

## Role for *Salmonella enterica* Enterobacterial Common Antigen in Bile Resistance and Virulence

Francisco Ramos-Morales,<sup>1\*</sup> Ana I. Prieto,<sup>1</sup> Carmen R. Beuzón,<sup>2†</sup> David W. Holden,<sup>2</sup> and Josep Casadesús<sup>1</sup>

*Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Seville 41080, Spain,<sup>1</sup> and Department of Infectious Diseases, Centre for Molecular Microbiology and Infection, Imperial College School of Medicine, London SW7 2AZ, United Kingdom<sup>2</sup>*

Received 17 March 2003/Accepted 2 June 2003

**Passage through the digestive tract exposes *Salmonella enterica* to high concentrations of bile salts, powerful detergents that disrupt biological membranes. Mutations in the *wecD* or *wecA* gene, both of which are involved in the synthesis of enterobacterial common antigen (ECA), render *S. enterica* serovar Typhimurium sensitive to the bile salt deoxycholate. Competitive infectivity analysis of *wecD* and *wecA* mutants in the mouse model indicates that ECA is an important virulence factor for oral infection. In contrast, lack of ECA causes only a slight decrease in *Salmonella* virulence during intraperitoneal infection. A tentative interpretation is that ECA may contribute to *Salmonella* virulence by protecting the pathogen from bile salts.**

Bile salts are detergent-like substances that aid in the digestion and absorption of lipids. Bile salts are secreted from the liver, stored in the gall bladder, and released through the bile duct into the intestine during food passage. The most abundant bile salts in humans are cholate and deoxycholate (DOC). Enteric bacteria are intrinsically resistant to bile salts, due both to the low permeability of the outer membrane bilayer to these lipophilic solutes and to active efflux mechanisms. Mutations that impair bile salt resistance in genes for lipopolysaccharide (LPS) synthesis (14), *tol* genes (16), efflux pump genes (12, 22), regulatory genes such as *marAB* (20) and *phoPQ* (25), and the DNA adenine methyltransferase gene (8, 17) have been previously described.

Here we show that mutations in the *wecD* and *wecA* genes of *Salmonella enterica* cause sensitivity to DOC. The *wec* gene cluster is required for synthesis of the enterobacterial common antigen (ECA), a glycolipid found in the external leaflet of the outer membrane in all species of the family *Enterobacteriaceae* (reviewed in reference 18). The ECA biosynthetic pathway is diagrammed in Fig. 1. The polysaccharide portion of ECA consists of a linear trimeric repeat with the following structure:  $\rightarrow 3)\alpha\text{-D-Fuc4NAc-(1}\rightarrow 4)\text{-}\beta\text{-D-ManNAc-(1}\rightarrow 4)\text{-}\alpha\text{-D-GlcNAc-(1}\rightarrow$ , where Fuc4NAc is 4-acetamido-4,6-dideoxy-D-galactose, ManNAc is *N*-acetyl-D-mannosaminuronic acid, and GlcNAc is *N*-acetyl-D-glucosamine. The initial step in the synthesis of the repeat unit is the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to undecaprenyl monophosphate to yield GlcNAc-pyrophosphorylundecaprenol (lipid I). The synthesis of lipid I is followed by the successive incorporation of ManNAc and Fuc4NAc from the donors UDP-ManNAc and TDP-Fuc4NAc to form lipid II and lipid III, respectively.

Subsequent steps involve polymerization, transfer of the polymer to a phospholipid aglycone, and translocation to the outer membrane. Below we show that *Salmonella* mutants unable to synthesize ECA are highly attenuated in oral infections and slightly attenuated in intraperitoneal infections. Hence, we propose that ECA plays a role in *Salmonella* virulence. Such a role can be tentatively correlated with bile salt resistance.

**MudJ mutagenesis identifies *wecD* as a locus necessary for resistance to DOC.** In an attempt to identify new genes potentially involved in bile salt resistance in *S. enterica* serovar Typhimurium, we performed a screen for mutants sensitive to sodium deoxycholate in strain 14028, which is virulent in mice. The strains used in this study are described in Table 1. MudJ mutagenesis was achieved by the method of Hughes and Roth (9). Five thousand  $\text{Km}^r$  colonies from 10 independent mutagenesis trials were patched in grids onto Luria-Bertani (LB) plates containing 1% DOC. Twenty-eight DOC-sensitive ( $\text{DOC}^s$ ) isolates were obtained. Reconstruction experiments showed that 19 of 28  $\text{DOC}^s$  isolates were resistant to lysis by P22 HT phage. These are probably LPS mutants, since P22 adsorbs to the O antigen (15), and *Escherichia coli* mutants lacking a complete O side chain in LPS (rough mutants) have been shown to display bile salt sensitivity (14). In addition, 3 out of 14 *Salmonella* mutants sensitive to bile salts obtained by Prouty et al. (16) had a rough LPS phenotype.

Chromosomal DNA from one  $\text{DOC}^s$  P22-sensitive mutant was prepared as previously described (4), digested with *Bam*HI, and ligated to plasmid pBluescript SK II(+). Transformants were selected on LB plates containing 40  $\mu\text{g}$  of kanamycin/ml. DNA sequencing with the MuL primer (23) revealed that the insertion was located in *wecD*. This gene is part of the *wec* gene cluster, which is involved in the biosynthesis and assembly of ECA. Two lines of evidence confirmed that the *wecD* mutant was sensitive to DOC. (i) Dilutions from exponential cultures of the *wecD* mutant and the wild-type strain were spread on LB plates supplemented with 1% DOC. Clear-cut differences between the strains under study were found (Fig. 2). (ii) The MIC of DOC was determined for each strain.

\* Corresponding author. Mailing address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apdo. 1095, 41080 Seville, Spain. Phone: 34 954557106. Fax: 34 954557104. E-mail: fframes@us.es.

† Present address: Dep. Biología Celular, Genética y Fisiología, Universidad de Málaga, Campus de Teatinos, 29071 Málaga, Spain.

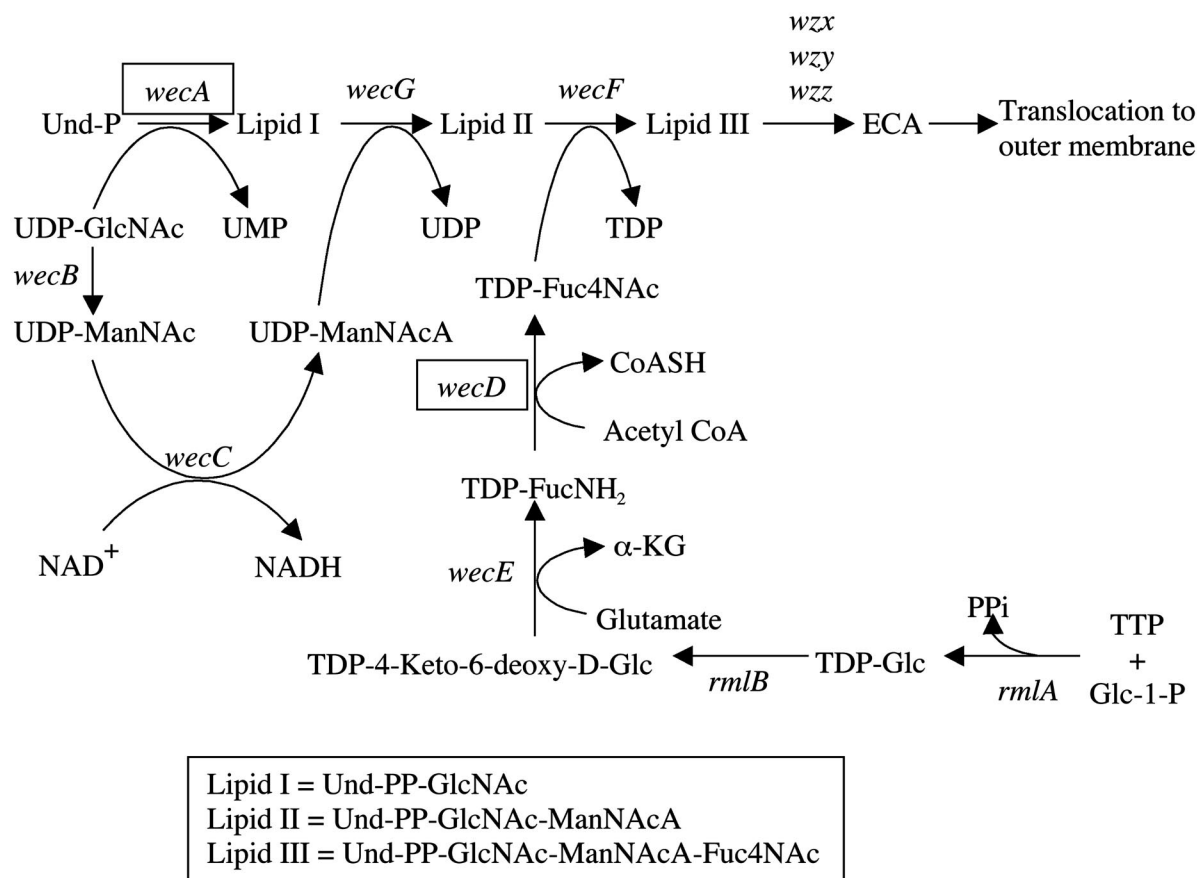


FIG. 1. The ECA biosynthetic pathway (based on reference 2). Individual genetic loci are shown next to each enzymatic reaction. Genes relevant for this work are boxed. Abbreviations: Und-P, undecaprenyl monophosphate; Glc, glucose; ManNAc, *N*-acetyl-D-mannosamine; Glc-1-P, glucose 1-phosphate; FucNH<sub>2</sub>, 4-amino-4,6-dideoxy-D-glucose; Fuc4NAc, 4-acetamido-4,6-dideoxy-D-galactose; CoASH, coenzyme A; α-KG, α-ketoglutaric acid; PPi, inorganic pyrophosphate.

For MIC determination, samples of 3 × 10<sup>3</sup> CFU/ml from stationary-phase cultures were subjected to various concentrations of DOC in polypropylene microtiter plates (Greiner, Frickenhausen, Germany). After an overnight incubation at 37°C, the MIC for the *wecD* mutant was found to be 0.1%, compared to 4% for the wild type.

**DOC sensitivity of *wec* mutants is due to failure to synthesize ECA.** Studies with *E. coli* have reported that null mutations in *wecE* or *wecF* confer sensitivity to MacConkey agar (which contains bile salts) and that this phenotype is caused by accumulation of lipid II, an ECA biosynthetic intermediate (5). According to the same study, the *wecE wecA* and *wecF wecA*

double mutants, which are unable to synthesize ECA but which do not accumulate lipid II, are able to grow on MacConkey agar. Results for the *wecA* single mutant were not shown in that study (5). A *wecD* mutant can be also expected to accumulate lipid II (Fig. 1). To investigate if the absence of ECA without accumulation of lipid II could cause DOC sensitivity in *Salmonella*, we tested a *wecA* mutant previously isolated in our laboratory (6, 11). The MIC determined for the *wecA* mutant was 0.2%, slightly higher than that for the *wecD* mutant but well below the MIC for the wild type (Fig. 2). A nearly identical MIC for the *wecD wecA* double mutant was obtained (Fig. 2). These experiments suggest that in *Salmonella* the absence of ECA, rather than the accumulation of lipid II, is the cause of DOC sensitivity.

***S. enterica wec* mutants are severely attenuated in the mouse model.** During a natural infection, *Salmonella* encounters DOC and other bile salts in the gut. Since *wec* mutants are 40-fold more sensitive to DOC than the wild type, we reasoned that *wec* mutations might cause attenuation specifically in orally inoculated mice. To test this hypothesis, 8-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were subjected to mixed infections with *wec* mutants. Groups of three or four animals were inoc-

TABLE 1. Strains used in this study

Strain	Description	Source
14028	Wild type	ATCC <sup>b</sup>
TT10288	<i>hisD9953::MudJ his-9944::MudI</i>	J. Roth
SV4429	<i>wecD::MudJ</i>	This work
SV4759	<i>wecA::Tn10dTet</i>	This work <sup>a</sup>
SV4801	<i>wecD::MudJ wecA::Tn10dTet</i>	This work

<sup>a</sup> This mutant was constructed by P22 HT-mediated transduction of strain 14028 with a lysate from mutant SV2078 (6).

<sup>b</sup> ATCC, American Type Culture Collection.

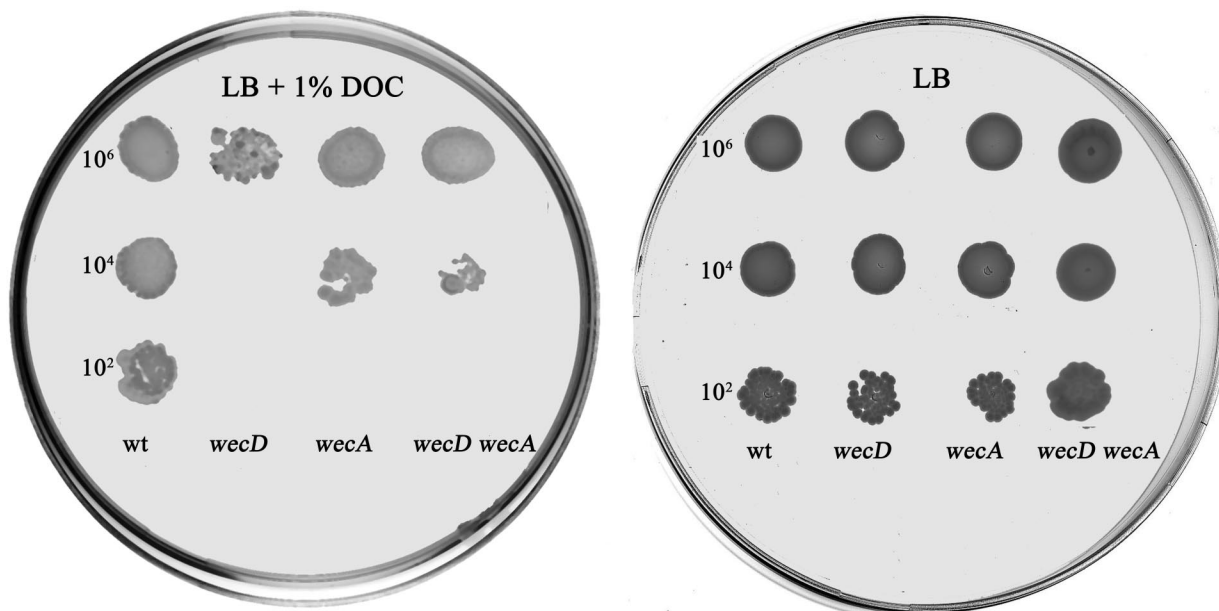


FIG. 2. DOC sensitivity assay for *wec* mutants. Five-microliter portions of the appropriate dilutions of exponential cultures of the wild-type and mutant strains were incubated for 24 h at 37°C in an LB plate (right) or an LB plate containing 1% DOC (left). The approximate numbers of bacteria present in the original drops are indicated on the left of each plate.

ulated with a 1:1 ratio of the mutant and the wild type. For oral inoculation, bacteria were grown overnight at 37°C in LB without shaking. For intraperitoneal inoculation, bacteria were grown overnight at 37°C in LB with shaking, diluted into fresh medium (1:100), and grown until an optical density at 600 nm of 0.35 to 0.6 was reached. Oral inoculation was performed by feeding the mice 25  $\mu$ l of saline containing 0.1% lactose and  $10^8$  bacteria. Intraperitoneal inoculation was performed with 0.2 ml of physiological saline containing  $10^5$  CFU. Bacteria were recovered from the spleen 48 h after intraperitoneal inoculation or 6 days after oral inoculation, and CFU were enumerated on selective medium (LB with 40  $\mu$ g of kanamycin/ml for *wecD* and 20  $\mu$ g of tetracycline/ml for *wecA*). A competitive index (CI) for each mutant, the ratio between the mutant and the wild-type strain in the output (bacteria recovered from the host after infection) divided by their ratio in the input (initial inoculum), was calculated (7, 21). The CI is a sensitive measure of the relative degree of virulence attenuation caused by a given mutation (3). *wecD* and *wecA* mutants were significantly outcompeted by the wild-type strain in both intraperitoneal and oral infections (Fig. 3). A detailed analysis of the CIs obtained indicates that both mutants are slightly attenuated in intraperitoneal infections (CI, 0.2 to 0.4) but severely attenuated in oral infections (CI < 0.005). The results obtained after intraperitoneal inoculations are consistent with an earlier report suggesting a small but significant difference in virulence between ECA-producing and ECA-deficient strains of *S. enterica* serovar Typhimurium (24). In that study, the difference in 50% lethal doses was about 10-fold. A subsequent study indicated that the apparently higher virulence of ECA-positive strains was due to their increased survival in mice and that this could be only partially attributed to their ECA-positive character (13). These data, together with the finding that ECA does not possess endotoxin-like activity (10) and the

failure of anti-ECA antibodies to protect against salmonellosis (19), prompted the view that ECA was not a significant determinant of virulence (18). In contrast, our results with oral infections show that *wec* genes have a significant role in virulence. The different behaviors exhibited by *wec* mutants in oral and intraperitoneal experiments are in agreement with the hypothesis that ECA may be required for resistance to DOC in the small intestine.

#### Different degrees of attenuation in *wecD* and *wecA* mutants.

The analysis of CIs of *wec* mutants against the wild-type strain yields another interesting conclusion. Although both *wecD* and *wecA* mutants were significantly outcompeted by the wild-type strain in oral infections, the CI of the *wecD* mutant in oral infections was lower than the CI of the *wecA* mutant. Statistical

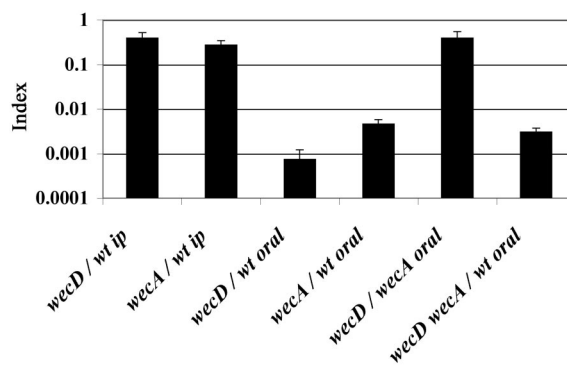


FIG. 3. CI analysis for *wecD* and *wecA* mutants. Shown is a graphical representation of CI analysis of strains carrying mutations in *wecD*, *wecA*, or both genes, after intraperitoneal (ip) or oral infections. The strains used in each mixed infection are indicated under the corresponding bars. The CIs are the means from at least three infections. Error bars represent the standard deviations. wt, wild-type strain.

TABLE 2. CIs for *wecD* and *wecA* mutants obtained in mixed infections with the wild-type strain<sup>a</sup>

Strain	Mutant gene(s)	Intraperitoneal inoculation		Oral inoculation	
		CI	<i>P</i>	CI	<i>P</i>
SV4429	<i>wecD</i>	0.39	0.0137	0.0007	<0.0001
SV4759	<i>wecA</i>	0.27	0.0030	0.0046	<0.0001
SV4801	<i>wecD, wecA</i>	ND	ND	0.003	<0.0001

<sup>a</sup> *P* values were obtained by Student's *t* test. The null hypothesis was a CI of 1. A *P* value  $\leq 0.05$  indicates that CI is significantly different from 1. ND, not determined.

analysis (Student's *t* test) showed that this difference was significant ( $P = 0.0009$ ). In pursuit of a more precise comparison of *wecD* and *wecA* mutants, we tested them in a direct-competition experiment. The CI for the *wecD* mutant versus the *wecA* mutant in oral infections was 0.39 (Fig. 3), and statistical analysis indicates that this value is significantly different from 1 ( $P = 0.0008$ ). This result confirms that a *wecD* mutant is more attenuated than a *wecA* mutant. One difference between these mutants is that the *wecD* strain is expected to accumulate lipid II (5) (Fig. 1). Altogether, these results suggest that ECA is an important virulence factor per se and that the accumulation of the lipid II intermediate might cause further attenuation. If this hypothesis is correct, a double mutant carrying null mutations in *wecD* and *wecA* should be as attenuated in oral infections as the *wecA* single mutant, since the double mutant does not accumulate lipid II. To test this prediction, we constructed a *wecD wecA* double mutant by P22 HT transduction and determined the CI value for this strain versus the wild type. Data shown in Table 2 and Fig. 3 indicate that the *wecD wecA* double mutant is highly attenuated in oral infections. However, the CI for this strain is significantly higher than the CI for the *wecD* single mutant and not significantly different from the CI for the *wecA* mutant (*P* values [Student's *t* test] of 0.0034 and 0.0952, respectively). These data support the view that lack of ECA causes a decrease in virulence and that a further decrease occurs if lipid II is accumulated.

**Role of ECA in *Salmonella* virulence.** Despite the unique and universal occurrence of ECA in the family *Enterobacteriaceae*, its biological function remains unknown. Several lines of evidence presented in this study support a role for ECA in both the resistance to bile salts and the virulence of *S. enterica* serovar Typhimurium in the mouse model. Our results also suggest that both traits may be related, since the attenuation of *wec* mutants is more significant in oral than in intraperitoneal infections. A recent study (1) suggested that extracellular polysaccharides of uropathogenic *E. coli* are virulence determinants in the murine urinary tract. However, the CI displayed by a *wecE* mutant against the wild-type strain was not significantly different from 1 (which indicates no attenuation) for the bladder or the kidney and around the limit of significance for the urine ( $P = 0.05$ ) (1). In contrast, the CIs displayed by *S. enterica wecA* and *wecD* mutants are extremely low and statistically significant, especially after oral inoculation ( $P < 0.0001$ ). This high degree of attenuation is consistent with the failure of *wec* mutants to resist the bactericidal effect of bile salts. Neither defect can be attributed to the accumulation of

lipid II since a *wecA* mutant, in which lipid II does not accumulate, is also attenuated in mice and sensitive to DOC.

This work was supported by grants QLRT-PL-00310 from the European Union and BIO2001-0232-C02-02 from the Ministry of Science and Technology of Spain. A stay of F.R.-M. at the Imperial College, London, was partially supported by a grant from the Regional Government of Andalusia. F.R.-M. is an investigator of the Ramón y Cajal program from the Ministry of Science and Technology of Spain.

## REFERENCES

- Bahrani-Mougeot, F. K., E. L. Buckles, C. V. Lockett, J. R. Hebel, D. E. Johnson, C. M. Tang, and M. S. Donnenberg. 2002. Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract. *Mol. Microbiol.* **45**:1079–1093.
- Barr, K., J. Klena, and P. D. Rick. 1999. The modality of enterobacterial common antigen polysaccharide chain lengths is regulated by *o349* of the *wec* gene cluster of *Escherichia coli* K-12. *J. Bacteriol.* **181**:6564–6568.
- Beuzon, C. R., and D. W. Holden. 2001. Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions in vivo. *Microbes Infect.* **3**:1345–1352.
- Cano, D. A., M. Martinez-Moya, M. G. Pucciarelli, E. A. Groisman, J. Casadesus, and F. Garcia-Del Portillo. 2001. *Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. *Infect. Immun.* **69**:6463–6474.
- Danese, P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy. 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*. *J. Bacteriol.* **180**:5875–5884.
- Flores, A., and J. Casadesus. 1995. Suppression of the pleiotropic effects of HisH and HisF overproduction identifies four novel loci on the *Salmonella typhimurium* chromosome: *osmH*, *sfiW*, *sfiX*, and *sfiY*. *J. Bacteriol.* **177**:4841–4850.
- Freter, R., P. C. O'Brien, and M. S. Macsai. 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. *Infect. Immun.* **34**:234–240.
- Heithoff, D. M., E. Y. Enioutina, R. A. Daynes, R. L. Sinsheimer, D. A. Low, and M. J. Mahan. 2001. *Salmonella* DNA adenine methylase mutants confer cross-protective immunity. *Infect. Immun.* **69**:6725–6730.
- Hughes, K. T., and J. R. Roth. 1988. Transitory *cis* complementation: a method for providing transposition functions to defective transposons. *Genetics* **119**:9–12.
- Mayer, H., and G. Schmidt. 1979. Chemistry and biology of the enterobacterial common antigen (ECA). *Curr. Top. Microbiol. Immunol.* **85**:99–153.
- Mousslim, C., D. A. Cano, and J. Casadesus. 1998. The *sfiX*, *rfe* and *metN* genes of *Salmonella typhimurium* and their involvement in the His<sup>c</sup> pleiotropic response. *Mol. Gen. Genet.* **259**:46–53.
- Nikaido, H., M. Basina, V. Nguyen, and E. Y. Rosenberg. 1998. Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those beta-lactam antibiotics containing lipophilic side chains. *J. Bacteriol.* **180**:4686–4692.
- Nnalue, N. A., and B. A. Stocker. 1987. The effects of O-antigen character and enterobacterial common antigen content on the in vivo persistence of aromatic-dependent *Salmonella* sp. live-vaccine strains. *Microb. Pathog.* **3**:31–44.
- Picken, R. N., and I. R. Beacham. 1977. Bacteriophage-resistant mutants of *Escherichia coli* K-12. Location of receptors within the lipopolysaccharide. *J. Gen. Microbiol.* **102**:305–318.
- Poteete, A. R. 1988. Bacteriophage P22, p. 647–682. *In* R. Calendar (ed.), *The bacteriophages*, vol. 2. Plenum Press, New York, N.Y.
- Prouty, A. M., J. C. Van Velkinburgh, and J. S. Gunn. 2002. *Salmonella enterica* serovar Typhimurium resistance to bile: identification and characterization of the *tolQRA* cluster. *J. Bacteriol.* **184**:1270–1276.
- Pucciarelli, M. G., A. I. Prieto, J. Casadesus, and F. Garcia-del Portillo. 2002. Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*. *Microbiology* **148**:1171–1182.
- Rick, P. D., and R. P. Silver. 1996. Enterobacterial common antigen and capsular polysaccharides, p. 104–122. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
- Saxén, H., and M. Hovi. 1985. The effect of antibodies to the enterobacterial common antigen (ECA) on experimental mouse salmonellosis. *FEMS Microbiol. Lett.* **27**:307–312.
- Sulavik, M. C., M. Dazer, and P. F. Miller. 1997. The *Salmonella typhimurium mar* locus: molecular and genetic analyses and assessment of its role in virulence. *J. Bacteriol.* **179**:1857–1866.
- Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos. 1987. Use of

- phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
22. **Thanassi, D. G., L. W. Cheng, and H. Nikaido.** 1997. Active efflux of bile salts by *Escherichia coli*. J. Bacteriol. **179**:2512–2518.
  23. **Torreblanca, J., and J. Casades.** 1996. DNA adenine methylase mutants of *Salmonella typhimurium* and a novel *dam*-regulated locus. Genetics **144**:15–26.
  24. **Valtonen, M. V., U. M. Larinkari, M. Plosila, V. V. Valtonen, and P. H. Makela.** 1976. Effect of enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. Infect. Immun. **13**:1601–1605.
  25. **van Velkinburgh, J. C., and J. S. Gunn.** 1999. PhoP-PhoQ-regulated loci are required for enhanced bile resistance in *Salmonella* spp. Infect. Immun. **67**:1614–1622.