated by SDS-PAGE or Tricine-SDS-PAGE, and detected by immunoblotting or Coomassie brilliant blue staining.

Immunofluorescence. A drop of bacterial culture prepared as described above was placed on a glass coverslip (13 mm diameter), and the bacteria were fixed by air drying in 4% paraformaldehyde for 50 min at 37 °C. The coverslips were washed twice with PBS, and blocked in PBS containing 4% BSA for 30 min at room temperature. The washed bacteria were then incubated with a rabbit polyclonal anti-EspA antibody for 1 h at 37 °C. After two washes, the coverslips were incubated with Alexa-488-labelled goat anti-rabbit secondary antibody for 1 h at 37 °C to stain EspA filaments.

The coverslips were washed twice, and mounted onto glass slides using 2 µl Vectashield (Vector Laboratories). The fluorescence was observed under a confocal laser scanning microscope, and the bacteria were examined with Nomarski differential interference contrast (DIC; Leica).

Measurement of the relative ATP concentration. The relative ATP concentration of the bacterial whole-cell lysate was measured using an ATP determination kit (Molecular Probes), as described in the manufacturer's instructions, with slight modification. A 10 µl volume of bacterial culture, prepared as described above, was mixed with 90 µl standard reaction solution containing polymyxin B sulfate (1 × 10⁴ units ml⁻¹) in a luminometer cuvette. The reaction mixture was vortexed briefly, and the luminescence was measured immediately in a Lumat LB9501 luminometer (Berthold). The luminescence readings were carried out for 10 s. The luminescence measurements were normalized to the bacterial density of each culture, which was measured as the OD₆₀₀ value.

Isolation of the TTS apparatus. The TTS apparatus was isolated by adapting a method described by Kubori *et al.* (1998). Overnight cultures (8 ml) of bacteria harbouring multiple copies of *grlA* grown in LB at 30 °C, with shaking, were diluted in 800 ml LB, both with and without an electron acceptor (TMAO and nitrate), and the bacteria were then grown without shaking for 4 h at 37 °C. The bacteria were collected by centrifugation, and gently suspended in 80 ml ice-cold sucrose solution (500 mM sucrose, 150 mM Tris, pH 8.0). The suspension was mixed with 4 ml 10 mg lysozyme ml⁻¹, and then with 320 µl 500 mM Na₂EDTA, and stirred on ice for 1 h. After incubation at 37 °C for 15 min with stirring, the suspension was mixed with 8 ml 3% Triton X-100, and incubated at room temperature until the solution became clear. After adding 400 µl 1 M MgSO₄ and 5.28 ml 5 M NaCl, the sample was spun at 20 000 g for

20 min at 4 °C to remove unlysed cells and cell debris, and then the supernatant was spun at 100 000 g for 1 h at 4 °C. The pellet was suspended in TET buffer (10 mM Tris, pH 8.0, 2 mM EDTA and 0.1% Triton X-100), and subjected to density-gradient centrifugation with 12 ml 36% CsCl for 17 h at 20 °C. Macromolecules in 10 ml of the middle portion of the gradient were spun down at 100 000 g for 1 h at 4 °C. After washing with TET buffer, the pellet was dissolved in sample buffer or TET buffer for immunoblotting.

RESULTS

Effect of electron acceptors on TTS in EHEC grown under anaerobic conditions

We compared the growth and expression of virulence factors in EHEC grown under two low O_2 conditions, anaerobic and standing, with those in EHEC grown under aerobic conditions. Under both of the low O_2 conditions, the growth rate of EHEC in the exponential phase was slower, and its cell density in the stationary phase was lower, than when it was grown under aerobic conditions. The expression level of the LEE-encoded virulence factors EspB and Tir was comparable between EHEC grown under low O_2 and aerobic conditions. However, the amounts of EspB and Tir in the culture supernatant of EHEC grown under the low O_2 conditions were much lower than in the supernatant of the aerobic culture (Fig. 1a). Since the growth of EHEC

under the low O_2 conditions was slower than under aerobic conditions, we added terminal electron acceptors to the medium, as an alternative to O_2 , and stimulated EHEC growth. All four electron acceptors tested, i.e. TMAO, DMSO, nitrate and fumarate, affected the growth in a similar manner: the growth rate in the exponential phase was not changed, whereas the growth after the transition from the exponential to the stationary phase was stimulated (Fig. 1b). We next examined the expression and secretion of virulence factors in EHEC grown in early stationary phase in the presence of an electron acceptor. Irrespective of the growth medium, the amount of EspB or Tir protein expressed in bacteria grown with an electron acceptor was almost the same as for bacteria grown without an electron acceptor. However, the amount of EspB and Tir in the culture supernatant of bacteria grown with an electron acceptor was greater than in the supernatant of bacteria grown without an electron acceptor (Fig. 1c). Although the effect on growth was not very different among the electron acceptors we tested, the effect of TMAO and nitrate on TTS was much stronger than that of the other electron acceptors. These results suggest that the increase in secretion is not the result of growth stimulation, but a result of the action of other mechanisms that specifically respond to TMAO and nitrate. The increase in EspB and Tir in the culture supernatant was not the result of cell lysis or

Fig. 1. Expression and secretion of type III secreted protein in EHEC grown under anaerobic conditions. (a) Expression and secretion of EspB and Tir in EHEC grown under aerobic, anaerobic and standing conditions. Bacteria were grown in LB with vigorous shaking (aerobic), in an anaerobic jar (anaerobic), and without shaking (standing), at 37 °C for 3 h. EspB and Tir in whole-cell extracts and culture supernatant were detected by immunoblotting with anti-EspB and anti-Tir antibodies, respectively. (b) Growth of EHEC in LB, both with and without an electron acceptor, at 37 °C. Bacterial cell densities were monitored by OD₆₀₀ readings. ◆, No electron acceptor; ■, TMAO; ▲, DMSO; ×, nitrate; ○, fumarate. (c) Effect of electron acceptors on the expression and secretion of EspB and Tir. Bacteria were grown in LB under anaerobic and standing conditions at 37 °C for 3 h, both with and without an electron acceptor. The proteins in the culture supernatant and whole-cell extract were analysed as described for (a). (d) Effect of electron acceptors in an EHEC TTSS-deficient mutant. The proteins in the culture supernatant and whole-cell extract were analysed as described for (a). $\Delta escD$, mutant lacking a functional TTSS; Whole, whole-cell extract; Sup, culture supernatant; T, TMAO; D, DMSO; N, nitrate; F, fumarate; –, no addition.

TTSS-independent secretion, because the cytoplasmic protein DnaK was not detected in the supernatant, and neither EspB nor Tir was present in the culture supernatant of the *escD* mutant of EHEC, which lacked a functional TTSS (Fig. 1d).

The increase of TTS in response to either TMAO or nitrate is a general phenotype among EHEC O157 strains, since the increase in TTS was observed in all of the eight strains tested (data not shown), which are representative of EHEC O157 subgroups (Ogura *et al.*, 2006; Ohnishi *et al.*, 2002).

Activation of anaerobic respiratory systems is necessary for TMAO- and nitrate-enhanced TTS

To explore the role of anaerobic respiratory systems in the enhancement of TTS, we examined the effect of a respiratory inhibitor on this process. Bacteria were grown in LB without an electron acceptor for 3 h, and then a respiration inhibitor, amytal, was added 30 min prior to the addition of the electron acceptor. Even though the EHEC was grown to the transition phase without an electron acceptor, further incubation with either TMAO or nitrate stimulated the secretion of EspB. In contrast, prior treatment with amytal completely abolished the stimulation of EspB secretion by TMAO and nitrate (Fig. 2a). Moreover, we examined the

effect of TMAO and nitrate on the TTS in a series of mutants that were deficient in quinone synthesis. Quinones are lipid-soluble molecules that are essential components in respiratory electron transfer chains. To explore the role of quinones in the enhancement of TTS, the TTS response of mutants defective in quinone synthesis was compared with that of the wild-type (WT) strain. In *E. coli*, the *ubiE* mutant is deficient in the synthesis of ubiquinone and menaquinone, whereas the *menA* mutation abolishes the synthesis of menaquinone and demethylmenaquinone (Lee *et al.*, 1997; Stevenson *et al.*, 1998; Wissenbach *et al.*, 1992). In EHEC, both the *ubiE* mutant and the *menA* mutant impaired the enhancement of EspB secretion in response to TMAO and nitrate (Fig. 2b). These results strongly suggest that activation of the respiratory system is necessary for activation of TTS in EHEC grown under anaerobic conditions.

A respiration system specific for TMAO or nitrate is necessary for enhanced TTS

At the final stage of respiration, electrons are transferred from quinones to the terminal electron acceptor by using an electron-acceptor-specific reductase. TMAO reductase, TorA, is encoded by the *torCAD* operon, and its expression is activated by the TorSTR signalling system (Ansaldi *et al.*, 1999; Mejean *et al.*, 1994; Pommier *et al.*, 1998). *E. coli* also possesses three distinct nitrate reductases. The *narGHJI* operon encodes the major respiratory nitrate reductase located in the cytoplasmic membrane, and its expression is activated by the NarXL signalling system (Darwin *et al.*, 1996; Walker & DeMoss, 1994). The *narVWYZ* operon encodes a second nitrate reductase, which is biochemically similar to the NarGHJI enzyme, but is constitutively produced at relatively low levels in the cell (Blasco *et al.*, 1990). A third nitrate reductase, encoded by the *napFDAGHBC* operon, is located in the periplasm, and its production is activated by the NarPQ signalling system, but not by NarXL (Darwin & Stewart, 1995; Darwin *et al.*, 1998; Stewart, 2003). To explore the role of electron acceptors in activation of TTS, we first examined a series of mutants that were deficient in either the TMAO- or the nitrate-specific signalling system, as this is necessary for activation of the corresponding expression of the gene encoding the reductase. The TTS response to TMAO or nitrate was abolished by the defect in the signaling system, i.e. the *torSTR* and *narXL* deletion mutations, respectively, without affecting the level of EspB expression in the cell (Fig. 3a). On the other hand, the response of TTS to nitrate was unaffected by deletion of the NarP–NarQ system. Deletion of the nitrate-specific signalling systems NarX–NarL and NarP–NarQ did not affect the response to TMAO, and vice versa (Fig. 3a). Next, we examined the response in a series of mutants that were deficient in either TMAO- or nitrate-specific reductase. The TTS response to TMAO was abolished completely by the *torCAD* mutation, but was not affected by the *narGHJI* mutation. In contrast, the nitrate-enhanced secretion was greatly decreased by the *narGHJI* mutation, but was not affected by the *torCAD* mutation (Fig. 3b). The NarVWYZ

Fig. 2. Roles of respiratory systems in the activation of TTS by electron acceptors. (a) Effect of a respiratory inhibitor on the activation of TTS. Bacteria were grown in LB under standing conditions at 37 °C for 3 h, without an electron acceptor, and then exposed to amytal (5 mM) for 30 min at 37 °C prior to incubation with an electron acceptor for 1 h. EspB protein in the culture supernatant and whole-cell extract was detected by immunoblotting using specific antibody. (b) Enhancement of TTS in mutants deficient in quinone synthesis. Bacteria were grown in LB under standing conditions at 37 °C, both with and without an electron acceptor. Abbreviations, see legend to Fig. 1.

Fig. 3. Role of the TMAO- and nitrate-specific respiration systems in the activation of TTS by an electron acceptor. (a) Role of TMAO- and nitrate-specific signal transduction systems. Bacteria were grown in LB under standing conditions at 37 °C for 3 h, with and without an electron acceptor (T, TMAO; N, nitrate). EspB in culture supernatant (Sup) and whole-cell extract (Whole) was detected by immunoblotting. $\Delta torSTR$, mutant lacking the TMAO-specific signalling system; $\Delta narQ$, mutant lacking the nitrate-specific sensor kinase; $\Delta narP$, mutant lacking the nitrate-specific response regulator; $\Delta narXL$, mutant lacking the nitrate-specific signalling system. (b) Role of the electron acceptor-specific reductase. $\Delta torCAD$, mutant lacking TMAO reductase; $\Delta narGHJI$, mutant lacking NarGHJI nitrate reductase; $\Delta narVWYZ$, mutant lacking NarVWYZ nitrate reductase.

enzyme, which is constitutively produced at low levels, had no effect on the nitrate-enhanced TTS (Fig. 3b). These results clearly indicate that the activation of a respiratory system specific for either TMAO or nitrate is essential for the enhancement of TTS.

Increase in ATP concentration by electron acceptors is not correlated with the level of TTS

The TTSS needs ATP as a driving force to secrete virulence factors (Eichelberg *et al.*, 1994; Hueck, 1998; Woestyn *et al.*, 1994). Since the activation of respiration could increase the yield of ATP, one explanation for the enhancement of TTS by activating the respiratory system is that the increased ATP concentration stimulates TTS. To examine the correlation between ATP and the level of TTS, we measured the ATP concentration in bacteria grown both with and without an electron acceptor. Although the ATP concentration was correlated with the growth rate in the late exponential to early stationary phase, no correlation with the amount of secreted protein was observed (Figs 4 and 1b). All four electron acceptors used in this study enhanced ATP production, while DMSO and fumarate enhanced TTS at a much lower level than TMAO and nitrate. We also increased

the ATP concentration in EHEC by using the *purK* mutant, and growing the bacteria in medium containing adenine. Although the concentration of ATP in the mutant grown with adenine was higher than in the WT grown with an electron acceptor (Fig. 4a), the secretion of EspB by the mutant remained very low, and was similar to that of the WT EHEC grown without an electron acceptor (Fig. 4b).

Electron acceptors activate an existing TTS apparatus

To determine whether the enhanced TTS required *de novo* protein synthesis, we examined the response after inhibiting protein synthesis. Bacteria were grown in LB without an electron acceptor for 3 h, and then a translation inhibitor, chloramphenicol, was added for 30 min prior to the addition of the electron acceptor. Albeit reduced in the level of secretion, bacteria treated with chloramphenicol showed the TTS response to TMAO and nitrate (Fig. 5). Moreover, the inhibition of respiratory activity by amytal completely abolished the response to electron acceptors (Fig. 5). These

Fig. 4. Changes in the cellular concentration of ATP, and TTS activity. WT EHEC was grown in LB under standing condition at 37 °C for 3 h, both with and without an electron acceptor (T, TMAO; D, DMSO; N, nitrate; F, fumarate). The *purK* mutant ($\Delta purK$) was grown in Dulbecco's modified Eagle's medium (DMEM) under standing condition at 37 °C for 3 h, with adenine (Ade) and guanine (Gua). (a) Cellular ATP levels in WT EHEC grown both with and without an electron acceptor, and in the *purK* mutant grown with adenine and guanine. The relative ATP concentration of the bacterial lysate was measured using an ATP determination kit. Mean ($\pm$ SD) values were obtained from three independent experiments. (b) Expression and secretion of EspB in WT EHEC grown with an electron acceptor, and in the *purK* mutant grown in DMEM with adenine and guanine. Whole, whole-cell extract; Sup, culture supernatant.

results support the idea that either TMAO or nitrate respiration is essential for the enhanced TTS described above, and they clearly indicate that the enhanced TTS could be achieved without *de novo* protein synthesis, including synthesis of the TTS apparatus. Thus, either TMAO or nitrate respiration activates an existing TTS apparatus.

TMAO and nitrate facilitate maturation of the TTS apparatus

The functional TTS apparatus of EHEC consists of a basal body with a needle structure attached to it by an EspA-sheath-like structure. The EspA-sheath-like structure protrudes from the bacterial surface, and forms a filament that serves as a bridge, tethering the bacterium to the host cell (Knutton *et al.*, 1998). To assess the effect of the electron acceptor on the formation of functional TTS apparatus, we observed the production of the EspA filament, and the adherence capacity of EHEC grown both with and without an electron acceptor. EspA filaments on the surface of EHEC were detected using immunofluorescence and an EspA-specific antibody (Fig. 6a). Compared with EHEC grown without an electron acceptor, EspA filaments on bacteria grown with TMAO and nitrate increased 5.4- and 3.5-fold, respectively. Because the adherence of EHEC to epithelial cells, and subsequent formation of microcolonies, depend mainly on TTSS activity, we next examined the adherence capacity of bacteria grown in LB with an electron acceptor (Fig. 6b). The adherence capacity of bacteria grown in LB with TMAO and nitrate increased 20.7- and 27.0-fold, respectively, over that of bacteria grown without an electron acceptor. The adherence capacity of the *escD* mutant was not increased by an electron acceptor, indicating that the increased adherence caused by TMAO and nitrate was dependent on the TTSS.

Since TTS was enhanced by the activation of a respiratory system without *de novo* protein synthesis, it seemed likely that the TTS apparatus changed from an inactive form to an

Fig. 5. Enhancement of TTS in EHEC, without translation activity. Bacteria were grown in LB under standing conditions at 37 °C for 3 h, without an electron acceptor, and then exposed to chloramphenicol (Cm; 100 µg ml⁻¹), or chloramphenicol (100 µg ml⁻¹) and amylal (5 mM), for 30 min at 37 °C. The electron acceptor was added (T, TMAO; N, nitrate), and the bacteria were incubated for a further 1 h at 37 °C. EspB in the whole-cell extract (Whole) or in the culture supernatant (Sup) was detected by immunoblotting.

Fig. 6. Enhancement of EspA filament formation and adherence capacity in EHEC grown with TMAO and nitrate. (a) Production of EspA filament in EHEC grown under anaerobic conditions. Bacteria were grown in LB under standing conditions at 37 °C for 3 h, both with and without an electron acceptor (T, TMAO; N, nitrate). Bacteria were fixed, and EspA was detected by immunofluorescent staining using anti-EspA antibody. The fluorescence was observed under a confocal laser scanning microscope. Bacteria were visualized by Nomarski DIC. (b) Adherence of bacteria to Caco-2 cells. Bacteria were grown in LB under standing condition at 37 °C for 3 h, both with and without an electron acceptor, and then allowed to infect Caco-2 cells for 3.5 h, which includes 1.5 h in fresh medium after the removal of non-infecting bacteria. Cells were fixed, and stained with Giemsa staining solution. The adherence efficiency was determined by counting the number of bacteria adhering to the Caco-2 cells. Means ($\pm$ SD) were obtained from three independent experiments. $\Delta escD$, mutant lacking functional TTSS.

active form in response to the activation of either TMAO or nitrate respiration. Recently, it was reported that there are two forms of TTS apparatus: the needle complex and the base complex in *Salmonella typhimurium* (Marlovits *et al.*, 2004). In contrast to the fully assembled needle complex, the base complex, which is thought to be the preassembled complex, lacks the needle substructure. As suggested by the differences in the appearance of EspA filament on the bacterial surface (Fig. 6a), the TTS apparatus that formed in EHEC grown without an electron acceptor could be the base complex lacking the needle substructure. To investigate the presence of the needle component in the TTS apparatus, we partially purified the TTS apparatus by preparing fractions containing macromolecules from the bacterial membrane.

We first tried to isolate the TTS apparatus in WT EHEC, but failed to detect any of the component proteins by using antibodies. We then increased the expression of the TTS apparatus by introducing multiple copies of the *grlA* gene, which encodes a positive transcriptional regulator for LEE genes (Deng *et al.*, 2004). Although overexpression of the *grlA* gene increased the amount of translocators and effectors, growth with either TMAO or nitrate further stimulated the secretion markedly (Fig. 7a). The amount of EscC, the outer-ring protein of the basal body of the TTS apparatus (Gauthier *et al.*, 2003), was also increased to a detectable level, and the amount of EscC was not altered by the presence of an electron acceptor (data not shown). The fraction containing the needle complex was prepared from EHEC overexpressing *grlA*, grown both with and without an electron acceptor, and it was analysed for the components of TTS apparatus, i.e. EscC and EscF. EscF is a needle structural protein (Sekiya *et al.*, 2001). EscC was detected at the same level in bacteria irrespective of the growth conditions, while the amount of EscF protein was much greater in preparations from bacteria grown with either TMAO or nitrate, than from those grown without an electron acceptor (Fig. 7b). These results strongly suggest that, under anaerobic growth conditions, formation of the fully assembled needle complex is stimulated by the activation of specific

respiratory systems, such as the TMAO and nitrate respiratory systems.

DISCUSSION

We demonstrated that the release of type III secreted proteins into the culture supernatant of EHEC grown under anaerobic conditions was much lower than that of EHEC cultured under aerobic conditions. The stimulation of anaerobic growth by adding a specific electron acceptor simultaneously stimulated the secretion of translocators and effectors, represented by EspB and Tir, respectively. The effect of the electron acceptor on EHEC growth appeared to be restricted to the transition phase of growth; the growth rate of EHEC grown with and without an electron acceptor was the same in the exponential phase, while the addition of an electron acceptor to the medium increased the growth rate in the late exponential to early stationary phase. Indeed, the growth rate in the exponential phase for a mutant lacking the gene encoding either TMAO or nitrate reductase was not altered from that of the WT, even in medium containing either TMAO or nitrate, in contrast to the loss of growth stimulation in these mutants at the transition stage (H. Ando & T. Tobe, unpublished results). The enhancement of TTS at the transition phase was dependent on the activity of the respiratory system specific for the available electron acceptor, given that a mutant lacking either TMAO or nitrate reductase was not able to enhance the secretion of EspB by either TMAO or nitrate, respectively. Furthermore, the inhibition of respiratory activity by amytal abolished the effect of TMAO and nitrate to enhance TTS. The expression of LEE genes, and the release of type III secreted proteins, are optimal in the late exponential phase. Therefore, the activity of the respiratory systems in the transition stage of growth may be critical for the production of functional TTS apparatus.

Although EHEC utilizes all four of the terminal electron acceptors we examined, the effect on the stimulation of TTS was different for each acceptor. Even though DMSO and fumarate could stimulate EHEC growth as much as TMAO and nitrate, the amount of secreted proteins elicited by either DMSO or fumarate was much lower than that elicited by either TMAO or nitrate. These results indicate that stimulation of growth by activating anaerobic respiration is not the reason for TTS activation. Indeed, the increase in ATP levels was not correlated with TTS activity, and the electron acceptor could activate TTS after blocking protein synthesis. It is likely that activation of respiratory systems affects the function of TTSS-associated proteins independently of growth stimulation. We found that EHEC grown without an electron acceptor produced TTS apparatus lacking the EspA filament and EscF needle components at a higher frequency than EHEC grown with either TMAO or nitrate. Since EscC was isolated with TTS apparatus that was partially purified from the membrane of EHEC grown without an electron acceptor, it is unlikely that the translocation of TTSS basal body components to the bacterial

Fig. 7. Maturation of TTS apparatus. (a) Enhancement of TTS by an electron acceptor in EHEC overexpressing *grlA*. Bacteria were grown in LB under standing conditions at 37 °C for 4 h, both with and without an electron acceptor (T, TMAO; N, nitrate). EspB in the culture supernatant was detected by Coomassie brilliant blue staining. (b) Amount of EscC and EscF in the TTS apparatus isolated from the bacterial membrane. EHEC overexpressing *grlA* was grown under standing conditions at 37 °C for 4 h, both with and without an electron acceptor. The TTS apparatus was isolated by adapting a method described by Kubori *et al.* (1998). The relative amounts of EscC and EscF were measured by immunoblotting with anti-EscC and anti-EscF antibodies, respectively. The relative amount of EscF in the TTS apparatus was determined by normalization to the amount of EscC, and it is represented as the fold increase over the amount of the EscC–EscF complex in EHEC grown without an electron acceptor. Δ escD, mutant lacking functional TTSS.

membrane was impaired when EHEC was grown without an electron acceptor. In *S. typhimurium*, two forms of TTS apparatus have been found: one is a mature complex with a needle structure, and the other is thought to be a premature form of the complex, lacking the needle structure (Marlovits *et al.*, 2004). Furthermore, assembly of the basal body, which contains outer rings and inner rings, is dependent on Sec machinery, while the next step to form the needle extension is dependent on the components of TTS apparatus (He *et al.*, 2004). Therefore, it is plausible that stimulation of the respiratory system by an electron acceptor enhances the maturation step of TTS apparatus formation, which is dependent on the components of TTS apparatus.

The analysis with mutations in genes encoding quinone synthesis indicates that quinones are necessary for the enhancement of TTS by TMAO and nitrate. Quinones have been shown to modulate the activity of membrane proteins. The oxidized forms of quinones inhibit the kinase activity of ArcB during aerobic growth through the oxidation of two cytosolic redox-active cysteine residues (Georgellis *et al.*, 2001; Malpica *et al.*, 2004). We think that it is likely that quinones play an important role in the formation of the mature TTS apparatus in EHEC by affecting the activity of a component. We propose that oxidized quinones promote conformational changes in a component of TTS apparatus that induces the recruitment of the needle structure to form the mature TTS apparatus. DsbA and DsbB, whose oxidation is dependent on quinones, are known to be required for TTSS activity in *Shigella flexneri*, *Salmonella typhimurium* and *Yersinia pestis* (Watarai *et al.*, 1995; Miki *et al.*, 2004; Jackson & Plano, 1999). In *Y. pestis* and *S. typhimurium*, DsbA is necessary for disulfide bond formation in YscC and SscC, respectively, which are outer-ring components of the TTS basal body. Since translocation of EscC, a homologue of YscC and SscC, into the macromolecule was observed in EHEC grown without an electron acceptor, it is unlikely that TTS apparatus formation is activated through the activity of DsbA and DsbB. Furthermore, amounts of the oxidized forms of DsbA and DsbB in EHEC were not changed by growth with either TMAO or nitrate, as compared with those with either DMSO or fumarate (H. Ando & T. Tobe, unpublished results), suggesting that the maturation of TTS apparatus is achieved through a mechanism independent of DsbA and DsbB activity. Since a respiratory system is essential for bacterial growth, it is difficult to distinguish the effect of a deficiency in a respiratory system on TTS function from its effect on protein synthesis or the expression of a functional TTS apparatus *in vivo*. The identification of a regulatory component that promotes maturation of TTS apparatus would be helpful for the further elucidation of these mechanisms.

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