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# Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7

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## Summary

**In general, wild *Escherichia coli* strains can grow effectively under moderately acidic organic acid-rich conditions. We found that the Shiga Toxin-producing *E. coli* (STEC) O157:H7 NGY9 grows more quickly than a K-12 strain in Luria–Bertani (LB)-2-morpholinoethanesulphonic acid (MES) broth supplemented with acetic acid (pH 5.4). Hypothesizing that the resistance of STEC O157:H7 to acetic acid is as a result of a mechanism(s) other than those known, we screened for STEC mutants sensitive to acetic acid. NGY9 was subjected to mini-Tn*5* mutagenesis and, from 50 000 colonies, five mutants that showed a clear acetic acid-sensitive phenotype were isolated. The insertion of mini-Tn*5* in three mutants occurred at the *fcl*, *wecA* (*rfe*) and *wecB* (*rffE*) genes and caused loss of surface O-polysaccharide, loss of both O-polysaccharide and enterobacterial common antigen (ECA) and loss of ECA respectively. The other two mutants showed inactivation of the *waaG* (*rfaG*) gene but at different positions that caused a deep rough mutant with loss of the outer core oligosaccharide of lipopolysaccharide (LPS) as well as phenotypic loss of O-polysaccharide and ECA. With the introduction of plasmids carrying the *fcl*, *wecA*, *wecB* and *waaG* genes, respectively, all mutants were complemented in their production of O-polysaccharide and ECA, and normal growth was restored in organic acid-rich culture conditions. We also found that the growth of *Salmonella* LPS mutants Ra, Rb1, Rc, Rd1, Rd2 and Re was suppressed in the presence of acetic acid compared with that of the parents. These results suggest that the full expression of LPS (including O-polysaccharide) and ECA is indispensable to the resistance against acetic acid and other short chain fatty acids in STEC O157:H7 and *Salmonella*. To the best of our knowledge, this is a newly identified physiological role for O-polysaccharide and ECA as well as an acid resistance mechanism.**

## Introduction

An important property of food-borne pathogens such as the Shiga Toxin-producing *Escherichia coli* (STEC) O157:H7 is the ability to survive in the extremely acidic environment of the stomach (pH 2.4), as well as moderately acidic environments containing various organic acids produced as a result of fermentation in the lower digestive tract. The low infectious dose associated with STEC O157:H7 is attributed to its acid-resistant nature, as many STEC strains are capable of surviving at a pH of lower than 2.5 for more than 2 h (Arnold and Kaspar, 1995; Benjamin and Datta, 1995; Meng and Doyle, 1998). This acid resistance capacity is also necessary for enteric bacteria to colonize and establish a commensal relationship with the mammalian hosts in such environments (Castanie-Cornet *et al*., 1999).

In general, acid stress is defined as the combined biological effect of low pH and organic acids present in the environment (Bearson *et al*., 1997). Although the lower intestinal tract is less acidic than the stomach, the presence of organic acids would enhance acid stress to a level potentially lethal to Enterobacteriaceae such as *E. coli* and the *Salmonella enterica* serovar Typhimurium (Salmond *et al*., 1984; Lin *et al*., 1996). These Gram-negative bacteria have developed various devices to protect themselves from acid stress.

Although the terminology for the acid survival mechanisms of bacteria is rather confusing because of differences in assay methods, three systems have been defined for acid survival response of *E. coli*, *Shigella* and *Salmonella*: acid tolerance response (ATR), acid resistance (AR) and acid habituation (AH).

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ATR protects cells at pH 3 for several hours and was first described in *Salmonella*. This protection against extremely acidic pH needs before exposure to a moderately acidic pH (adaptive pH). *Escherichia coli* also possesses both exponential phase and stationary phase ATR systems. It is thought that the mechanisms of *E. coli* ATR would be similar to those described for *Salmonella* (Foster, 1999; 2000). Acid-adapted *E. coli* cells maintain an internal pH ($pH_i$) that is close to neutral in extremely acidic environments. The involvement of proton flux in ATR, whereby adapted cells do not allow protons to flow in as easily as unadapted cells do, was suggested.

Exponential phase ATR in *E. coli* is known as AH. It is tested through exposure of bacteria in nutrient broth, at pH 3–3.5, and measuring survival of cells after 7 min. Many substances have been found to affect AH. These include glucose, glutamate, aspartate, $FeCl_3$, KCl, and L-proline, which can induce habituation at a neutral external pH ($pH_o$). On the other hand, phosphate and cAMP reduce the induction of AH (Rowbury and Goodson, 1998). The control of AH is dependent on CysB (Rowbury and Goodson, 1997).

The major difference between these closely related species in acid resistance is found in stationary phase cells in which *E. coli* becomes markedly more resistant than *Salmonella* when challenged by low pH in complex media. This resistance, known as AR, protects cells at pH 2 for several hours. It is usually tested through exposure of stationary phase cells to pH 2–2.5 in minimal media and measuring the survival of cells after 2–4 h. Three distinct, efficient and low pH-inducible stationary phase resistance systems in AR were identified in the Enterobacteriaceae. These include the $\sigma^s$ and cAMP receptor protein-dependent oxidative system, the glutamate-dependent system, and an acid-induced arginine-dependent system (Lin *et al*., 1995). Stationary phase sigma factor $\sigma^s$, which is encoded by *rpoS*, is now known to play a major role when bacteria are exposed to a variety of environmental stresses, including acid stress (Price *et al*., 2000).

Short-chain fatty acids (SCFAs) have antibacterial activities and have been used for many years as food and drink preservatives. However, very little information has been available concerning the resistance of bacteria to these organic acids. The question of whether organic acid (e.g. acetate) stress is different from inorganic acid (e.g. HCl) stress, as a result of the fact that organic acids not only acidify the $pH_i$ of the cell but also accumulate as intracellular anions, remains. The protonated form of these organic acids permeates the cytoplasmic membrane and deprotonates in the cytoplasm based on the $pH_i$ and $pK_a$ of the acid (Freese *et al*., 1973; Salmond *et al*., 1984; Cherrington *et al*., 1991). Bearson *et al.* (1998) recently indicated that there are distinct tolerance systems for each type of acid stress in *S. typhimurium*. The RpoS-dependent system is clearly required for protection against organic acid stress whereas PhoPQ plays a major role in the protection against inorganic acid stress (Baik *et al*., 1996; Bearson *et al*., 1998; Kwon and Ricke, 1998; Foster and Moreno, 1999). In addition, benzoic acid-induced proteins have also been identified in *E. coli*, although the function of these proteins in the protection against organic acid stress has not been fully elucidated (Lambert *et al*., 1997).

Although most of the acid resistance mechanisms described above were identified in laboratory *E. coli* and *Salmonella* strains, clinically isolated bacteria tend to be even more resistant to acid stress. In this study, we tried to screen for other mechanisms of resistance against organic acids in wild *E. coli*. Out of 50 000 Tn*5*-inserted mutants of STEC O157:H7, we isolated five mutants that became sensitive to acetic acid. These mutants had a defect in the expression of surface polysaccharides. Therefore, intact surface polysaccharides play a role in the natural resistance against organic acids. This is the first study to show the involvement of surface polysaccharides in the acid resistance of wild, Gram-negative bacteria.

## Results

### *Isolation of mutants susceptible to acetic acid and other short chain fatty acids (SCFA)*

As Fig. 1 shows, the wild *E. coli* strain STEC O157:H7

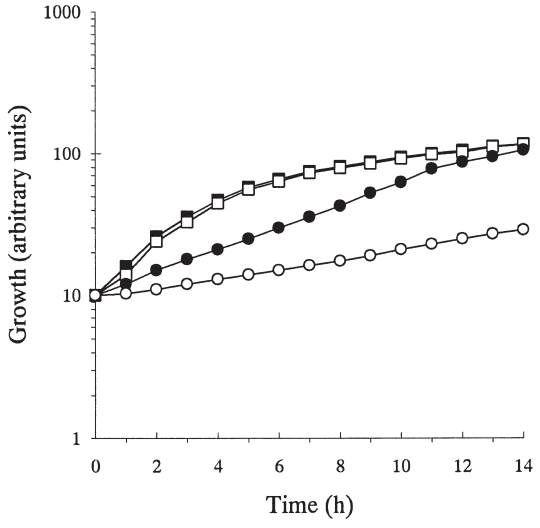


**Fig. 1.** Growth of NGY9 and MC4100 in the presence of 12 mM acetic acid. Cells were initially grown overnight and diluted 1:100 in LB-MES (50 mM of 2-morpholinoethanesulphonic acid) medium, pH 5.4, without NGY9 (■) and MC4100 (□), or with NGY9 (●) and MC4100 (○), containing 12 mM acetic acid. Growth [optical density at 600 nm ($OD_{600}$)] was then determined every hour at 37°C. One optical density unit at 600 nm corresponds to 100 arbitrary units. Values are representatives of two to three experiments.

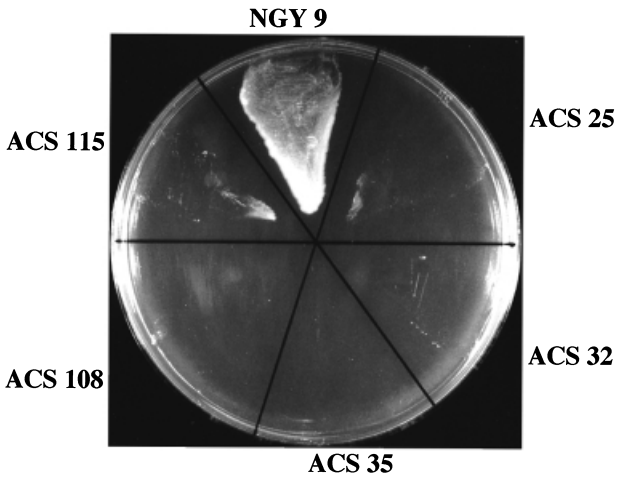


**Fig. 2.** Phenotype of acetic acid-sensitive (ACS) mutants. NGY9 and its ACS mutants were grown on an LB-MES plate (pH 5.4) containing 12 mM acetic acid. Mini-Tn*5* insertion mutants of NGY9 were negatively screened on the same medium.

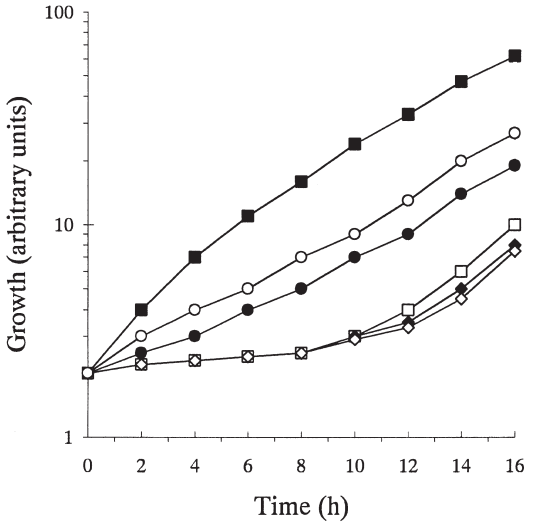


**Fig. 3.** Growth kinetics of NGY9 and ACS mutants in the broth culture supplemented with acetic acid. NGY9 (■), ACS25 (□), ACS32 (◆), ACS35 (◇), ACS108 (●) and ACS115 (○) were initially grown overnight and diluted 1:100 in LB-MES medium (pH 5.4) containing 12 mM acetic acid. Growth (optical density at $OD_{600}$) was then determined every hour at 37°C. One optical density unit of 600 nm corresponds to 100 arbitrary units. Values are representatives of two to three experiments.

NGY9 grows much faster than the laboratory K-12 strain MC4100 in the presence of 12 mM acetic acid (pH 5.4) in a rich medium. This growth suppression was not as a result of the low pH of the medium, as both strains grew similarly in LB-MES medium (pH 5.4). However, the survival rate of NGY9 in an extremely acidic environment (pH 1.9, adjusted with HCl at final concentration of 225 mM) was not higher than that of MC4100 and, moreover, an *rpoS*-deficient mutant of NGY9 showed no growth retardation in the presence of 12 mM acetic acid (pH 5.4) (unpublished observations), suggesting that the organic acid resistance of NGY9 was different from previously described AR systems.

To identify specific genes involved in the resistance of NGY9 to acetic acid at moderately low pH, NGY9 was mutagenized with the transposon mini-Tn*5*(Tc1) and transposon-inserted chromosomal mutants were replica-plated on to LB-MES agar with 12 mM acetic acid (pH 5.4). Approximately 50 000 mini-Tn*5*(Tc1)-inserted colonies were screened, and, finally, five mutants, ACS25, 32, 35, 108 and 115 (acetic acid-sensitive mutant), which showed poor growth or no growth on LB-MES agar with 12 mM acetic acid, were isolated (Fig. 2). All of them grew well in minimal media (pH 7.2) at 30°C and 45°C (data not shown). Figure 3 shows the growth of these mutants as well as NGY9 in LB-MES broth supplemented with 12 mM acetic acid (pH 5.4). All ACS mutants showed slower growth than NGY9 under these conditions, although the rate of growth of each mutant was different. The lag phase of mutants ACS25, 32 and 35, was prolonged. However, the doubling times of these mutants were probably similar to those of the parent strain after the prolonged lag phase. On the other hand, the doubling times of ACS108 and 115 were longer than those of the parent strain, and no prolongation of the lag phase was observed (Fig. 3). It is noteworthy that the growth of ACS32 and 35 was severely impaired on the solid medium and no growth was observed after 3 days' incubation at 37°C. However, mutants ACS115, 108 and 25 eventually grew on the solid medium after three overnights' culture (data not shown). All these ACS mutants were also susceptible to other SCFAs such as propionic acid and butyric acid in the same assay (data not shown).

### *Identification of genes inactivated by mini-Tn5(Tc1) integration in ACS mutants*

We determined the positions at which mini-Tn*5*(Tc1) was integrated into the chromosomes of ACS mutants. Chromosomal DNA from the mutants was isolated, partially digested with the restriction endonuclease *Sau*3AI and ligated into the *Bam*HI site of cloning plasmid pSTV29. Tc-resistant clones were then isolated. These plasmids contained the DNA fragments of mini-Tn*5*(Tc1) and its flanking chromosomal region. Sequencing of these DNA inserts was performed from Tn*10 tetA* downstream of mini-Tn*5*(Tc1). The sequences were compared with the DNA database of *E. coli* K-12 and other DNA via BLAST through the GenomeNet WWW server (http://www.genome. ad.jp/). Strains ACS32 and 35 were found to contain a single mini-Tn*5*(Tc1) insertion in the same gene showing a high degree of homology with the

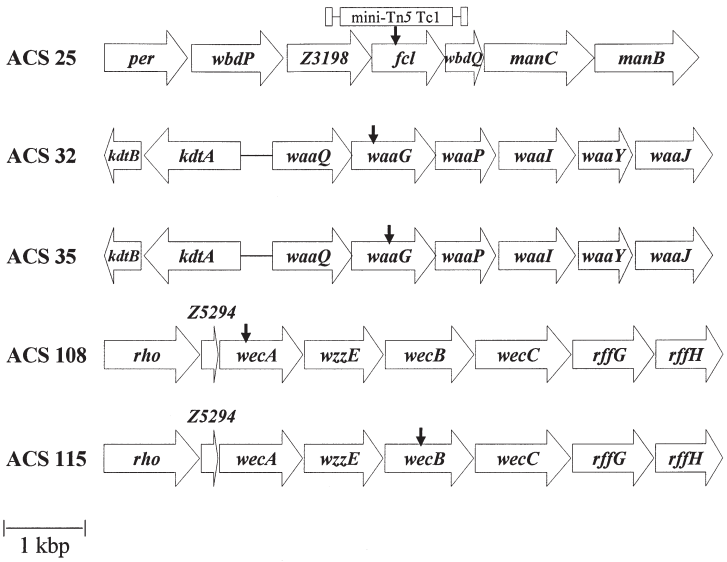


**Fig. 4.** Insertion points of mini-Tn*5*(Tc1) in the genome of the ACS mutants. Whole genomic DNA of ACS mutants was partially digested with *Sau*3AI restriction enzyme and cloned into pSTV29. Plasmids that showed the Tc-resistant phenotype were sequenced with a known primer from the *tetA* downstream region of mini-Tn*5*. For details, see *Experimental procedures*.

*E. coli* K-12 *waa*G (formerly *rfa*G: Reeves *et al*., 1996) gene, a component of the *waa* locus. This gene encodes the enzyme UDP-glucose: lipopolysaccharide (LPS) $\alpha$1,3-glucosyltransferase that adds glucose I to heptose II of the core oligosaccharide (OS) of LPS (Schnaitman and Klena, 1993; Raetz, 1996; Heinrichs *et al*., 1998). Similarly, the gene disrupted in ACS25 by mini-Tn*5*(Tc1) was *fcl* of the O157 O-polysaccharide gene cluster encoding an enzyme of the GDP-L-fucose pathway that is involved in the biosynthesis of O-polysaccharide (Andrianopoulos *et al*., 1998; Wang and Reeves, 1998). We further identified the insertion points of ACS108 and 115 as *wecA* (formerly *rfe*) and *wecB* (formerly *rffE*) respectively. In the *wec* gene cluster, *wecA* encodes a key enzyme UDP-GlcNAc:undecaprenylphosphate GlcNAc-1-phosphate transferase for the biosynthesis of both enterobacterial common antigen (ECA) and O-polysaccharide of Gram-negative bacteria (Ohta *et al*., 1991; Schnaitman and Klena, 1993; Rick and Silver, 1996). The *wecB* gene encodes UDP-GlcNAc-2-epimerase and is involved in the biosynthesis of UDP-ManNAcA of the ECA synthetic pathway (Schnaitman and Klena, 1993; Rick and Silver, 1996). All of the genes inactivated in ACS mutants are summarized in Fig. 4. The expected phenotypic expression of the surface polysaccharides in the *waaG*-deficient mutants ACS32 and ACS35, the *wecA* mutant ACS108, the *wecB* mutant ACS115, and the *fcl* mutant ACS25 are shown in Fig. 5. ACS115 lost ECA and ACS25 lost O-polysaccharide, whereas ACS108 lost both ECA and O-polysaccharide. ACS32 and ACS35 lost the outer core OS of LPS and so the expression of both O-polysaccharide and most of the ECA molecules that are bound to the non-reducing terminus of the outer core OS would be lost in these mutants. The phenotypic characteristics of the

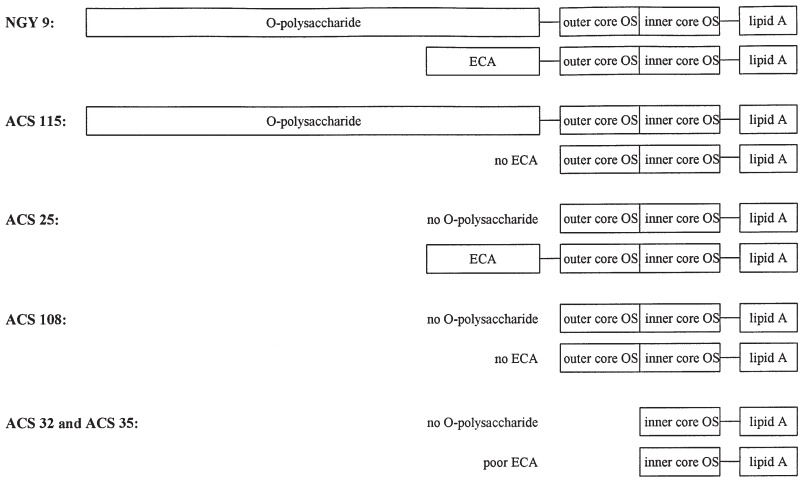


**Fig. 5.** LPS and ECA structure of ACS mutants deduced from their mutations. LPS, lipopolysaccharide; ECA, enterobacterial common antigen; OS, oligosaccharide.

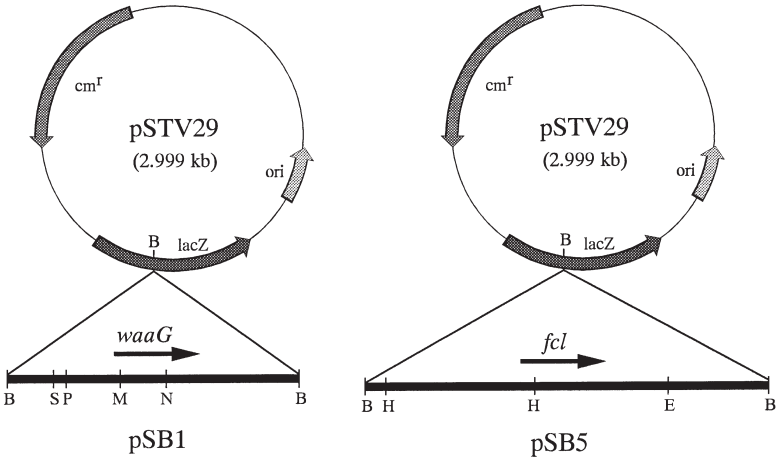


**Fig. 6.** Plasmids constructed for complementation study. The *waaG* gene and the *fcl* gene were inserted at the *Bam*HI site downstream of the *lac* promoter of pSTV29 to form pSB1 and pSB5 respectively. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Nsp*V; P, *Pst*I; and S, *Sal*I.

surface polysaccharides of these ACS mutants, detected by immunoblotting, are described in the next section.

### *Complementation of the resistance against acetic acid and the surface polysaccharide expression of ACS mutants*

To confirm the involvement of the surface polysaccharides in the resistance of NGY9 to acetic acid, a complementation study of the ACS mutants was performed. The newly constructed plasmids containing relevant genes for the complementation were pSB1 for the *waaG* gene and pSB5 for the *fcl* gene (Fig. 6). For the complementation of both ACS108 and ACS115, pHSF131 (Ohta *et al.*, 1991) was used. This low-copy plasmid carries the entire *wec* gene cluster and, thereby, complements both *wecA* and *wecB*. The transcription of *waaG* on pSB1 and *fcl* on pSB5 was under the control of the *lac* promoter of the vector. We found that low-level transcription, without an inducer of the *lac* promoter, was sufficient for the functional expression of *waaG* and *fcl* in these plasmids. As Fig. 7 shows, the growth of ACS32 and 35 was complemented by the introduction of pSB1, and the growth of ACS25 was complemented by pSB5 on LB-MES agar supplemented with 12 mM acetic acid (pH 5.4). Similarly, the growth of ACS108 and ACS115, under the same culture conditions, was restored by pHSF131. The loss of surface O157 LPS and its complementation in these ACS

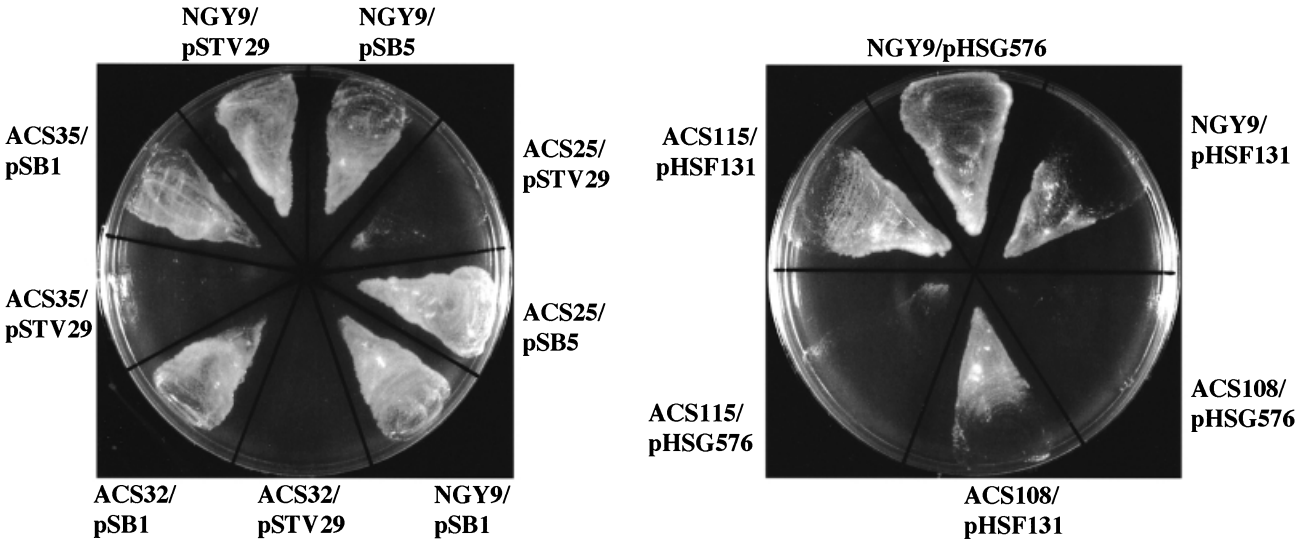


**Fig. 7.** Complementation of the growth of ACS mutants. Bacteria were grown on LB-MES plates (pH 5.4) containing 12 mM acetic acid. The acid-resistant phenotype of all mutants was restored by the introduction of the relevant genes. Plasmid pSB1(*waaG*) was introduced into ACS32 and ACS35, and pSB5(*fcl*) into ACS25. Plasmid pHSF131(*wecA* and *wecB*) was introduced into ACS108 and ACS115. Plasmids pSTV29 and pHSG576 were vectors.

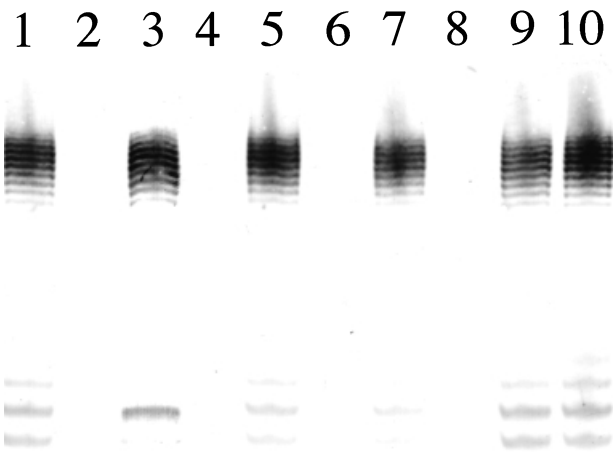


**Fig. 8.** Expression of O157 polysaccharide and its complementation in ACS mutants. Immunoblot analysis of LPS preparations of NGY9 and its ACS mutants was performed with rabbit anti-O157 serum. LPS preparations were separated on SDS-polyacrylamide gel, transferred on to nitrocellulose membranes and detected with anti-O157 serum. Lanes contain LPS preparations from NGY9 (lane 1), ACS25 (lane 2), ACS25/pSB5 (lane 3), ACS32 (lane 4), ACS32/pSB1 (lane 5), ACS35 (lane 6), ACS35/pSB1 (lane 7), ACS108 (lane 8), ACS108/pHSF131 (lane 9) and ACS115 (lane 10).

mutants is shown in Fig. 8. Electrophoresis of LPS preparations followed by immunoblotting with anti-O157 serum revealed that ACS25, ACS32, ACS35 and ACS108 completely lost the expression of O157 polysaccharide of LPS. This defect in ACS32 and ACS35 was complemented by pSB1, whereas that of ACS25 was complemented by pSB5. We initially supposed that Tn*5*-insertion would abolish the genes downstream *fcl* or *waaG* of the mutants by the polar effect. Therefore, we tried to introduce longer DNA fragments, which contained entire downstream genes from the insertion positions. However, as our results show, shorter DNA fragments, which contain only the *fcl* or the *waaG* gene, are enough for the complementation of the synthesis of the full architecture of LPS. These results suggest to us the presence of additional promoters in the downstream region of the Tn*5*-insertion sites in these mutants. A *wecA* mutant ACS108 lost the expression of O157 polysaccharide, which was restored by pHSF131, suggesting that the biosynthesis of this O polysaccharide is dependent on *wecA*. These results indicate that the expression of O157 polysaccharide is indispensable for resistance to acetic acid.

Figure 9 shows that mutants ACS32, ACS35, ACS108 and ACS115 lost the expression of ECA associated with core OS of LPS, although the phenotypic expression of ECA in ACS108 was weak. The expression of ECA of ACS32 and ACS35 was complemented by pSB1, as the complete core OS structure of LPS is necessary for the connection to ECA polysaccharide. As Fig. 9 shows, the expression of ECA in ACS108 and ACS115 was recovered by the introduction of pHSF131. As ACS115 was defective only in the expression of ECA and the introduction of pHSF131 recovered the expression of ECA and the growth on LB-MES agar supplemented with 12 mM acetic acid (pH 5.4), the acetic acid-sensitive character of this mutant is as a result of the loss of expression of ECA. In other words, cell surface ECA plays a role in the protection of bacteria against the action of acetic acid.

### *SCFA sensitivity of* Salmonella *LPS mutants*

A series of *Salmonella* LPS mutants have been well characterized and used for research (Nikaido, 1996). As described above, we showed the O157 polysaccharide to be involved in the resistance of STEC O157 to acetic acid and other SCFAs. To examine the protective effect of LPS in greater detail, *Salmonella* LPS mutants with polysaccharides of various lengths were tested for acetic acid-resistance. Figure 10 shows that the growth of LPS mutants of *Salmonella* [chemotypes Rd1(SL1032), Rd2(SL1181) and Re(SL1102)], which do not contain residues beyond heptose, were inhibited by acetic acid. Similar results were obtained with other SCFAs in these LPS mutants (data not shown). The growth of LPS mutants with chemotypes Ra(TV119), Rb1(TV148) and Rc(SF1195) was also slightly suppressed on LB-MES agar with 12 mM acetic acid (pH 5.4), compared with that of the wild-type *Salmonella enterica* serovar Typhimurium

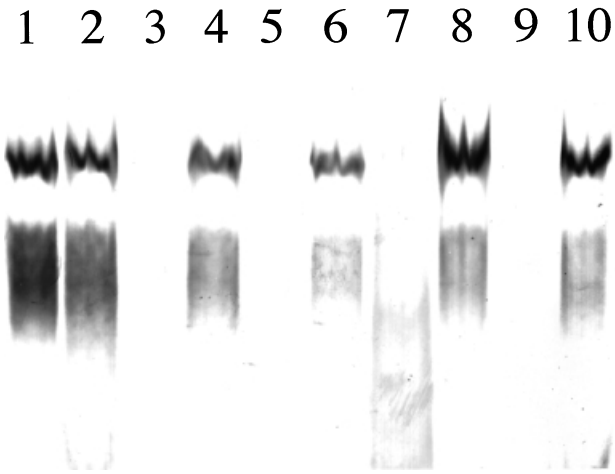


**Fig. 9.** Expression of ECA and its complementation in ACS mutants. Immunoblot analysis of ECA preparations (same as LPS preparations) of NGY9 and its ACS mutants was performed with rabbit anti-ECA serum. Electrophoresis and immunoblotting were performed as in Fig. 8. Lanes contain ECA preparations from NGY9 (lane 1), ACS25 (lane 2), ACS32 (lane 3), ACS32/pSB1 (lane 4), ACS35 (lane 5), ACS35/pSB1 (lane 6), ACS108 (lane 7), ACS108/pHSF131 (lane 8), ACS115 (lane 9) and ACS115/pHSF131 (lane 10).

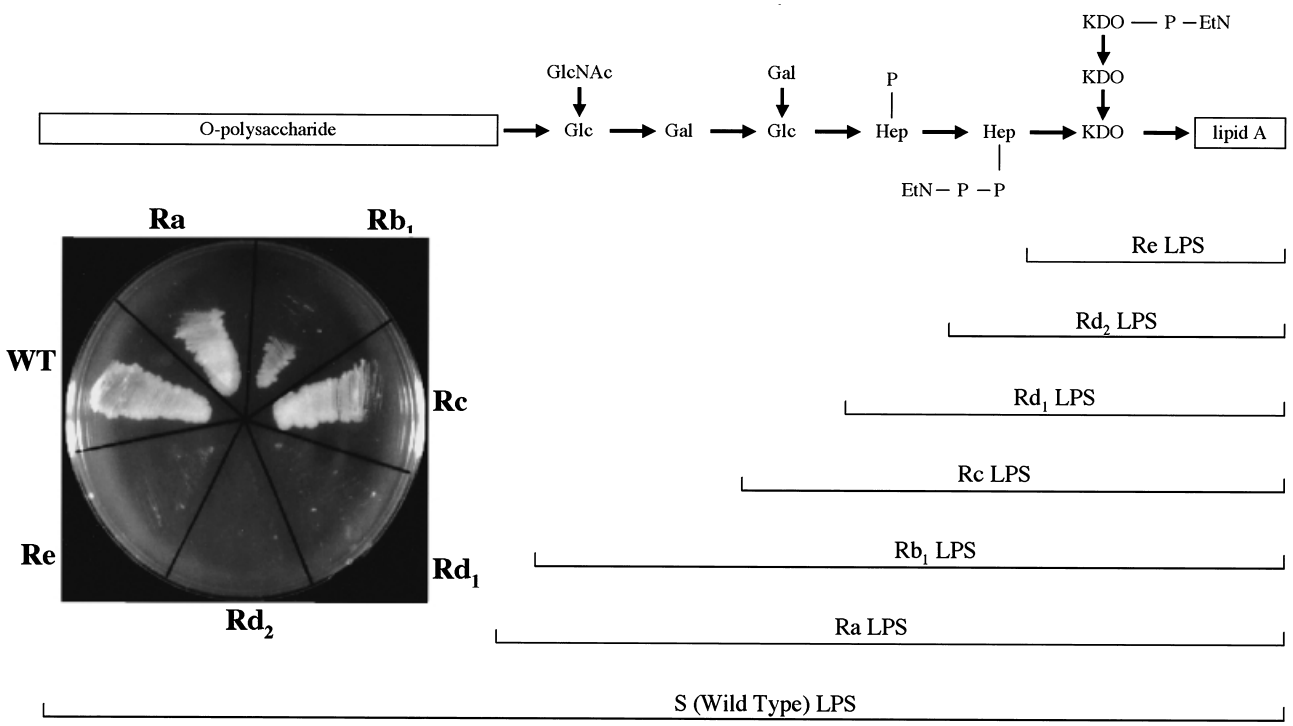


**Fig. 10.** Acetic acid-sensitive phenotype of *Salmonella typhimurium* LT2 and a series of LPS mutants. Bacteria were grown on an LB-MES plate (pH 5.4) containing 12 mM acetic acid. LPS chemotypes of the mutants are shown for convenience (Nikaido, 1996). WT, *Salmonella typhimurium* LT2; Ra, TV119; Rb1, TV148; Rc, SF1195; Rd1, SL1032; Rd2, SL1181; Re, SL1102. Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-galactose; Hep, L-glycero-D-manno-heptose; P, phosphate; EtN, ethanolamine; KDO, 2-keto-3-deoxy-octonic acid.

(Fig. 10). All these results indicate that LPS of sufficient length protects against the action of acetic acid and other SCFAs in some species of Enterobacteriaceae, and some types of O-polysaccharide might be more protective than others.

## Discussion

Wild bacteria are generally refractory to environmental stress compared with laboratory strains. STEC O157:H7 is tolerant to acidic environments (Conner and Kotrola, 1995; Doyle *et al*., 1997; Meng and Doyle, 1998). STEC O157:H7 can survive in fermented sausage (pH 4.5) for up to 2 months (Glass *et al*., 1992), in mayonnaise (pH 3.6–3.9) for several weeks (Zhao and Doyle, 1994) and in apple cider (pH 3.6–4.0) for up to one month (Zhao *et al*., 1993). We also found that STEC isolates grew well in media supplemented with acetic acid, in which laboratory K-12 strains could not grow, although the degree of resistance to acetic acid varied (Horii *et al*., 1998). The resistance of STEC O157:H7 to acetic acid (and other SCFAs) is likely to be different from mechanisms such as AR, ATR and AH, because even acetic acid-sensitive K-12 strains are equipped with the machinery for all these forms of resistance (Lin *et al*., 1995; Foster, 2000). Alternatively, wild STEC O157:H7 could express these mechanisms of resistance to acetic acid more efficiently than laboratory strains. We have found that an RpoS-deficient mutant of STEC grew at a similar rate to the parent strain in a medium supplemented with acetic acid and, therefore, RpoS was not involved in this resistance. Diez-Gonzalez and Russell (1997) recently mentioned that the acetate tolerance of STEC O157:H7 could be achieved by decreasing the intracellular pH and thereby decreasing ΔpH to prevent toxic accumulations of intracellular acetate anion, possibly because of the production of large amounts of D-lactate. Relevant genes for this function, however, have remained to be identified. Hypothesizing that the resistance to acetic acid of STEC O157:H7 is not owing to known mechanisms, we screened for STEC mutants sensitive to acetic acid. Through random transposon insertion mutagenesis and selection of acetic acid-sensitive mutants from 50 000 colonies with no other selection pressures, we isolated several mutants that showed poor or no growth on a medium supplemented with acetic acid. We found that they had mutations affecting not previously reported acid-resistance machineries, but surface O-polysaccharide and ECA expression. The introduction of relevant genes into these mutants restored both the expression of the polysaccharides and the resistance to acetic acid. We thus insist that the contribution of the surface polysaccharides, O-polysaccharide and

ECA, to the resistance against acetic acid and SCFAs is vital for STEC O157:H7. To the best of our knowledge, this is a newly identified physiological role for O-polysaccharide and ECA, and a mechanism of acid resistance. *Salmonella* also became sensitive to acetic acid on losing the expression of O-polysaccharide, but the contribution of O-polysaccharide to acetic acid resistance seems smaller than that of O157 polysaccharide, although quantitative evaluation is difficult.

The structure of the O157 polysaccharide was determined by Perry *et al.* (1986) to be unbranched and linear with a tetrasaccharide repeating unit. The published structure of the repeating unit is as follows: [-3)-α-D-GalNAc*p*-(1–2)-α-D-PerNAc*p*-(1–3)-α-L-Fuc*p*-(1–4)-β-D-Glc*p*-(1-]$_n$. The presence of PerNAc (D-perosamine) confers the bacterium a unique antigenic character, but this O-polysaccharide probably does not neutralize acetic acid on the cell surface. The physiological roles of O-polysaccharide of Gram-negative bacteria may be (i) to make the cell surface hydrophilic and protect cells from dehydration; (ii) to hinder the access to hydrophobic compounds; (iii) to catch toxic divalent cations by chelation; (iv) to stabilize and maintain the full architecture of the outer membrane; (v) to shield cells from attack by bacteriophages; (vi) to give bacteria antigenic diversity; and (vii) to protect cells from host phagocytosis. Loss of O-polysaccharide generally results in loss of virulence. In a low pH medium, acetic acid remains in the molecular form and thus hydrophobic. Molecular acetic acid can easily penetrate membranes with hydrophobic layers and dissociates into ionic form in the cytoplasm of bacteria. An excess amount of acetate anion in the cytoplasm may disturb physiological conditions such as the osmolarity and metabolic activity of bacteria and cause cell death. The fully expressed O-polysaccharide of STEC O157:H7 may hinder the access of molecular acetic acid, and moreover, prevent disruption of the membrane by the osmotic pressure of the cytoplasm, although no experimental evidence to this effect has been presented.

*Salmonella* mutants that lost the outer core sugar residues of OS of LPS became more sensitive to acetic acid. These mutants (called deep rough mutants) are much more sensitive to hydrophobic dyes, detergents and antibiotics. They are also hypersensitive to fatty acids, phenol and polycyclic hydrocarbons (Nikaido and Vaara, 1985; 1987; Nikaido, 1996). An increase in the phospholipid content of the outer membrane in these deep rough mutants may result in the formation of a phospholipid bilayer. Hydrophobic molecules are hypothesized to penetrate through the phospholipid bilayer (Nikaido and Vaara, 1985; 1987; Nikaido, 1996). Acetic acid molecules may also penetrate through the same pathway into the cytoplasm in deep rough mutants. This speculation explains our observation that although the mutants with simple loss of O-antigen and ECA showed slow growth on LB-MES agar with 12 mM acetic acid (pH 5.4), no growth of deep rough mutants ACS32 and 35 and those of *Salmonella* was visible even after 3 days of incubation on the same medium. The involvement of LPS in the resistance to acid stress was also shown in *Helicobacter pylori* by McGowan and colleagues (1998). Qualitative differences in LPS profiles were observed in *H. pylori* cells grown at pH 5 compared with pH 7, suggesting that *H. pylori* may alter its LPS structure in response to acidic pH. Although the bacterium and the acid stress used in their assay were different from our conditions, their results were supportive to our results.

ECA is a surface polysaccharide whose chemical structure is, unlike LPS, common among all members of the family Enterobacteriaceae. It is composed of the trisaccharide repeating unit –3)-α-D-Fuc4NAc-(1–4)-β-D-ManNAcA-(1–4)-α-D-GlcNAc(1- and linked to both the anchor phosphodiacylglycerol and the core OS of LPS (Rick and Silver, 1996). The amount of ECA expressed on the surface of cells is believed to be much smaller than that of O-polysaccharide in most bacteria. Although the expression of ECA is highly conserved in wild bacteria, the physiological functions of ECA have remained unknown (Rick and Silver, 1996). The present study showed that ACS115, which is deficient only in the expression of ECA polysaccharide, became sensitive to acetic acid. This unexpected result suggests that ECA has some protective role in the survival of bacteria against environmental stress. This may be the reason why ECA is conserved in a broad range of wild bacteria, although the mechanism(s) of the protective function of ECA against acetic acid and other SCFAs is still unknown.

The lower digestive tract is rich in SCFAs produced by the fermentative activities of enteric bacteria. A small amount of these organic acids is metabolized via the bacterial β-oxidation cycle, but excess amounts would suppress the growth of bacteria. Most species of Enterobacteriaceae colonize in such a harsh environment with the aid of O-polysaccharide and ECA.

## Experimental procedures

### *Bacterial strains, plasmids and growth conditions*

The bacterial strains and plasmids used in this study are listed in Table 1. NGY9 was an isolate of the Sakai outbreak and the isolation of an isogenic *rpoS*-deficient mutant from NGY9 was performed as described previously (Yokoyama *et al.*, 2000). All strains were stored at –80°C in 20% glycerol. *Escherichia coli* strains were routinely grown in Luria–Bertani (LB) medium. *Escherichia coli* XL-1Blue and JA221 served as cloning hosts. For the measurement of acid sensitivity, the media were prepared as follows: LB broth was buffered at pH 5.4 with 50 mM 2-morpholinoethanesulphonic acid (MES), to prepare LB-MES. To measure the effect of

**Table 1.** Characteristics of bacterial strains and plasmids used in this study.

| Strain/plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strain | | |
| *E. coli* O157:H7 (NGY9) | A clinical isolate of Sakai outbreak, Japan | Laboratory stocks |
| *S. typhimurium* LT2 (LPS mutants) | Ra to Re chemotypes | Laboratory stocks |
| *E. coli* MC4100 | Δ(*argF*$^-$*lac*)*U169 araD rpsL relA flbB deoC ptsF rbsR* | Laboratory stocks |
| *E. coli* XL-1 Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI* ZΔM15 Tn*10* [Tet$^r$]) | Stratagene |
| *E. coli* JA221 | F$^-$ *leuB6 trpE5 hsdR(rk*$^-$ *mk*$^+$*) recA1 lacY* | Laboratory stocks |
| Plasmid | | |
| pUT::mini-Tn*5*(Tc1) | *Kpn*I–*Not*I fragment of mini-Tn*5*(Km2) was replaced by a 2.8 kb *Bgl*I–*Bgl*I fragment encoding the *tetAR* segment of Tn*10* | T. Tobe |
| pSTV29 | Cloning vector; Cm$^R$ | Takara Biomedicals |
| pHSG576 | Cloning vector; Cm$^R$, derivative of pSC101 containing polycloning sites of pUC8 | Ohta *et al*. (1991) |
| pSB1 | Contains the 1.4 kb *Bam*HI fragment from NGY9, derivative of pSTV29; Cm$^R$ | This study |
| pSB5 | Contains the 1.94 kb *Bam*HI fragment from NGY9, derivative of pSTV29; Cm$^R$ | This study |
| pHSF131 | Contains the 12 kb *Sal*I–*Eco*RI fragment of entire *wec* gene cluster; derivative of pHSG576; Cm$^R$ | Ohta *et al*. (1991) |

acetic acid, acetic acid at final concentration of 12 mM was added to LB-MES after the autoclaving of LB-MES and then pH was adjusted to 5.4 with NaOH. For the measurement of the growth of bacteria, cultures were performed in 5 ml of LB-MES with 12 mM of acetic acid in 16 × 160 mm test tubes with vigorous shaking at 200 rpm at 37°C. Acid-sensitive mutants were screened at 37°C on a basal medium containing: $K_2HPO_4$, 7 g; $KH_2PO_4$, 2 g; $(NH_4)_2SO_4$, 1 g; sodium citrate, 0.5 g; 10 mM $MgSO_4$; thiamine (2 μg ml$^{-1}$); and glucose (0.6%) per litre. Solid (1.4% agar) and liquid media were supplemented, when required, with 50 μg ml$^{-1}$ of ampicillin; 25 μg ml$^{-1}$ of chloramphenicol; 10 μg ml$^{-1}$ of tetracycline; 40 μg ml$^{-1}$ of Xgal and 100 mM of IPTG. *Escherichia coli* S17–1 λ*pir* harbouring a suicide vector, pUT::mini-Tn*5*(Tc1) was used as a conjugal transposon donor. pUT::mini-Tn*5*(Tc1) was kindly provided by Dr T. Tobe (Tokyo University). It was constructed by him from pUT::mini-Tn*5*(Km2) (de Lorenzo and Timmis, 1994). Briefly, the *Kpn*I–*Not*I fragment of the Km$^r$ segment of mini-Tn*5*(Km2) was excised and replaced with a 2.8 kb *Bgl*I–*Bgl*I fragment encoding the *tetAR* segment of Tn*10*.

### *Random transposon mutagenesis and screening for acid-sensitive mutants*

STEC NGY9 was mutagenized with mini-Tn*5*(Tc1) as follows. Briefly, conjugation was performed by mixing mid-exponential phase cells of the donor [*E. coli* S17–1 λ*pir* carrying pUT::mini-Tn*5*(Tc1)] and the recipient (NGY 9) and then by a filter mating technique (de Lorenzo and Timmis, 1994). Exconjugants were resuspended in 10 mM $MgSO_4$ and plated on selective basal agar supplemented with tetracycline (10 μg ml$^{-1}$). The plates were incubated at 37°C until colonies became visible.

NGY9 carrying mini-Tn*5* insertions were screened for acid sensitivity by the replica plating method on LB-MES agar plates containing tetracycline, with or without acetic acid. The LB-MES was adjusted to pH 5.4 with 12 mM acetic acid. Transconjugants that grew poorly or not at all on acidified medium, but grew well on conventional LB agar plates supplemented with tetracycline, were retested for the confirmation of a stable mini-Tn*5* insertion and selected for further analysis. Approximately 50 000 colonies were screened for sensitivity to acetic acid.

### *DNA manipulation and sequencing*

Plasmid and chromosomal DNAs were isolated as described previously (Shibayama *et al*., 1998). Recombinant DNA methods, including the restriction endonuclease digestion, ligation, transformation of competent bacterial cells, and agarose gel electrophoresis, were performed according to the methods of Sambrook *et al.* (1989). Restriction endonucleases and T4 DNA ligase were purchased from Takara Biomedicals and were used according to the instructions of the supplier.

To sequence the regions flanking the mini-Tn*5*(Tc1)-insertion, genomic DNA from each mutant was partially digested with the restriction endonuclease *Sau*3AI, and subsequently ligated into the *Bam*HI site of plasmid pSTV29, and recombinant plasmids that carried both tetracycline resistance and chloramphenicol resistance genes were selected. The sequence of the flanking region was obtained by sequencing from the Tn*10 tetA* down-end of the transposon with the primer GTGATGATAAAAGGCACC. The Big-dye cycle sequencing kit (Applied Biosystems) was used, and the resulting reactions were analysed at the Nagoya University Biotechnology Center. Sequence analysis was carried out by using BLASTN (Perna *et al*., 2001).

### *Complementation of the mutants*

Complementation of *wecA* and *wecB* mutants was performed by the introduction of pHSF131 that encodes the *wecA* gene

and the entire *rff* gene cluster (Ohta *et al*., 1991). Complementation of *waaG* and *fcl* mutants was accomplished by cloning the relevant genes into a broad host-range plasmid pSTV29 downstream of the *lac* promoter. The oligodeoxyribonucleotide primers used to amplify the *waaG* gene were *waaG*-5′-ATGGATCCATAACGTGGCAAACGG and *waaG*-3′-GCGGATCCGCAGCAGTTAATACGC and the primers used to amply the *fcl* gene were *fcl*-5′-CTGGATCCAGAA TATACAGCCGATG and *fcl*-3′-TCGGATCCGAACCAATAGC CCTGTG. The underlined nucleotides represent *Bam*HI restriction sites added for convenience of cloning. The *waaG* and *fcl* genes were PCR-amplified from wild NGY9 with a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycler and subjected to a 5 min denaturation step at 94°C followed by 30 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 3 min. The reaction mixture was then held at 72°C for 3 min. The generated DNA fragment was purified by electroelution, digested with *Bam*HI, and ligated with *Bam*HI-cleaved pSTV29 at the multicloning site for *waaG* and *fcl* respectively. The resulting plasmids, designated as pSB1 and pSB5, were used to complement the mutants.

### *Electrophoretic analysis of lipopolysaccharide (LPS) and enterobacterial common antigen (ECA) and immunoblotting*

Rabbit antiserum against *E. coli* O157 was prepared by immunization of rabbits with NGY9 as described previously (Yokoyama *et al*., 2000). Anti-ECA rabbit antiserum was prepared by Ohta *et al.* (1991). LPS and ECA were extracted by the modified phenol–water method described previously (Westphal and Jann, 1965; Shibayama *et al*., 1998). Briefly, 1.5 ml of overnight bacterial culture was precipitated by centrifugation and the cells were suspended in 0.5 ml of physiologic saline. The cell suspensions were mixed well with 0.5 ml of 90% phenol at room temperature. After centrifugation, the aqueous phase was transferred to a new tube and mixed with 1 ml of absolute ethanol. The LPS and ECA were precipitated by centrifugation, and the precipitate was washed with 70% and 99% ethanol, serially, before being air-dried for further analysis. This simple extraction procedure minimizes the contamination of intracellular components. Electrophoresis and immunoblotting of LPS and ECA were performed as described previously (Yokoyama *et al*., 2000). Briefly, after electrophoresis on 10% SDS-polyacrylamide gels in Tris-glycine buffer, LPS on the gel was electrophoretically transferred on to a nitrocellulose membrane (Advantec Toyo) by using a semi-dry blotting system (Nippon Eido). The membrane was soaked in 50 mM Tris-HCl saline buffer (TBS) containing 0.1% skimmed milk for 1 h at room temperature. The membrane was then incubated with the anti-O157 serum or anti-ECA serum (diluted 1:500 with TBS) overnight at room temperature and extensively washed with TBS. Horseradish peroxidase-conjugated goat anti-rabbit IgG (MBL), diluted 1:500 with TBS, was added and the membrane was incubated for 2 h at room temperature on a shaker and then washed with TBS. The immunoreaction on the membrane was visualized with 4-chloro-1-naphthol (0.2 mg $ml^{-1}$ in TBS) containing 0.2% of hydrogen peroxide.

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