APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2006, p. 946–949 Vol. 72, No. 1
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.1.946–949.2006

Butyrate Specifically Down-Regulates *Salmonella* Pathogenicity Island 1 Gene Expression

I. Gantois,[1]* R. Ducatelle,[1] F. Pasmans,[1] F. Haesebrouck,[1] I. Hautefort,[2] A. Thompson,[2] J. C. Hinton,[2] and F. Van Immerseel[1]

Department of Pathology, Bacteriology and Avian Diseases, Research Group Veterinary Public Health and Zoonoses, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium,[1] and Molecular Microbiology Group, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom[2]

Received 12 August 2005/Accepted 5 October 2005

Invasion of intestinal epithelial cells by *Salmonella enterica* is decreased after exposure to butyric acid. To understand the molecular mechanisms of this phenomenon, a comparative transcriptomic analysis of *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium grown in medium supplemented with butyrate was performed. We found that butyrate down-regulated the expression of 19 genes common to both serovars by a factor of twofold or more, and 17 of these genes localized to the *Salmonella* pathogenicity island 1 (SPI1). These included the SPI1 regulatory genes *hilD* and *invF*. Of the remaining two genes, *ampH* has 91% homology to an *Escherichia coli* penicillin-binding protein and *sopE2* encodes a type III-secreted effector protein associated with invasion but located at a separate site on the chromosome from SPI1.

Non-host-specific *Salmonella enterica* subspecies *enterica* serovars, such as *Salmonella* serovar Enteritidis and *Salmonella* serovar Typhimurium, are the leading cause of self-limiting nontyphoidal gastroenteritis in humans (4). The interaction between bacteria and host cells is crucial in *Salmonella* pathogenesis. During infection of the host, *Salmonella* bacteria encounter several environmental triggers, including the acidity of the stomach and the anoxia of the gut. In the intestine, the anaerobic environment is dominated by fermentative bacteria, which mainly accumulate three organic acids, acetate, propionate, and butyrate (6). These acids play a vital role in maintenance of colonic integrity and metabolism. Butyrate is of particular importance because it has nutritious properties for the healthy and injured colon epithelium (16) and is able to induce apoptosis in colon cancer cells (29). In addition, a shortage of butyrate can result in intestinal inflammation (18). Recently, butyrate was also shown to affect the interaction of *Salmonella enterica* with the intestinal epithelium, characterized by a reduction of invasion (34, 35).

Invasion of intestinal epithelial cells is an important step in the pathogenesis of *Salmonella*-mediated enteritis and requires a set of genes encoded on the *Salmonella* pathogenicity island 1 (SPI1). Bacterial internalization occurs through the type III secretion system (TTSS), which injects bacterial effector proteins directly into the cytosol of epithelial cells, inducing actin rearrangements leading to the uptake of the bacteria (5, 7). Currently, it is known that acetate, when converted to acetylphosphate, increases SPI1 gene expression through activation of the sensor kinase/response regulator system BarA/SirA (22). However, the molecular mechanism underlying the invasion-suppressive activity of butyrate is unknown. To elucidate this mechanism, we compared the gene expression profiles of two *Salmonella enterica* serovars grown in Luria-Bertani (LB) medium versus LB medium supplemented with 10 mM butyrate by using cDNA microarray technology.

In our experiments, we used two well-characterized strains, *Salmonella* serovar Typhimurium SL1344 (20) and *Salmonella* serovar Enteritidis 76Sa88 (10). In order to determine the timing of the largest butyrate-mediated reduction in invasion, a HeLa cell invasion assay was performed. Briefly, HeLa cells (European Collection of Cell Cultures) were grown in six-well plates containing HEPES-buffered Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum. Bacteria were grown overnight in LB medium. The bacterial suspension was diluted 1:50 in 50 ml of either LB medium supplemented with 10 mM butyrate or control LB medium in 250-ml flasks. The pH of all media was brought to pH 6 and the osmolarity to 600 mmol/kg. The suspensions were incubated statically at 37°C. After 2, 4, and 6 h of incubation, HeLa cells were infected using a multiplicity of infection of 100:1 under 10% CO_2 atmosphere. Invasion was limited to 30 min, and extracellular bacteria were killed in medium containing 30 μg/ml gentamicin for 30 min. The gentamicin concentration was subsequently reduced to 5 μg/ml for the remaining time of the experiment. At appropriate time points, the infected cells were lysed in 0.1% sodium dodecyl sulfate and intracellular populations determined by viable counts. Exposure of the bacteria to butyrate for 4 h at 37°C resulted in the lowest invasion percentage of the HeLa cells for both serovar Typhimurium and serovar Enteritidis (data not shown). Furthermore, to confirm our findings, SPI1 expression in individual bacteria was measured in a green fluorescent protein-based system using a chromosomally integrated *prgH-gfp*$^+$ fusion (19). PrgH is a structural component of the needle complex of the SPI1 type III secretion system and is regulated by HilA (24). The level of *prgH* expression in SL1344 was measured hourly using flow cytometric analysis as previously reported (19). The lowest expression of *prgH* was observed after 4 h of

* Corresponding author. Mailing address: Department of Pathology, Bacteriology and Avian Diseases, Research Group Veterinary Public Health and Zoonoses, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Phone: (32) 09 264 77 40. Fax: (32) 09 264 77 89. E-mail: inne.gantois@UGent.be.

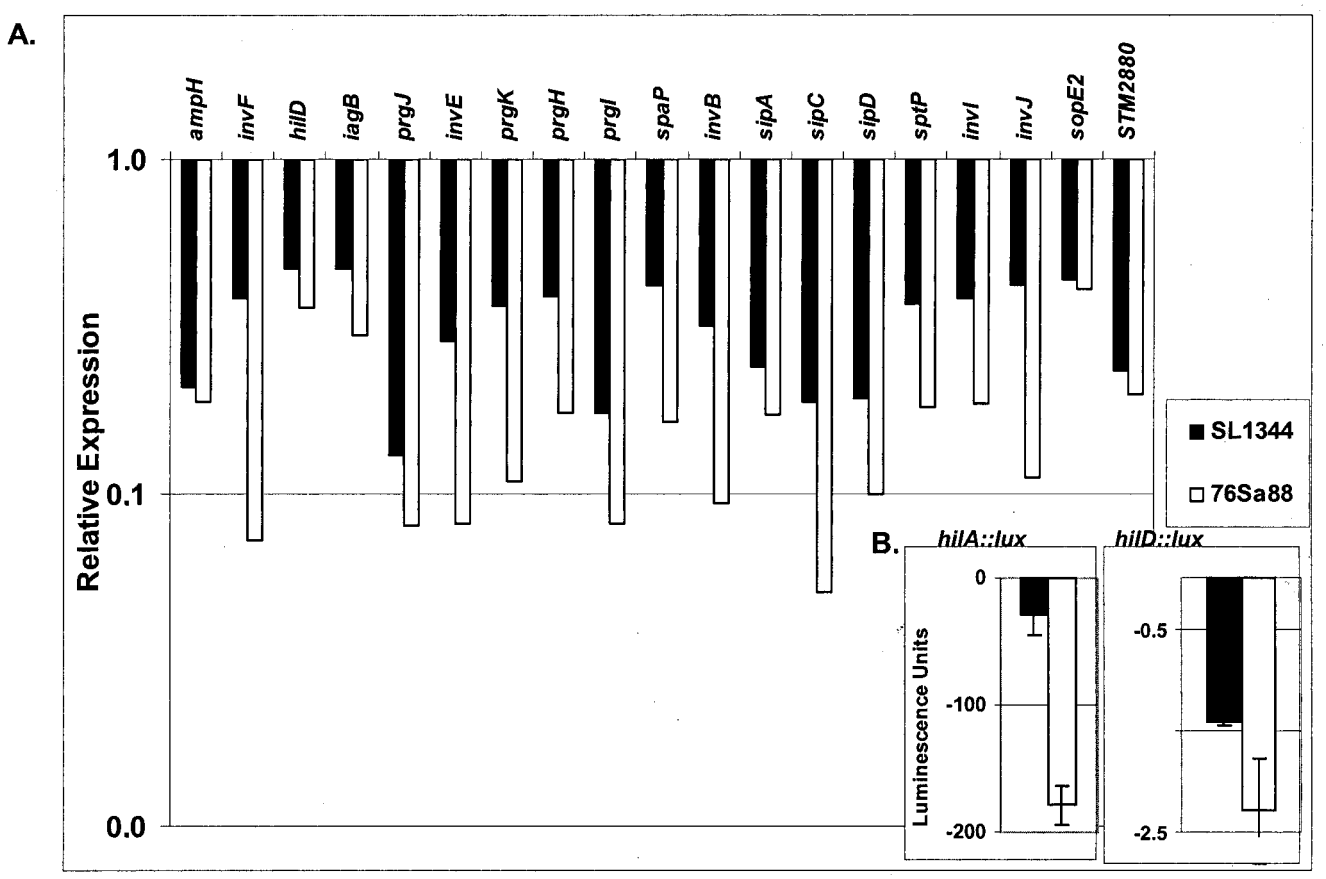

FIG. 1. (A) List of genes that were down-regulated more than twofold in *Salmonella* serovar Typhimurium SL1344 and *Salmonella* serovar Enteritidis 76Sa88 bacteria grown in LB medium supplemented with 10 mM butyric acid relative to the control condition. Expression data were normalized to the control condition (LB), and the ratios for butyrate-treated LB to control LB were calculated. (B) Strains were grown overnight at 37°C and diluted 50-fold in LB and LB supplemented with 10 mM butyrate. Light emission was measured every 15 min, and the graph shows the difference observed at the time point when the highest level of expression was normally observed in LB.

incubation in media supplemented with butyrate. On the basis of these findings, we extracted bacterial RNA from the strains serovar Typhimurium SL1344 and serovar Enteritidis 76Sa88 after 4 h of growth in LB medium with and without butyric acid. The pHs of both media did not vary during the 4-h incubation. In order to provide robust and reproducible gene expression data, microarray experiments were performed with at least two biological and technical replicate samples for all conditions examined. An indirect comparison experimental design was used for the microarray hybridizations (36). The reference channel was Cy3-labeled genomic DNA isolated from either serovar Typhimurium or serovar Enteritidis (for all microarray protocols, see www.ifr.ac.uk/safety/microarrays/protocols.html). RNA was isolated from cultures and labeled with Cy5-dCTP during reverse transcription. The microarray used in this study was the whole-genome *Salmonella* serovar Typhimurium/Enteritidis SALSA cDNA microarray which carried 5,877 coding sequences (13). Features with a reference signal lower than background plus 2 standard deviations were excluded from subsequent analysis. The microarray data were normalized to the untreated control for each serovar and statistically filtered by applying a *t* test with a false discovery rate of $P = 0.05$. The data were further filtered to exclude genes that changed in expression by less than twofold relative to the untreated control samples. In serovar Typhimurium SL1344 treated with butyrate, only 49 genes were down-regulated more than twofold. Interestingly, 23 of these genes were invasion-associated SPI1 genes, and 15 genes were of unknown function, among which 3 belong to the SPI1 gene cluster (STM2868, STM2870, and STM2880). Genes encoding SPI1 effector proteins were also down-regulated upon exposure to butyrate (i.e., STM4257 for SPI4, *pipC* and *sopB* for SPI5, and *sopE2*). The remaining genes were *iacP* (encoding an acyl carrier protein); *hycB*, *hycC*, *hycE*, and *hycG* (coding for a hydrogenase enzyme); *ampH* (encoding a penicillin-binding protein); and *hupB* (encoding a nucleoid-associated protein).

The gene expression profile from serovar Enteritidis 76Sa88 grown in LB containing butyrate showed a similar down-regulation of SPI1. Of the 90 genes that were down-regulated more than twofold, 24 were SPI1 genes and 25 had an unknown function, including the STM2880 gene located within SPI1. The comparative transcriptomic analysis revealed that 19 genes were down-regulated by butyrate in both strains (Fig. 1B). Of these genes, 17 were SPI1 associated, and a description of their functions can be found in Table 1.

The key regulator of SPI1 is HilA, a member of the OmpR/

TABLE 1. List of functions of the SPI1 and SPI1-associated genes which are down-regulated more than two-fold in *Salmonella* serovar Typhimurium SL1344 and *Salmonella* serovar Enteritidis 76Sa88 with butyrate

Gene type and name	Function
Regulators	
invF	Transcriptional activator
hilD	Mediates derepression of *hilA*
iagB	Acts downstream of *hilA*, but function is unknown
Apparatus genes	
prgJ	Structural component of the SPI1-encoded TTSS
invE	Structural component of the TTSS
invI	Function still unknown
prgH	Major component of TTSS; membrane-associated lipoprotein
prgK	Major component of TTSS; membrane-associated lipoprotein
prgI	Major component of TTSS; membrane-associated lipoprotein
spaP	Putative inner membrane component of the TTSS
Secreted proteins and their chaperones	
invB	TTSS-associated chaperone of SopE
sipD	Required for the translocation of SipB, SipC, and other putative effector proteins
sipA	Large effector protein; polymerizes G-actin
sptP	Secreted effector protein tyrosine phosphatase, cytoskeletal disruption
invJ	Required for the secretion of several other proteins (SipB and SipC)
sopE2 (not SPI1 encoded but SPI1 associated)	Effector protein that mediates cytoskeletal rearrangements
STM2880 (FUN gene on SPI1)	Function unknown

ToxR family of transcriptional activators (2). It has been shown that a null mutation in *hilA* causes a dramatic attenuation of invasion, demonstrating the importance of HilA for entry into epithelial cells (14, 27). As HilA is crucial for invasion, its regulation has been intensively studied. The complex and coordinated mechanism of *hilA* expression is regulated by environmental and regulatory factors. Invasion is tightly orchestrated by several environmental signals and shows a significant decrease under conditions of high oxygen, low pH, and low osmolarity (3, 17). Numerous regulators of *hilA*, both activators and repressors, have been identified. BarA/SirA (1), HilD (14, 30), HilC/SirC/SprA (11, 12, 28, 30), CsrAB (1), PhoB, FadD, and FliZ (25) have been shown to activate *hilA* expression. The negative regulators, identified through transposon mutagenesis, include HilE, Ams, and Pag, whereas Hha, a small nucleoid-associated protein, was identified by introduction of a serovar Typhimurium chromosomal DNA gene bank into a serovar Typhimurium *hilA*:Tn*5*lacZY reporter strain (14, 15). Of the small nucleoid-binding proteins that affect *hilA* expression, H-NS acts as a repressor, but Fis and HU activate *hilA* by changing the expression of *hilD* (32).

Apart from *hilD*, none of these regulators were down-regulated by butyrate in both serovars. However, *hilA* expression was down-regulated sixfold in serovar Enteritidis but changed by less than twofold in serovar Typhimurium. In order to check the specificity of this change for serovar Enteritidis, we monitored the expression of plasmid-borne *hilA*::*luxCDABE* and *hilD*::*luxCDABE* transcriptional fusions both in serovar Typhimurium SL1344 and in serovar Enteritidis 76Sa88 (33). Our results are illustrated in Fig. 1B. These experiments confirmed that the addition of butyrate to both serovar Typhimurium and serovar Enteritidis grown in LB as described above resulted in down-regulation of *hilA* and *hilD*.

Two members of the AraC/XylS family of transcription activators, HilC and HilD, can independently derepress *hilA* expression by binding directly to the upstream repressing sequence and counteracting its repressing effect in vitro and in vivo (24, 30, 31). A cascade of transcriptional activation has been unraveled in which HilD, in response to particular environmental signals, directly affects *hilA* expression by binding to the upstream repressing sequence, thereby derepressing *hilA*. By binding upstream of *invF* and *prgH*, HilA directly activates the *invF* and *prgH* operons. InvF, in conjunction with SicA and PrgH, activates the *sip* genes encoding the TTSS effector proteins (8, 9, 11, 21, 23, 24). Studies suggested that HilC is not required to affect the HilA activity by any environmental conditions or inputs, except by the EnvZ/OmpR regulatory pathway (26). In contrast, all of the other environmental and regulatory inputs modulate *hilA* expression through transcriptional and/or posttranscriptional alteration of HilD or by modulating the activity of an unknown repressor (26).

Remarkably, we observed that most genes that showed significant changes after contact of both *Salmonella* strains with butyrate were genes of the *Salmonella* pathogenicity island 1. Thus, butyric acid specifically down-regulates SPI1 genes in both serovars. Our findings are consistent with the butyrate-mediated reduction in invasion reported previously (22, 34). The primary target of butyrate in the bacterial cell remains unknown, but our data suggest that butyrate could interfere with HilA-dependent regulation of SPI1 by altering the regulation of *hilD* transcription.

We show that butyrate targets a narrow and specific set of genes in *Salmonella*. This underlines the importance of butyrate as a signaling molecule in the intestine playing a crucial role in the maintenance of gut health. Since butyrate is a fermentation product of the intestinal flora, it is tempting to speculate that modification of gut flora to produce more bu-

tyric acid (e.g., by probiotics) could reduce invasive infections by *Salmonella* serovars in farm animals.

We are grateful to Vittoria Danino for commenting on the manuscript.

This project was funded by a Marie Curie Fellowship from the European Commission (contract number QLK2-GH-01-60081-09). Filip Van Immerseel is funded by a research grant from Ghent University (BOF).

REFERENCES

1. **Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer.** 2000. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. Mol. Microbiol. **35:**1872–1882.
2. **Bajaj, V., C. Hwang, and C. A. Lee.** 1995. HilA is a novel ompR/toxR family member that activates the expression of *Salmonella* Typhimurium invasion genes. Mol. Microbiol. **18:**715–727.
3. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella* Typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Mol. Microbiol. **22:**703–714.
4. **Chalker, R. B., and M. J. Blaser.** 1988. A review of human salmonellosis. III. Magnitude of *Salmonella* infections in the United States. Rev. Infect. Dis. **10:**111–124.
5. **Collazo, C. M., and J. E. Galán.** 1996. Requirement for exported proteins in secretion through the invasion-associated type III system of *Salmonella* Typhimurium. Infect. Immun. **64:**3524–3531.
6. **Cummings, J. H., E. W. Pomare, W. J. Branch, C. P. Naylor, and G. T. Macfarlane.** 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut **28:**1221–1227.
7. **Daoguo, Z., and J. E. Galán.** 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. **3:**1293–1298.
8. **Darwin, K. H., and V. L. Miller.** 1999. InvF is required for expression of genes encoding proteins secreted by the SPI-1 type III secretion apparatus in *Salmonella typhimurium*. J. Bacteriol. **181:**4949–4954.
9. **Darwin, K. H., and V. L. Miller.** 2000. The putative invasion protein chaperone SicA acts together with InvF to activate the expression of *Salmonella typhimurium* virulence genes. Mol. Microbiol. **35:**949–960.
10. **Desmidt, M., R. Ducatelle, and F. Haesebrouck.** 1997. Pathogenesis of *Salmonella enteritidis* phage type four after experimental infection of young chickens. Vet. Microbiol. **56:**99–109.
11. **Eichelberg, K., and J. E. Galán.** 1999. Differential regulation of *Salmonella typhimurium* type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and HilA. Infect. Immun. **67:**4099–4105.
12. **Eichelberg, K., W. D. Hardt, and J. E. Galán.** 1999. Characterization of sprA, an AraC-like transcriptional regulator encoded within the *Salmonella* Typhimurium pathogenicity island 1. Mol. Microbiol. **33:**139–152.
13. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the biology of the macrophage infection by gene expression profiling of intracellular *Salmonella* enterica. Mol. Microbiol. **47:**103–118.
14. **Fahlen, T. F., N. Mathur, and B. D. Jones.** 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella* Typhimurium. FEMS Immunol. Med. Microbiol. **28:**25–35.
15. **Fahlen, T. F., R. L. Wilson, J. D. Boddicker, and B. D. Jones.** 2001. Hha is a negative modulator of transcription of *hilA*, the *Salmonella enterica* serovar Typhimurium invasion gene transcriptional activator. J. Bacteriol. **183:**6620–6629.
16. **Frankel, W., J. Lew, B. Su, A. Bain, D. Klurfeld, E. Einhorn, R. MacDermott, and J. Rombeau.** 1994. Butyrate increases colonocyte protein synthesis in ulcerative colitis. J. Surg. Res. **57:**210–214.
17. **Galán, J. E., and R. I. Curtis.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:**1879–1885.
18. **Harig, J. M., K. H. Soergel, R. A. Komorowski, and C. M. Wood.** 1998. Treatment of diversion colitis with short-chain fatty acid irrigation. N. Engl. J. Med. **320:**23–28.
19. **Hautefort, I., M. J. Proença, and J. C. D. Hinton.** 2003. Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression in vitro and during infection of mammalian cells. Appl. Environ. Microbiol. **69:**7480–7491.
20. **Hoiseth, S. K., and B. A. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
21. **Kaniga, K., J. C. Bossio, and J. E. Galán.** 1994. The *Salmonella* Typhimurium invasion genes *invF* and *invG* encode homologues of the AraC and PulD family of proteins. Mol. Microbiol. **13:**555–568.
22. **Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier.** 2002. Intestinal short-chain fatty acids alter *Salmonella* Typhimurium invasion gene expression and virulence through BarA/SirA. Mol. Microbiol. **46:**1451–1464.
23. **Lostroh, J. P., V. Bajaj, and C. A. Lee.** 2000. The cis requirements for transcriptional activation by hilA, a virulent determinant encoded on SPI-1. Mol. Microbiol. **37:**300–315.
24. **Lostroh, J. P., and C. A. Lee.** 2001. The *hilA* box and sequences outside it determine the magnitude of *hilA*-dependent activation of P_{prgH} from *Salmonella* pathogenicity island 1. J. Bacteriol. **183:**4876–4885.
25. **Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee.** 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**1872–1882.
26. **Lucas, R. L., and C. A. Lee.** 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **183:**2733–2745.
27. **Penheiter, K. L., N. Mathur, D. Giles, T. Fahlen, and B. D. Jones.** 1997. Non-invasive *Salmonella* Typhimurium mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. Mol. Microbiol. **24:**697–709.
28. **Rakeman, J. L., H. R. Bonifield, and S. I. Miller.** 1999. A HilA-independent pathway to *Salmonella typhimurium* invasion gene transcription. J. Bacteriol. **181:**3096–3104.
29. **Ruemmele, F. M., S. Dionne, I. Qureshi, D. S. Sarma, E. Levy, and E. G. Seidman.** 1999. Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP). Cell Death Differ. **6:**729–735.
30. **Schechter, L. M., S. M. Scott, and C. A. Lee.** 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the hilA promoter. Mol. Microbiol. **32:**629–642.
31. **Schechter, L. M., and C. A. Lee.** 2001. AraC/XylS family members, hilC and hilD, directly bind and derepress the Salmonlla Typhimurium hilA promoter. Mol. Microbiol. **40:**1289–1299.
32. **Schechter, L. M., S. Jain, S. Akbar, and C. A. Lee.** 2003. The small nucleoid-binding proteins H-NS, HU, and Fis affect *hilA* expression in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **71:**5432–5435.
33. **Van Immerseel, F., J. De Buck, F. Boyen, L. Bohez, F. Pasmans, J. Volf, M. Sevcik, I. Rychlik, F. Haesebrouck, and R. Ducatelle.** 2004. Medium-chain fatty acids decrease colonization and invasion through *hilA* suppression shortly after infection of chickens with *Salmonella enterica* serovar Enteritidis. Appl. Environ. Microbiol. **70:**3582–3587.
34. **Van Immerseel, F., J. De Buck, I. Desmet, F. Pasmans, F. Haesebrouck, and R. Ducatelle.** 2004. Interactions of butyric acid- and acetic acid-treated *Salmonella* with chicken primary cecal epithelial cells in vitro. Avian Dis. **48:**384–391.
35. **Van Immerseel, F., V. Fievez, J. De Buck, F. Pasmans, A. Martel, F. Haesebrouck, and R. Ducatelle.** 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with *Salmonella* Enteritidis in young chickens. Poult. Sci. **83:**69–74.
36. **Yang, Y. H., and T. Speed.** 2002. Design issues for cDNA microarray experiments. Nat. Rev. Genet. **3:**579–588.