

Genetic Analysis of Non-Hydrogen Sulfide-Producing *Salmonella enterica* Serovar Typhimurium and *S. enterica* Serovar Infantis Isolates in Japan

Chieko Sakano,^a Makoto Kuroda,^b Tsuyoshi Sekizuka,^b Taisei Ishioka,^a Yukio Morita,^{a,c} Akihide Ryo,^d Hiroyuki Tsukagoshi,^a Yuko Kawai,^a Nobuko Inoue,^a Hayato Takada,^a Yumiko Ogaswara,^b Atsuyoshi Nishina,^e Masa-aki Shimoda,^a Kunihisa Kozawa,^a Kazunori Oishi,^{f,g} Hirokazu Kimura^{a,f}

Gunma Prefectural Institute of Public Health and Environmental Sciences, Maebashi-shi, Gunma, Japan^a; Pathogen Genomics Center, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan^b; College of Nutritional Science, Tokyo Kasei University, Kaga, Itabashi-ku, Tokyo, Japan^c; Department of Molecular Biodefense Research, Yokohama City University Graduate School of Medicine, Fukuura, Kanazawa-ku, Yokohama, Kanagawa, Japan^d; Yamagata Prefectural Yonezawa Women's Junior College, Yonezawa-shi, Yamagata, Japan^e; Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Gakuen, Musashimurayama-shi, Tokyo, Japan^f; Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan^g

Whole-genome sequencing of non-H₂S-producing *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Infantis isolates from poultry meat revealed a nonsense mutation in the *phsA* thiosulfate reductase gene and carriage of a CMY-2 β -lactamase. The lack of production of H₂S might lead to the incorrect identification of *S. enterica* isolates carrying antimicrobial resistance genes.

Salmonella enterica subsp. *enterica* serovar Typhimurium and *S. enterica* serovar Infantis of the genus *Salmonella* cause acute enterocolitis. Generally, H₂S production is considered to be indicative of the presence of pathogenic food-borne *Salmonella* serovars and is an important index for screening and confirmation of *Salmonella* using various agars, including DHL (deoxycholate hydrogen sulfide lactose), SS (*Salmonella-Shigella*), and TSI (triple sugar iron) agars.

We isolated non-H₂S-producing *S. Typhimurium* and *S. Infantis* strains from retail chicken meats in Gunma prefecture, Japan, in 2010. Forty-seven *Salmonella* spp. isolates were obtained from 95 poultry meat samples (49.5%) between June and December 2010. Four (8.5%) were non-H₂S-producing *Salmonella* (*S. Typhimurium* isolates GST-106, GST-108, and GST-204; *S. Infantis* isolate GSI-9) isolates. H₂S production and lysine decarboxylase testing of *Salmonella* on TSI and LIM (lysine indole motility) agar was subsequently conducted (see Fig. S1 in the supplemental material). The other isolates found to be H₂S producing were as follows: *S. enterica* serovar Enteritidis (1 strain), *S. enterica* serovar Yovokome (1 isolate), *S. enterica* serovar Livingstone (1 isolate), *S. enterica* serovar Schwarzengrund (3 isolates), *S. enterica* serovar Manhattan (3 isolates), *S. Infantis* (38 isolates), and *S. Typhimurium* (2 isolates).

Pulsed-field gel electrophoresis (PFGE) analysis (1) suggested that the profile of the *S. Infantis* and *S. Typhimurium* strains were similar but not identical among these serovars (see Fig. S2 in the supplemental material). Multilocus sequence typing (MLST) for *S. enterica* (<http://mlst.ucc.ie/mlst/dbs/Senterica/>) suggested that the *S. Typhimurium* strains belonged to ST328, while the *S. Infantis* strains belonged to ST32 (see Table S1 in the supplemental material).

To elucidate the genetic alteration responsible for H₂S production, Illumina GAIIX 81-mer paired-end short read sequencing of *S. Infantis* and *S. Typhimurium* isolates was performed (see Table S1 in the supplemental material), followed by short-read mapping (2) against the corresponding reference genome sequences of *S. Infantis* SIN (downloaded from the Wellcome Trust Sanger Insti-

tute [<ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/SIN.dbs>]) and *S. Typhimurium* T000240 (3), respectively. Whole-genome sequencing suggested that some nucleotide variations were present between H₂S-producing and non-H₂S-producing isolates (see Tables S2 and S3 in the supplemental material). Notably, the *phsA* gene, encoding the thiosulfate reductase precursor, was terminated in the coding sequence by an alteration to the stop codon (nonsense mutation) in both *S. Infantis* (GSI-9) and *S. Typhimurium* (GST-106, GST-108 and GST-204) (Fig. 1; also, see Tables S2 and S3 in the supplemental material). The *phs* genes are essential for the dissimilatory anaerobic reduction of thiosulfate to H₂S in *S. Typhimurium* (4, 5), suggesting that the disruption of the *phsA* gene could be involved in H₂S production. Intriguingly, a nonsense mutation in the *phsA* gene was also identified in low-H₂S-producing *S. enterica* serovar Paratyphi A (AKU_12601 and ATCC 9150) (6); thus, comparative genomic analysis also supports our findings.

H₂S is highly toxic to mammalian cells, including those of humans, and the cecal mucosa detoxifies H₂S by converting it to thiosulfate (S₂O₃²⁻) (7). Luminal inflammation generates reactive oxygen species, leading to the conversion of thiosulfate to tetrathionate (S₄O₆²⁻). *S. enterica* utilizes tetrathionate as an electron acceptor in anaerobic respiration using TtrABC and TtrRS located on a SPI2 pathogenicity island (8). It is possible that non-H₂S-

Received 20 August 2012 Returned for modification 11 September 2012

Accepted 27 October 2012

Published ahead of print 7 November 2012

Address correspondence to Hirokazu Kimura, kimhiro@nih.go.jp, or Makoto Kuroda, makokuro@nih.go.jp.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.02225-12>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.02225-12

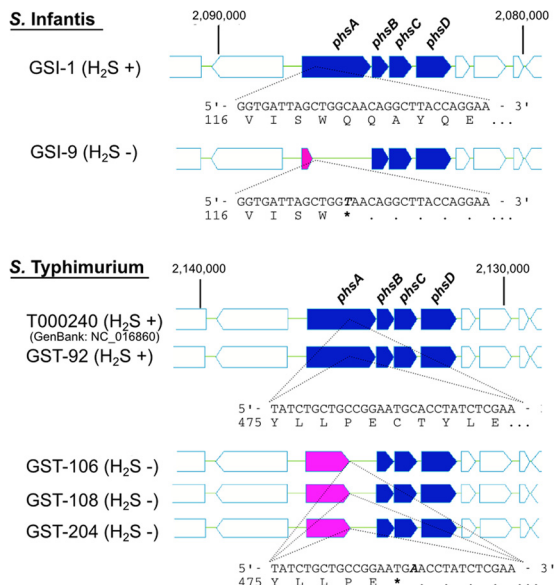


FIG 1 Schematic representation of the nonsense mutation in the *phsA* gene encoding the thiosulfate reductase subunit. Nucleotide variations and corresponding amino acid mutations are shown below the gene organization schemes.

producing phenotypes generate more thiosulfate, leading to enough substrate for TtrABCERS anaerobic respiration in *Salmonella* spp. Indeed, recent studies have suggested that TtrABCERS anaerobic respiration based on tetrathionate contributes to competitiveness with other microbiota in intestinal inflammation (9, 10, 11), implying that reduced H₂S production might lead to increased TtrABCERS anaerobic respiration that promotes growth and colonization in the gut.

Furthermore, the non-H₂S-producing *S. Infantis* and *S. Typhimurium* isolates carried CMY-2 β -lactamase (Table 1) and demonstrated reduced susceptibility to cefazolin (data not shown). Aminoglycoside and trimethoprim resistance genes were also identified (Table 1). A plasmid carrying the *bla*_{CMY-2} gene was detected by S1 nuclease-PFGE and Southern blot analysis (12), indicating that it appears to have been an independent acquisition by two different serovars (Fig. 2). The CMY enzyme is the enzyme most frequently detected in *E. coli*, *Klebsiella pneumoniae*, and *Salmonella* spp. (13), and poultry meat is most frequently contaminated with CMY-2-producing *Salmonella* (14). Indeed, CMY-2-producing *S. Infantis* and *S. Typhimurium* isolates have been found in retail chicken and cattle in Japan (15, 16). Because of the clinical importance of

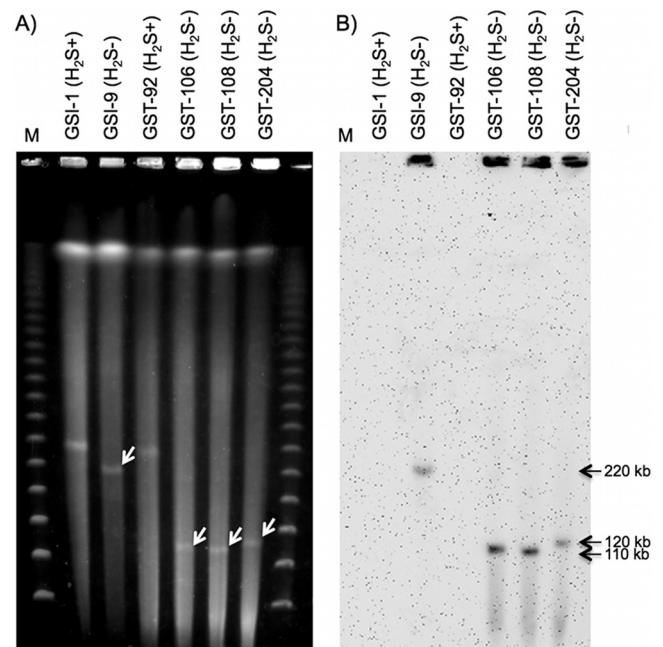


FIG 2 PFGE-Southern blot hybridization for *bla*_{CMY-2} detection. (A) S1 nuclease-PFGE analysis of the *Salmonella* isolates. (B) Southern blot obtained using a *bla*_{CMY-2}-specific DNA probe. M, lambda ladder marker for PFGE.

third- and fourth-generation cephalosporin in human and veterinary medicine, it is of further concern that insufficient testing might lead to antimicrobial-resistant *Salmonella* isolates being missed.

In conclusion, we isolated non-H₂S-producing *S. enterica* isolates. Whole-genome analysis suggested that this lack of H₂S production may be associated with a *phsA* gene mutation and that these strains may be disseminated in poultry. Although we did not examine the pathogenicity of these strains in experimental animals, this defect may be associated with energy production and gut colonization. In addition, such strains may slip unnoticed past screening methods, resulting in incorrect identification of *S. enterica*, including antimicrobial resistant isolates. Comprehensive testing will be required to detect unique isolates and to map the overall epidemiology of *S. enterica*.

Nucleotide sequence accession number. Obtained short reads have been deposited in the DDBJ Sequence Read Archive of Japan (accession number DRA000592).

TABLE 1 Antimicrobial resistance genes in *Salmonella* isolates^a

Isolate	Aminoglycosides			β -Lactams			Trimethoprim— <i>dfrA14</i>
	<i>aadA1</i>	<i>aac(6')-Iaa</i>	<i>aph(3')-Ic</i>	<i>bla</i> _{CMY-2}	<i>bla</i> _{TEM-1}	Tetracycline— <i>tet(B)</i>	
GSI-1 (H ₂ S ⁺)	+	—	—	—	—	—	+
GSI-9 (H ₂ S ⁻)	—	+	—	+	—	—	—
GST-92 (H ₂ S ⁺)	+	+	+	—	+	—	+
GST-106 (H ₂ S ⁻)	—	+	—	+	—	+	—
GST-108 (H ₂ S ⁻)	—	+	—	+	—	+	—
GST-204 (H ₂ S ⁻)	—	+	—	+	—	+	—

^a Antimicrobial resistance genes were identified in a ResFinder web search (<http://www.cbs.dtu.dk/services/ResFinder/>).

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare, Japan (H24 Shokuhin-Ippan-008).

REFERENCES

- Gerner-Smidt P, Hise K, Kincaid J, Hunter S, Rolando S, Hyytia-Trees E, Ribot EM, Swaminathan B. 2006. PulseNet U. S. A.: a five-year update. *Foodborne Pathogens Dis.* 3:9–19.
- Li H, Durbin R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 26:589–595.
- Izumiya H, Sekizuka T, Nakaya H, Taguchi M, Oguchi A, Ichikawa N, Nishiko R, Yamazaki S, Fujita N, Watanabe H, Ohnishi M, Kuroda M. 2011. Whole-genome analysis of *Salmonella enterica* serovar Typhimurium T000240 reveals the acquisition of a genomic island involved in multidrug resistance via IS1 derivatives on the chromosome. *Antimicrob. Agents Chemother.* 55:623–630.
- Clark MA, Barrett EL. 1987. The *p*hs gene and hydrogen sulfide production by *Salmonella typhimurium*. *J. Bacteriol.* 169:2391–2397.
- Alami N, Hallenbeck PC. 1992. Mutations that affect the regulation of *p*hs in *Salmonella typhimurium*. *J. Gen. Microbiol.* 138:1117–1122.
- McClelland M, Sanderson KE, Clifton SW, Latreille P, Porwollik S, Sabo A, Meyer R, Bieri T, Ozersky P, McLellan M, Harkins CR, Wang C, Nguyen C, Berghoff A, Elliott G, Kohlberg S, Strong C, Du Carter F J, Kremizki C, Layman D, Leonard S, Sun H, Fulton L, Nash W, Miner T, Minx P, Delehaanty K, Fronick C, Magrini V, Nhan M, Warren W, Florea L, Spieth J, Wilson RK. 2004. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. *Nat. Genet.* 36:1268–1274.
- Levitt MD, Furne J, Springfield J, Suarez F, DeMaster E. 1999. Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. *J. Clin. Invest.* 104:1107–1114.
- Hensel M, Hinsley AP, Nikolaus T, Sawers G, Berks BC. 1999. The genetic basis of tetrathionate respiration in *Salmonella typhimurium*. *Mol. Microbiol.* 32:275–287.
- Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW, Russell JM, Bevins CL, Adams LG, Tsois RM, Roth JR, Baumler AJ. 2010. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* 467:426–429.
- Thiennimitr P, Winter SE, Winter MG, Xavier MN, Tolstikov V, Huseby DL, Sterzenbach T, Tsois RM, Roth JR, Baumler AJ. 2011. Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. *Proc. Natl. Acad. Sci. U. S. A.* 108:17480–17485.
- Rohmer L, Hocquet D, Miller SI. 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. *Trends Microbiol.* 19:341–348.
- Shahada F, Sekizuka T, Kuroda M, Kusumoto M, Ohishi D, Matsumoto A, Okazaki H, Tanaka K, Uchida I, Izumiya H, Watanabe H, Tamamura Y, Iwata T, Akiba M. 2011. Characterization of *Salmonella enterica* serovar Typhimurium isolates harboring a chromosomally encoded CMY-2 β -lactamase gene located on a multidrug resistance genomic island. *Antimicrob. Agents Chemother.* 55:4114–4121.
- Li XZ, Mehrotra M, Ghimire S, Adewoye L. 2007. β -Lactam resistance and β -lactamases in bacteria of animal origin. *Vet. Microbiol.* 121:197–214.
- Arlet G, Barrett TJ, Butaye P, Cloeckert A, Mulvey MR, White DG. 2006. *Salmonella* resistant to extended-spectrum cephalosporins: prevalence and epidemiology. *Microbes Infect.* 8:1945–1954.
- Taguchi M, Seto K, Yamazaki W, Tsukamoto T, Izumiya H, Watanabe H. 2006. CMY-2 β -lactamase-producing *Salmonella enterica* serovar Infantis isolated from poultry in Japan. *Jpn. J. Infect. Dis.* 59:144–146.
- Sugawara M, Komori J, Kawakami M, Izumiya H, Watanabe H, Akiba M. 2011. Molecular and phenotypic characteristics of CMY-2 β -lactamase-producing *Salmonella enterica* serovar Typhimurium isolated from cattle in Japan. *J. Vet. Med. Sci.* 73:345–349.