

Full Length Research Paper

Analysis of the protein profiles of the antibiotic-resistant *Salmonella typhimurium* definitive phage type (dt) 104

Yakubu B. Ngwai^{1,a,*}, Kozo Ochi², Yasuki Ogawa³ and Yoshikazu Adachi³

¹Department of Microbiology, Human Virology and Biotechnology, National Institute for Pharmaceutical Research and Development, P.M.B. 21 Garki, Abuja, Nigeria.

²National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki, 305-8642, Japan.

³Animal Health Laboratory, School of Agriculture, Ibaraki University, 3-21-1 Ami, Ami-machi, Ibaraki-ken, 300-0393, Japan.

Accepted 18 May, 2020

The emergent *Salmonella typhimurium* definitive phage type (DT) 104 is of particular global concern due to its frequent isolation and multiple antibiotic resistances. There is thus a need to know the kind of proteins expressed by *S. typhimurium* DT104 so as to provide a basis for developing an intervention. This study examined the protein profiles of a few isolates of *S. typhimurium* DT104 and a non-DT104 strain *S. typhimurium* L1388 (ST). Crude SDS-soluble whole cell and outer membrane protein (OMP) extracts revealed similar protein profiles for both phage types. A single major protein band (28.4 kDa) was observed in periplasmic fractions from both phage types. However, proteins released into growth medium was variable; one of the DT104 isolates had common proteins with the non-DT104 strain ST. Similar SDS-soluble whole cell protein profiles were observed for both phage types grown in a low-iron Medium A at 37°C; but a 38.5 kDa protein (observed in TSB-grown cells) was observed only in the temperature-tolerant DT104 isolate. The protein contents of cell-free ultracentrifuge supernatants of sonically disrupted cells of each of the DT104 isolates were significantly ($P < 0.05$) more than that from ST L1388, but the latter expressed a 51-kDa protein absent in the supernatants of all DT104 isolates. The higher protein content of DT104s provides possible indication of increased production of protein-like metabolites. Although the N-terminal sequence of the first twenty amino acids of the 51-kDa protein (Ala-Gln-Val-Ile-Asn-Thr-Asn-Ser-Leu-Ser-Leu-Leu-Thr-Gln-Trp-Ala-Ala-Ala-Ala) showed 14-amino acid overlap and resemblance with the flagillin, FLIC, only fourteen of its 104 trypsin digests were homologous with those of FLIC. Further work is being done to characterize this protein and to investigate its potential for use as vaccine target through antigenicity tests.

Key words: *Salmonella typhimurium* DT104, protein profile.

INTRODUCTION

In human, swine, avian and bovine salmonellosis, *Salmonella typhimurium* is the most frequently isolated serotype out of the over 2,400 serotypes in the genus *Salmonella* (Schwartz, 1999; Popoff, 1997). It is thus a common cause of bacterial gastroenteritis worldwide,

with contaminated food of animal origin being the major source of these pathogens (Fierer and Swancutt, 2000; D'Aoust, 1994). The emergence and spread of the definitive phage type strain (DT104) is an important human and veterinary concern globally, because of the strain's multiple-drug resistances and extensive host range (Baggessen et al., 2000; Sameshima et al., 2000; Poppe et al., 1998; Threlfall et al., 1996; Akkina et al., 1999; Besser et al., 1997). An infection with this strain, in humans, is associated with common symptoms such as diarrhea, fever, abdominal pain, vomiting, and blood in the stool (Poppe et al., 1998). Some reports have associated *S. typhimurium* DT104 with a possible higher

*Corresponding author. E-Mail: ybngwai@yahoo.com, Tel: +234-80-43296945.

^aThis study was done while at the Animal Health Laboratory, School of Agriculture, Ibaraki University, Ami-machi, Ibaraki, Japan.

Table 1. Relevant characteristics of *S. typhimurium* test strains.

Strains	Source	Country of isolation	Minimum inhibitory concentration (MIC) in $\mu\text{g ml}^{-1}$										Resistance
			A	T	C	F	G	K	S	Su	Tmp	Na	
306-98	Human	CDC (U.S.A)	> 512	128	256	64	1	2	512	> 512	0.5	4	ACFSSuT
T980018	Human	CDC (U.S.A)	> 512	64	256	64	0.5	2	256	> 512	0.5	4	ACFSSuT
T980021	Human	CDC (U.S.A)	> 512	256	256	128	1	2	>512	> 512	1	4	ACFSSuT
T980042	Human	CDC (U.S.A)	> 512	64	256	64	1	2	128	> 512	0.5	4	ACFSSuT
T980043	Human	CDC (U.S.A)	> 512	128	256	64	1	2	256	> 512	0.5	4	ACFSSuT
ST ^a	Chick	Japan	1	2	8	8	0.5	2	32	64	0.5	4	SSu

A, Ampicillin; T, tetracycline; C, chloramphenicol; F, florfenicol; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfamethoxazole; TMP, trimethoprim; and NA, nalidixic acid. ^aST, *S. typhimurium* L1388. MIC was determined by the doubling agar dilution method of NCCLS, U.S.A.

virulence than the non-multi-resistant strains (Akkinä et al., 1999; Szmolleny et al., 2000), but other studies using assays based on stress response, tissue invasion, macrophage survival and mortality rate in animals, have indicated otherwise (Allen et al., 2001; Jorgensen et al., 2000; Carlson et al., 2000a; Carlson et al., 2000b; Threlfall et al., 1998).

The overall gene complex in DT104 is comprised of a sequence of approximately 1.4 kb containing two integrons and intervening plasmid-derived sequences coding for resistance to chloramphenicol and tetracyclines (Briggs et al., 1999). There is evidence to suggest a relationship between the expression of antibiotic resistance and bacterial virulence (Bjorkman et al., 1998). Resistant genes usually mediate changes in the cell surface physicochemical properties, such as hydrophobicity and charge, which are relevant to the initial stages of bacterial attachment to tissues (Onalapo et al., 1987; Onalapo and Klemperer, 1986). Bacterial cell surface physicochemical properties are determined by the components of the cell surface, such as proteins, lipopolysaccharides (LPS) and phospholipids, which interact with host cells (Williams et al., 1986). No study has been reported on the array of proteins produced by DT104. In this study, we examined the profiles of five human isolates of the multi-resistant strain *S. typhimurium* DT104 and a non-multi-resistant strain *S. typhimurium* L1388, with the hope of finding differences or otherwise in the protein profiles. To our knowledge, no similar report has preceded this one.

MATERIALS AND METHODS

Bacterial isolates and culture media

Five isolates of the multi-resistant strain *S. typhimurium* DT104 (Mr-DT104) from human (306-98, T980018, T980021, T980042, and T980043) sources, and a non-multi-resistant strain *S. typhimurium* L1388 (ST), were used in this study. Relevant characteristics of the isolates are given in Table 1. All the isolates were propagated in trypticase soy broth (TSB; BBL, U.S.A.) or as otherwise indicated. Unless otherwise indicated, all chemicals used were from Wako Chemical Company, Japan.

Whole cell (WC) protein

Whole cell lysates were prepared by a method adapted from Hitchcock and Brown (1983). Briefly, a colony was grown in TSB for 24 h at 37°C; cells were harvested by centrifugation (3000 rpm x 5 min) at room temperature, washed at least three times with, and re-suspend in, 10 ml of PBS (0.9 g NaCl, 0.02 g KCl, 0.02 g KH₂PO₄, 0.29 g Na₂HPO₄, distilled water to 100 ml, pH 7.2). Cells from 1.5 ml of the washed culture were harvested in a microfuge tube, re-suspended in 100 μl of single strength SDS-PAGE sample lysis buffer (62.5 mM Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue, pH 6.8) and heated at 100°C for 10 min. The lysed samples were then centrifuged (5000 rpm x 10 min) at room temperature (RT), and 10 μl of the filtrates were loaded on a SDS- 10% or 12% PAGE gel and electrophoresed using Tris-Glycine-SDS buffer system (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) developed by Laemmli (1970). Molecular weights were determined by regression analysis of a standard molecular weight marker (Broad-range marker: New England BioLabs Inc., UK) separated on the same gel parallel to the samples. Gels were stained for 1 h in 0.25% Coomassie Brilliant Blue R-250 (CBB; SIGMA, U.S.A.) in 50% methanol: 10% acetic

acid: 40% distilled water, and de-stained in 5% methanol: 10% acetic acid: 85% distilled water.

Outer membrane protein (OMP)

Crude OMP extracts were prepared by the as described by Filip et al. (1973) with some modifications. Briefly, one colony of each isolate was grown in 30 ml of TSB for 24 h at 37°C, and cells were harvested by centrifugation (6000 rpm x 10 min x 4°C), washed three times with, and re-suspend in, 10 ml of PBS. The cell suspension was sonicated over ice at constant maximum pulse pressure for 3 min, with 30 s cooling interval between the bursts. To the broken cell suspension was added 10 ml of 4% solution of sodium N-lauroyl sarcosine (SARKOSYL: SIGMA, U.S.A) in PBS to a final 2% SARKOSYL concentration; and the mixture was left to stand at room temperature for 1 h. Unbroken cells and cellular debris were removed by centrifugation (9,500 rpm x 10 min x 4°C), and the cell -free supernatant was ultra-centrifuged (40,000 rpm x 1 h x 4°C) to pellet the OMP. The OMP pellets of each isolate were re-suspended in 100 µl of distilled water; 10 µl aliquots were mixed with double strength SDS-PAGE sample lysis buffer (125 mM Tris-HCl, 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue, pH 6.8), and heated at 100°C for 10 min. Samples were loaded on SDS-10% PAGE, electrophoresed, and stained as described for whole cell protein.

EDTA-soluble extract

EDTA-soluble proteins were prepared as described previously by Poxton and Byrne (1981) with minor modifications. Cells were harvested (8000 rpm x 20 min x RT) from 25 ml of 24-h TSB culture and washed three times with PBS. The washed cells were re-suspended in 0.4 ml of PBS-10 mM 2Na.EDTA.2H₂O, heated (45°C, 20 h), vortex-mixed briefly, and centrifuged (15,000 rpm x 10 min x 4°C) to remove the cells. Aliquots were mixed with equal volumes of double strength SDS-PAGE lysis buffer and loaded on SDS-12% PAGE gel for electrophoresis as already described.

Periplasmic proteins

Periplasmic proteins were released by osmotic shock treatment based on the method of Nossal and Heppel (1966) with modifications. Briefly, bacteria were grown in 25 ml of TSB at 37°C for 24 h with shaking; cells were harvested by centrifugation (8000 rpm x 30 min x 4°C) and washed two times with PBS (pH 7.2). The washed cell pellet was resuspended in 0.5 ml of fractionation buffer (20% sucrose, 1 mM EDTA, 30 mM Tris-HCl; pH 8.0) and incubated on ice for 10 min. Cells were then harvested (5000 rpm x 10 min x 4°C); the pellet was re-suspended in 0.5 ml ice-cold 5 mM MgSO₄ with shaking at room temperature for 10 min, and the supernatant containing periplasmic proteins was collected by centrifugation (5000 rpm x 10 min x 4°C). Both the supernatant (after extraction with the fractionation buffer) and the periplasmic fractions from each of the isolates were mixed with equal volumes of double strength SDS-PAGE lysis buffer, boiled (100°C, 5 min) and analyzed on 12% SDS-PAGE.

Cell-free culture supernatant (CFCS) analysis

Proteins secreted by each of the isolates into culture medium during growth were analyzed. Briefly, 50 ml of TSB was inoculated with one colony of a bacterium and incubated (37°C, 24 h) with shaking. Culture supernatant was collected after centrifugation (6000 rpm x 20 min x 4°C), and filtered through 0.4 µm-cellulose acetate filter

ADVANTEC: Toyo Roshi Kaisha, Ltd., Japan) to remove bacterial cells. The protein in CFCS was precipitated on ice (for 45 min) with 10% trichloroacetic acid, washed three times with ice-cold 100% acetone, and air-dried. The protein pellet was dissolved in 1X SDS-PAGE lysis buffer, heated (100°C, 5 min), and run on 8% SDS-PAGE gel as earlier described.

Flagillin analysis

Flagillin proteins were extracted with acidified PBS as described by Ibrahim et al. (1985) with certain modifications. One colony of each strain was taken from 24-h DHL agar culture, inoculated into two 250 ml of TSB in separate 500-ml flasks, and incubated (37°C, 18-24 h) with shaking at 65 rpm. The two cultures were pooled together, and the cells harvested (7000 rpm, 4°C, 20 min), washed two times with PBS (pH 7.2). The washed cell pellet was re-suspended in 40 ml of acidified PBS (pH 1.9, adjusted with HCl), then stirred for 50 min at room temperature, and centrifuged (7000 rpm, 4°C, 20 min). The supernatant was ultracentrifuged (35,500 rpm, 4°C, 1 h) to further remove cellular and insoluble materials. The pH of the supernatant was adjusted to 7.2 with 1 N NaOH, and soluble protein was precipitated with solid ammonium sulfate (added to a final 2.67 M concentration) at 4°C overnight. The protein was then collected by centrifugation, dried and stored at -20°C for future use or boiled in SDS sample buffer in preparation for SDS-PAGE.

Analysis of cell-free ultracentrifuge supernatant (CFUS) protein

Cell-free centrifuge supernatant was prepared based on the method of Chopra et al. (1987) with certain modifications. Briefly, each bacterial strain was grown in 50 ml TSB at 37°C for 24 h with shaking (65 tows per minute). The cells were harvested (6000 rpm x 10 min x 4°C), washed three times with, and re-suspended in 20 ml of, phosphate-buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄, distilled water to 1000 ml; pH 7.2). The washed cell suspension was sonicated (three bursts at 30 s interval for cooling) on ice at constant maximum pulse pressure using a 250D Branson Sonifier (Branson, Japan). Unbroken cells and cellular debris were removed by centrifugation (9500 rpm x 10 min x 4°C), and the supernatant was ultra-centrifuged (45,000 rpm x 3 h x 4°C) to remove cell membranes and other particulate matter. The protein content of the supernatant was assayed by spectrophotometric measurement of absorbance at 280 nm (A₂₈₀) using bovine serum albumin (BSA) as standard. Results are means of four experiments. The protein in the supernatant was precipitated with 10% trichloroacetic acid, and analyzed by 12% SDS-PAGE as earlier described.

Protein profiles following growth in low iron medium

Whole cell protein preparations of bacteria grown in low-iron Medium A (0.7 g K₂ HPO₄, 0.4 g KH₂PO₄, 0.2 g (NH₄)₂SO₄, 0.02 g MgSO₄.7H₂O, 0.5% D-glucose, and distilled water to 100 ml; pH 6.95) (Pollack et al., 1970) were compared by SDS-PAGE analysis as earlier described.

Two-dimensional electrophoresis (2DE)

TSB-grown whole cell, flagillin, CFUS and low iron (Medium A)-grown whole cell protein samples of selected strains were analyzed by two-dimensional SDS-PAGE (shortened to 2DE) using tube gels based on O' farrell (1975) as described by Ames and Nikaido (1976) with modifications. Briefly, the gel mixture containing 1.92 g

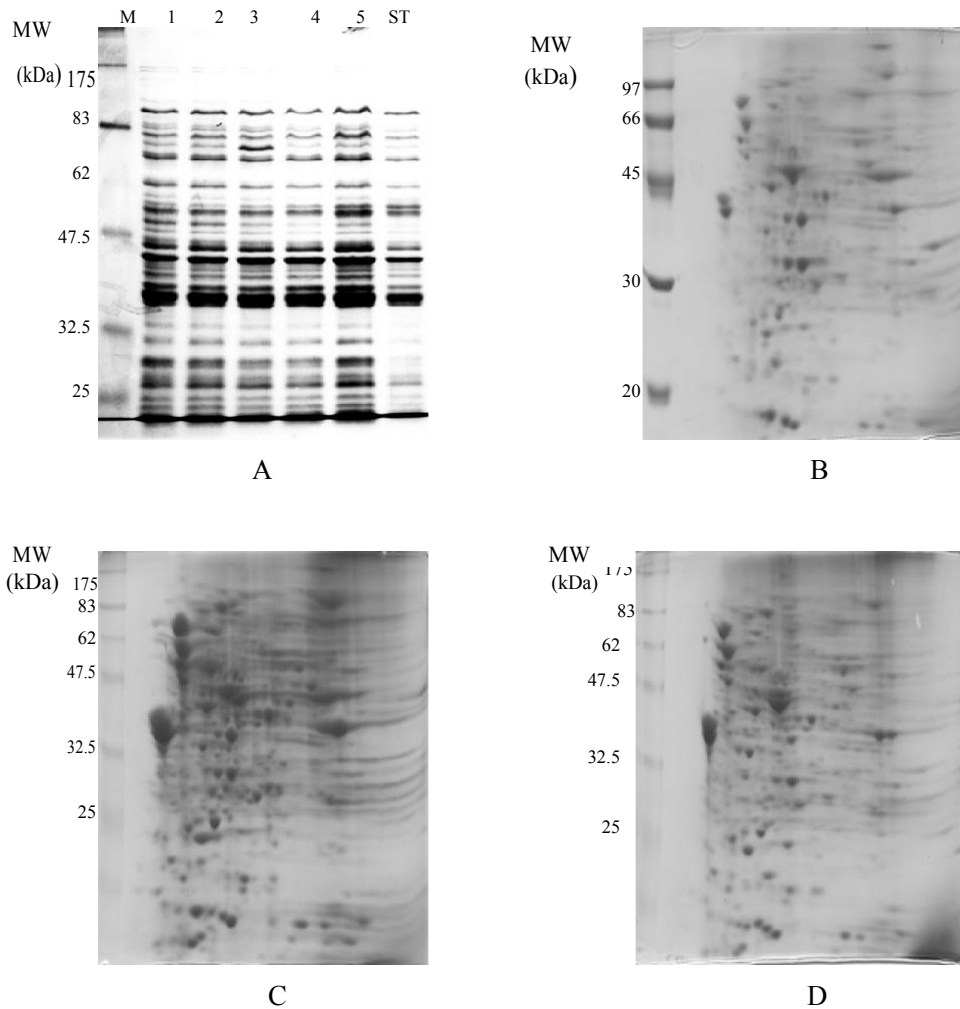


Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the whole cell (WC) proteins of *S. typhimurium* DT104 and *S. typhimurium* L1388 (ST) strains. A: one-dimensional SDS- 10% PAGE of WC proteins prepared by boiling (100°C, 10 min) PBS-washed, TSB-grown cell pellets in SDS sample buffer; 1-5 are respectively 306-98, T980018, T980021, T980042, and T980043; B, C, and D are two-dimensional (2D) SDS-12% PAGE of T980021, T980043 and ST respectively. Samples for 2D were pre- extracted by boiling in SDS boiling (100°C, 10 min) buffer and then acid-precipitated and re-solubilized in urea sample buffer as described in *Materials and Methods*. MW (in kilo dalton) are A, C and D: prestained broad range (New England BioLabs, UK), and B: Low Molecular Weight Calibration Kit (Amersham-Biotech, Sweden).

urea, 0.53 ml of 30% acrylamide stock (29 g acrylamide: 1 g N, N'-Methylene-bis (acrylamide), 0.8 ml of 10% Triton X-100, 160 µl of 40% ampholyte (pH 5~7: Ampholine[®], Pharmacia Biotech, Sweden), 40 µl of 40% ampholyte (pH 3.5~10): Ampholine[®], Amersham Biosciences, Sweden) and distilled water to 4 ml was prepared. Ammonium persulfate (8 µl of 10% stock) and 5.6 µl of N, N', N'- N'-tetramethylethylenediamine (TEMED) were subsequently added in succession, and the final gel mixture was poured into tubes to the 7-cm mark measured from the bottom making sure that bubbles were excluded. The gels were overlaid with 2 M urea and the tubes left to stand vertically overnight to allow complete polymerization of the acrylamide gel matrix.

Whole cell protein samples for 2DE were prepared by boiling (100°C, 5 min) PBS-washed cells (25 ml culture-equivalent) in SDS-boiling buffer (5% SDS, 5% 2- mercaptoethanol, 10% glycerol, 60 mM Tris-HCl, pH 6.8). The supernatants were collected by centrifugation (16,000 rpm x 4°C, 5 min) and the protein content

precipitated with 10% trichloroacetic acid on ice for 30 min. The proteins were pelleted by centrifugation, washed three times with ice-cold 100% acetone and air-dried. The dried whole cell proteins and other samples (flagillin, CFUS) for 2DE were digested (100°C, 5 min) in 4 µl of 10% SDS and dissolved in 100 µl of urea sample buffer containing 8 M urea, 4% Triton X-100, 20 mM dithiothreitol, 2% ampholyte (pH 3.5~10) and traces of bromophenol blue.

The gel overlays were removed, and the gels loaded with 50- 60 µl of samples; the samples were re-overlaid successively with 20 µl of 2 M urea and 0.2 M NaOH (upper buffer). The tubes were then assembled in the isoelectric focusing unit (ATTO Corporation, Japan) and focused (10 min at constant 50 V, 10 min at constant 100 V, and 205 min at constant 300 V) in accordance with manufacturer's instructions using 6 mM H₃PO₃ and 0.2 M NaOH as lower and upper buffers respectively. After focusing (also called 'first dimension'), the gels expelled from the tubes were rinsed with 'distilled water and then transferred into an equilibration

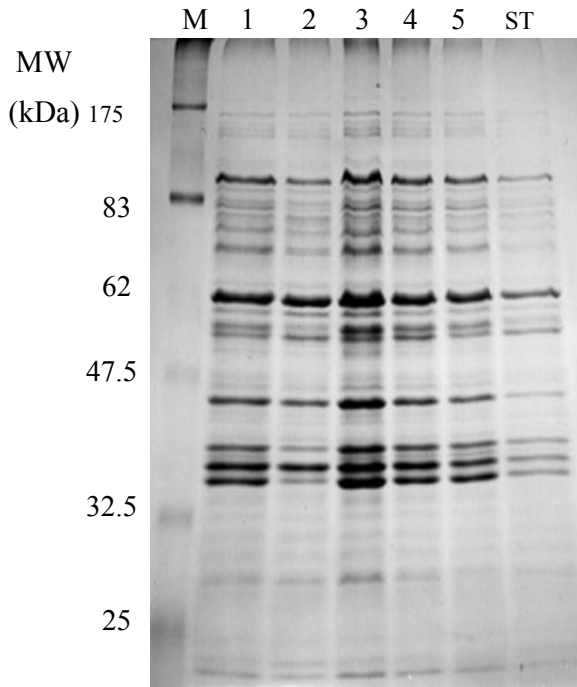


Figure 2. Outer membrane protein (OMP) of *S. typhimurium* DT104 (306-98 [1], T980018 [2], T980021 [3], T980042 [4], T980043 [5]) and *S. typhimurium* L1388 (ST) strains analyzed by 10% SDS-PAGE. Bacterial cells suspended in PBS were disrupted on ice by sonication at maximum pulse for 2 min and unbroken cells/cell debris was removed by centrifugation. The filtrate was mixed with aqueous solution of Sarkosyl to final 2%, allowed to stand at room temperature for 45 min, and subsequently ultracentrifuged to pellet the OMP. The pellets were then analyzed by SDS-PAGE. M: prestained broad range molecular weight marker (BioLabs).

buffer (2% SDS, trace quantity of bromophenol, 50 mM Tris-HCl, pH 6.8) for 30 min. The equilibrated gels were laid over the stacking gels of normal slab SDS-PAGE gels with the help of 0.5% agarose dissolved in single-strength electrophoresis buffer and run in the so-called 'second dimension' at a constant 100 V until the dye eluted from the gel. The gels were stained and de-stained as in normal SDS-PAGE.

N-Terminal amino acid sequencing by Edman degradation

CFUS protein sample of the non -DT104 strain ST was separated by 10% SDS- PAGE as usual, and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon- P: Millipore Corp., U.S.A) using the BioRad's Transblot[®] equipment at a constant voltage of 20V for 30 min in accordance with manufacturer's instructions, with the exception that PVDF was pre- wetted for 10 s in 100% methanol prior to equilibration in blotting buffer for 5 min. After transfer, the membrane was stained for 5 min in CBB dye and de-stained as usual to reveal the bands. The blot was then washed in distilled water and air-dried. The band of interest was excised from the blot for N-terminal amino acid sequencing using the PTH sequencer (Hitachi Co., Japan).

Peptide mass fingerprinting

Peptide analysis of the 51-kDa protein from CFUS of the non-DT104 strain ST was done by In-Gel digestion as described in the Applied Biosystems protocol manual. The protein band was excised from the Coomassie brilliant blue (CBB)-stained SDS-PAGE gel, size-reduced and transferred to a clean 1.5-ml microfuge tube. The gel slice was washed three times with 50% acetonitrile- 25 mM ammonium bicarbonate (pH 8.0) to remove excess CBB and then soaked for 5 min in 100% acetonitrile (ACN) to dehydrate the gel. The acetonitrile was removed completely from the microfuge tube and the gel was dried in a Speed-Vac. The dried gel was digested at 37°C for 18 h with sufficient volume of ice-cold trypsin (Stock: 12.5 µg ml⁻¹ in 25 mM ammonium bicarbonate, pH 8.0). The resulting peptides were extracted by soaking the digested gel pieces in two successive 100 µl of 50% ACN-5% trifluoroacetic acid for 1 h each and the supernatants recovered into a clean microfuge tube. The combined supernatant was dried by Speed-Vac. The dried peptide residue was reconstituted in 5 µl of 0.1% trifluoroacetic acid (TFA) . 2 µl of the reconstituted peptide was mixed on matrix-assisted laser desorption ionization (MALDI) plate with 1 µl of fresh cyano matrix suspended in a solution containing two volumes of 0.1% TFA and one volume of CAN, and analyzed with a MALDI time-of-flight (TOF) spectrometer (Applied Biosystems) in accordance with manufacturer's instructions. Similarity search was done using the Mascot database (<http://www.matrixscience.com>).

Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) using WebStat 2.0 (West, W.R. and Ogden, T.R. October 6, 2000, posting date. [Online] Department of Statistics, University of South Carolina, Columbia, U. S. A. <http://www.stat.sc.edu/webstat/>; June 20, 2002 [date last accessed]), and significance of results determined at the 5% probability level (that is, at $P = 0.05$).

RESULTS

Whole cell (WC) protein

SDS- soluble whole cell proteins profile of DT104s and ST is as shown in Figure 1. SDS- soluble whole cell lysates (representing total cell protein) of all the DT104s and ST indicated similar banding patterns. 2DE maps of WC proteins of selected isolates grown in the same media were also similar (Figures 1B, C and D).

Outer membrane protein (OMP)

OMP profiles of all the isolates were also similar (Figure 2) , with sharp protein bands between 34.7 and 94.9 kDa in each of the DT104s and ST.

EDTA-soluble extract

EDTA-soluble fractions of DT104s and ST were similar (Figure 3). Sharp bands of 28 and 39 kDa proteins were observed in the EDTA-soluble extracts from all the DT104s and ST.

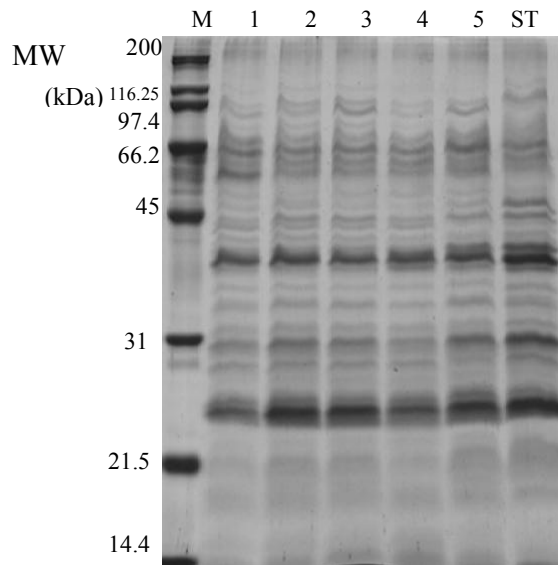


Figure 3. EDTA-soluble proteins of *S. typhimurium* DT104 (306-98 [1], T980018 [2], T980021 [3], T980042 [4], T980043 [5]) and *S. typhimurium* L1388 (ST) strains analyzed by 12% SDS-PAGE. EDTA-soluble proteins extracted (45^o C, 20 h) from PBS-washed cell pellets with PBS-10 mM EDTA. M: Broad range molecular weight marker (BioRad).

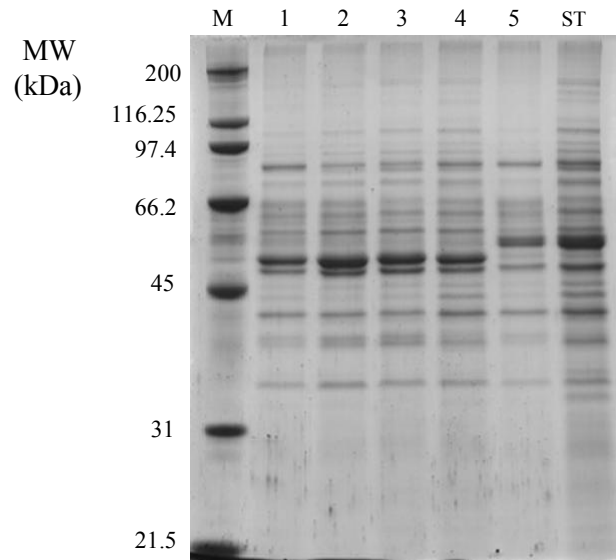


Figure 5. Cell-free culture supernatant (CFCS) protein of *S. typhimurium* DT104 (306-98 [1], T980018 [2], T980021 [3], T980042 [4], T980043 [5]) and *S. typhimurium* L1388 (ST) strains analyzed by SDS-10% PAGE. 24 h TSB-culture supernatants were collected and filtered (0.4 μ m pore size filter). Protein was precipitated with 10% TCA and analyzed by SDS-PAGE. M: Broad range molecular weight Marker (BIORAD).

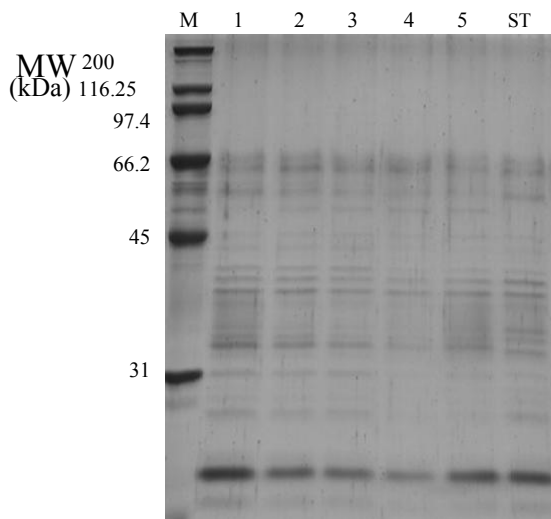


Figure 4. Periplasmic proteins of *S. typhimurium* DT104 (306-98 [1], T980018 [2], T980021 [3], T980042 [4], T980043 [5]) and *S. typhimurium* L1388 (ST) strains analyzed by 10% SDS-PAGE. Periplasmic fractions were prepared by cold-shock extraction of 24-h TSB-grown, PBS-washed cells. M: Broad range molecular weight marker (BioRad).

Periplasmic proteins

A single major band (28.4 kDa) was observed in the periplasmic fractions of all the isolates (Figure 4)

Cell-free culture supernatant (CFCS) analysis

Proteins secreted into culture medium during growth are presented in Figure 5. All DT104s and ST secreted a common distinct protein (47 kDa); another sharp band (49 kDa) seen in samples from all DT104s (with the exception of T980043) was however, absent in the culture supernatant of ST. Two proteins (53 and 54 kDa), absent in all but one DT104 isolate (T980043), were also secreted into the culture medium by ST during growth.

Flagillin analysis

As shown in Figure 6A, all the isolates expressed a common 48-kDa flagillin. One of the DT104 isolates (306-98) appeared to have expressed an additional 53-kDa flagillin absent in samples from other DT104s as well as the non-DT104 strain (ST). 2DE of samples from representative isolates have confirmed only one major protein spot between the 48-kDa and 62-kDa positions (Figures 6B, C and D).

Analysis of cell-free ultracentrifuge supernatant (CFUS) protein

The protein content and composition of crude cell-free extracellular fraction was determined, and the results are

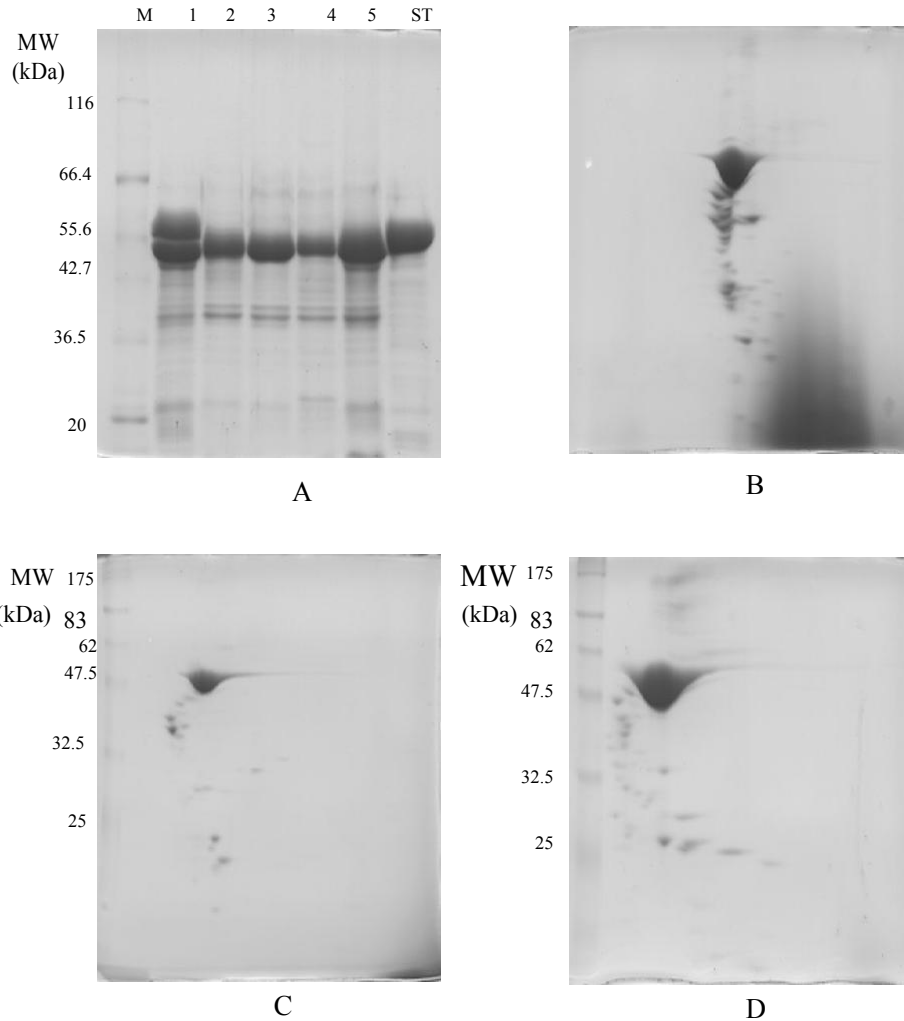


Figure 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the crude flagillin (FLA) proteins of *S. typhimurium* DT104 and *S. typhimurium* L1388 (ST) strains. FLA proteins were acid-extracted at pH 2.0 from 24-h TSB-grown and PBS-washed cells as described in *Materials and Methods*. A: One-dimensional SDS-10% PAGE; 1-5 are respectively 306-98, T980018, T980021, T980042, and T980043. B, C, and D are two-dimensional (2D) SDS-12% PAGE of 306-98, T980021 and ST respectively. MW (in kilo dalton) are A: non-prestained broad range molecular weight marker (New England BioLabs, UK), C and D: prestained broad range (New England BioLabs, UK).

presented in Figures 7 and 8, respectively. The protein contents of samples from all the DT104 isolates were significantly ($P < 0.05$) higher than that from ST; but the latter expressed a 51-kDa protein absent in samples from DT104s.

N-terminal sequence and peptide mass fingerprinting

The N-terminal sequence of first twenty amino acids in the 51-kDa protein from CFUS of ST was Ala-Gln-Val-Ile-Asn-Thr-Asn-Ser-Leu-Ser-Leu-Leu-Thr-Gln-Trp-Ala-Ala-Ala-Ala-Ala; this showed 14-amino acid overlap and resemblance with the flagillin, FLIC. However, of the 104 peptides generated by trypsin digestion, only fourteen resemble those of FLIC.

Protein profile after growth in low-iron Medium A

The SDS-soluble protein profile following growth in low-iron medium is given in Figure 9. There was no difference in the SDS-soluble protein extracts between the phage types when grown at 37°C, except for the lack of presence of the 38.5 kDa protein (observed in cells grown in nutrient-rich medium) by all the isolates except the temperature-tolerant DT104 isolate T980043.

DISCUSSION

Like in many bacteria, the cell surface is important to virulence of salmonellae, as most virulence factors are either found on the surface or secreted into their

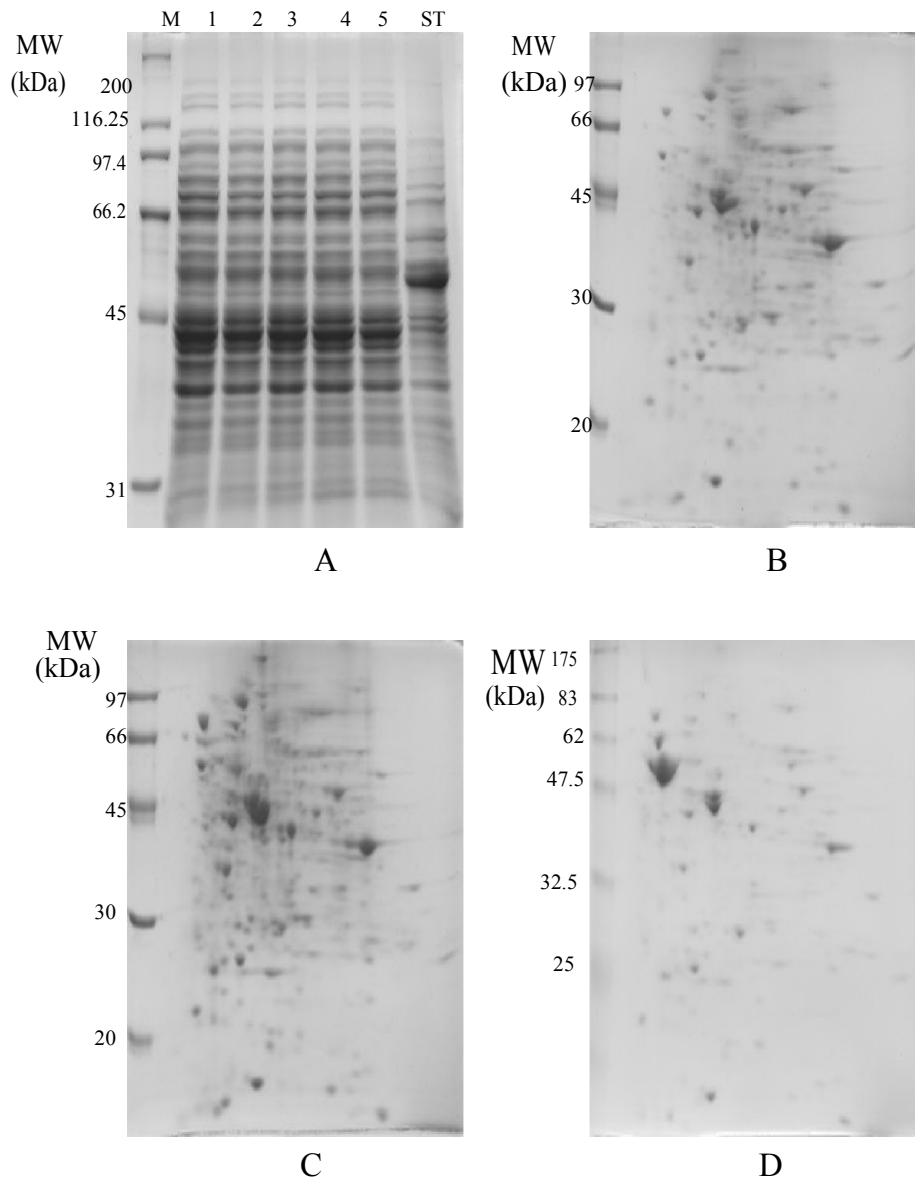


Figure 7. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) of the cell-free ultracentrifuge supernatant (CFUS) proteins of *S. typhimurium* DT104 and *S. typhimurium* L1388 (ST) strains. CFUS protein was prepared from 24-h TSB-grown and PBS-washed cells as described in *Materials and Methods*. A: One-dimensional SDS-10% PAGE; 1-5 are respectively 306-98, T980018, T980021, T980042, and T980043. B, C, and D are two-dimensional (2D) SDS-12% PAGE of T980021, T980043 and ST respectively. MW (in kilo dalton) are A: broad range molecular weight marker (BioRad), B and C: Low Molecular Weight Calibration Kit (Amersham-Biotech, Sweden), and D: prestained broad range (New England BioLabs, UK).

immediate environment (Finlay and Falkow, 1997). Our study have shown that the human isolates of DT104 and the strain ST examined do not differ in their total cell protein, OMP, EDTA-soluble protein extract, shock-released periplasmic protein following a SDS-PAGE analysis (Figures 1-4). By-products of metabolism, the nature of which depends on the bacteria, the nutritional state of the culture medium, and the incubation condition into the culture medium, are released during bacterial

growth. Some of these metabolites are protein-like, and account wholly or partly, for the virulence of the bacteria. The release of a 47-kDa protein by all the isolates into the culture medium indicated that DT104 still share a common protein metabolite with ST, despite the antibiotic sensitivity differences between them. However, that one of the DT104 isolates did not secrete another protein (49 kDa) observed in culture supernatants from the other DT104 isolates being investigated, as is the case with

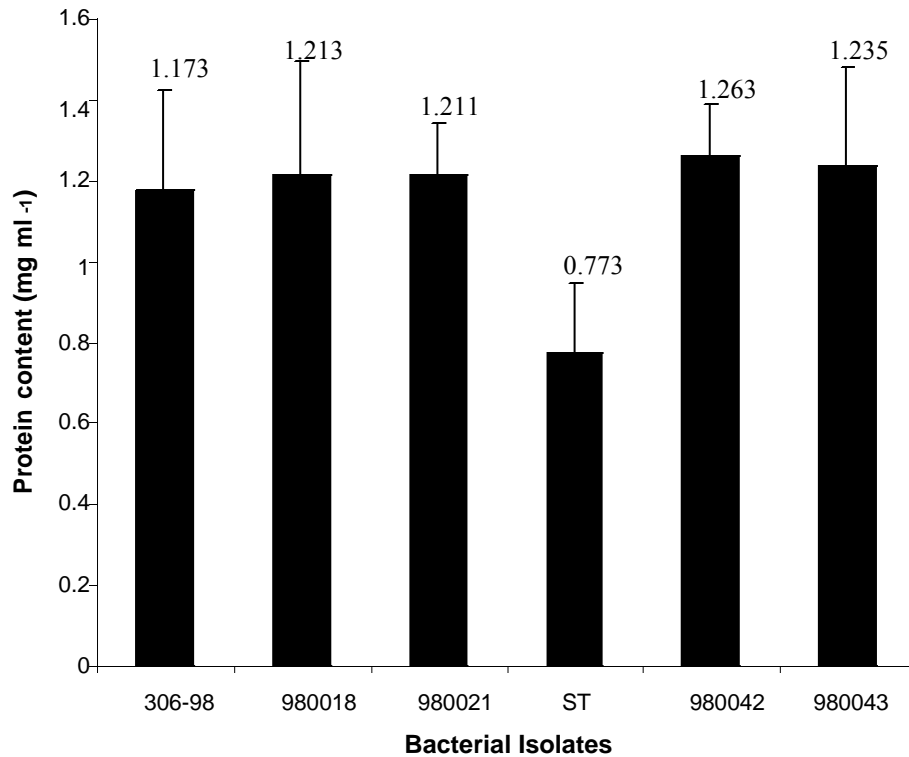


Figure 8. Protein content of the cell-free ultracentrifuge supernatant (CFUS) samples of *S. typhimurium* DT104s (306-98, T980018, T980021, T980042, T980043) and a non- DT104 strain *S. typhimurium* L1388 (ST). CFUS samples, prepared as described by Chopra et al. (1987), were analyzed by spectrophotometric measurement of absorbance at 280 nm (A_{280}) using bovine serum albumin (BSA) as standard. Error bars are standard deviations of the means of four independent experiments. DT104s differ significantly (ANOVA; $P < 0.05$) from ST.

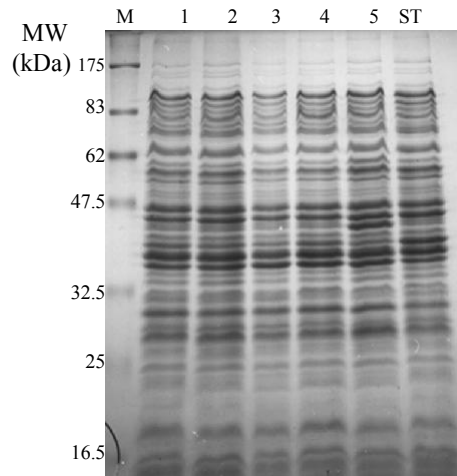
ST, is an indication that the variability in protein release may be independent of the phage type. This was further proved by secretion of two other proteins (53 and 54.0 kDa) by ST and a DT104 isolate (the one which did not secrete the 49 kDa), but not by the other DT104 isolates. It thus appears that the composition of protein-like factors secreted into culture medium during growth was not altered in DT104 isolates.

The method used to prepare the cell-free ultracentrifuge supernatant was adapted from that earlier described for toxin preparation from *S. typhimurium* by Chopra et al. (1987). CFUS samples from DT104 isolates were all of higher protein content than that from ST, but lack a 51-kDa protein observed in the sample from ST. There is thus, the possibility of an increased production of protein-like determinants of virulence in DT104 isolates. However, we do not yet know the significance of the non-expression of the 51-kDa protein by the DT104 isolates.

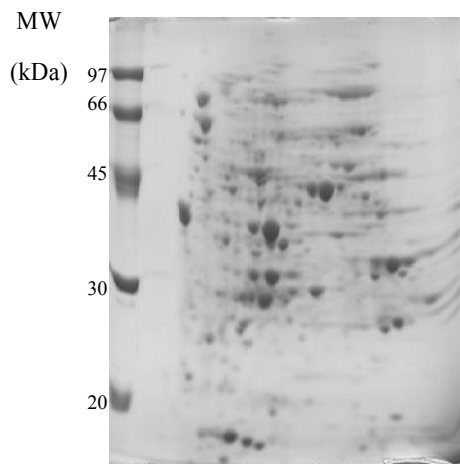
The mechanisms that aid in the acquisition of iron from host tissues can contribute to the virulence of microorganisms (Weinberg, 1978). Available reports have indicated proteins of a similar size in outer membranes prepared from cultures of *S. typhimurium* grown in low-

iron medium (Ernst, 1978; Bennett and Rothfield, 1976). We have also observed in this study that the protein profiles of SDS- soluble extracts of the whole bacterial cell prepared from both low-iron- grown- DT104 and ST isolates were generally similar; the only exception being the absence of the 38.5 kDa protein (seen in nutrient-rich TSB grown bacteria) in all but the DT104 isolate (T980043) whose growth was not significantly reduced at elevated temperature (unpublished observation).

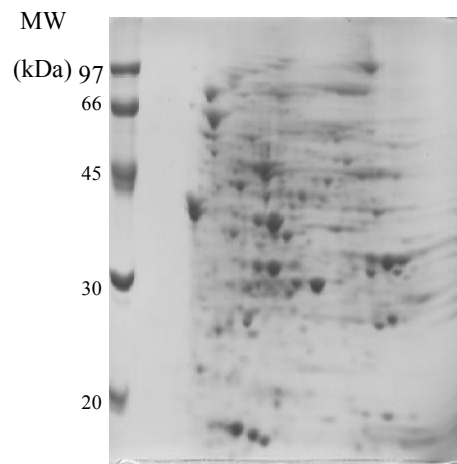
From our results, the protein profiles of all the DT104s isolates and the non-DT104 strain were similar, except for the presence of a 51 kDa protein in the CFUS sample of the latter strain and not in the former strain. However, there is a possibility of increased production of protein-like metabolites by DT104s. In addition, only the temperature-tolerant strain of the DT104s produced a 38.5 kDa protein when grown in low-iron medium; the relationship between this protein and the survival of this particular DT104 isolate will need to be further investigated. Although in a small sample size, the observations from this study should provide a basis for a more extensive study using a larger number of isolates.



A



B



C

Figure 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) of the total protein of low iron-grown cells (WC-ma) of *S. typhimurium* DT104 and *S. typhimurium* L1388 (ST) strains. WC-ma protein was prepared from 24-h Medium A-grown and PBS-washed cells as described in *Materials and Methods*. A: one-dimensional SDS-10% PAGE of WC-ma proteins prepared by boiling (100°C, 10 min) PBS-washed, Medium A (Pollack et al. 1970)-grown cell pellets in SDS sample buffer; 1-5 are respectively 306-98, T980018, T980021, T980042, and T980043. B and C are two-dimensional (2D) SDS-12% PAGE of T980021 and T980043 respectively. Samples for 2D were pre-extracted by boiling in SDS boiling (100°C, 10 min) buffer, then acid-precipitated and re-solubilized in urea sample buffer as described in *Materials and Methods*. MW (in kilo dalton) are A: prestained broad range (New England BioLabs, UK), B and C: Low Molecular Weight Calibration Kit (Amersham- Biotech, Sweden).

ACKNOWLEDGEMENTS

We are grateful to Rikkyo University, Japan for The Bishop William's Fellowship support to Yakubu Ngwai. Part of this work was carried out in the Microbial Function Laboratory, National Food Research Institute, Tsukuba, Japan. Strains used in this study were provided by Dr. Kazumitsu Tamura (formerly of the National Institute of Infectious Disease, 1-23-1, Toyama, Shinjuku, Tokyo, 102-8640, Japan).

REFERENCES

- Akkina JE, Hougue AT, Angulo RJ, Johnson R, Petersen KE, Saini PK, Fedorka-Cray PJ, Schlosser WE (1999). Epidemiologic aspects, control, and importance of multi-drug resistant *Salmonella typhimurium* DT104 in the United States. *J. Am. Vet. Med. Assoc.* 214: 790-798.
- Allen CA, Fedorka-Cray PJ, Vazquez-Torres A, Suyemoto M, Altier C, Ryder LR, Fang FC, Libby SJ (2001). *In Vitro* and *In Vivo* Assessment of *Salmonella enterica* Serovar Typhimurium DT104 Virulence. *Infect. Immun.* 69: 4673-4677.
- Ames GFL, Nikaido H (1976). Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* 15: 616-623.

- Baggessen DL, Sandvang D, Aarestrup FM (2000). Characterization of *Salmonella enterica* Serovar Typhimurium DT104 Isolated from Denmark and Comparison with Isolates from Europe and United States. *J. Clin. Microbiol.* 38: 1581-1586.
- Bennett RL, Rothfield LI (1976). Genetic and physiological regulation of intrinsic proteins of the outer membrane of *Salmonella typhimurium*. *J. Bacteriol.* 127: 498-504.
- Besser TE, Gay CC, Gay JM, Hancock DD, Rice D, Pritchett LC, Erickson ED (1997). Salmonellosis associated with *S. typhimurium* DT104 in the USA. *Vet. Rec.* 140: 75.
- Bjorkman J, Hughes D, Andersson DI (1998). Virulence of antibiotic-resistant *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA.* 95: 3949-3953.
- Briggs CE, Fratamico PM (1999). Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob. Agents Chemother.* 43: 846-849.
- Carlson SA, Browning AM, Ferris KE, Jones BD (2000a). Identification of diminished tissue culture invasiveness among multiple antibiotic resistant *Salmonella typhimurium* DT104. *Microb. Pathog.* 28: 37-44.
- Carlson SA, Willson RM, Crane AJ, Ferris KE (2000b). Evaluation of invasion-conferring genotypes and antibiotic-induced hyperinvasive phenotypes in multiple antibiotic resistant *Salmonella typhimurium* DT104. *Microb. Pathog.*, 28: 373-378.
- Chopra AK, Houston CW, Peterson JW, Prasad R, Mekalanos JJ (1987). Cloning and expression of the *Salmonella* enterotoxin gene. *J. Bacteriol.* 169: 5095-5100.
- D'Aoust JY (1994) *Salmonella* and the international food trade. *Int. J. Food Microbiol.* 24: 11-31.
- Ernst JF, Bennett RL, Rothfield LI (1978). Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. *J. Bacteriol.* 135: 928-934.
- Fierer J, Swancutt M (2000). Non-typhoid *Salmonella*: a review. *Curr. Clin. Top. Infect. Dis.* 20: 134-157.
- Filip C, Fletcher G, Wulff JL, Earhart CF (1973). Solubilization of the Cytoplasmic Membrane of *Escherichia coli* by the Ionic Detergent Sodium-Lauroyl Sarcosinate. *J. Bacteriol.* 115: 717-722.
- Finlay BB, Falkow S (1997). Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* 61: 136-169.
- Hitchcock PJ, Brown TM (1983). Morphological heterogeneity among *Salmonella* ipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* 154: 269-277.
- Jorgensen F, Leach S, Wilde SJ, Davies A, Stewart GSAB, Humphrey T (2000). Invasiveness in chickens, stress resistance and RpoS status of wild-type *Salmonella enterica* subsp. *enterica* serovar Typhimurium definitive type 104 and serovar Enteritidis phage type 4 strains. *Microbiology* 146: 3227-3235.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227: 680-685.
- Nossal NG, Heppel LA (1966). The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. Biol. Chem.* 241: 3055-3062.
- O' Farrell PH (1975). High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.
- Onaolapo JA, El-Haffar MIA, Townley D, Klemperer RMM (1987). R-plasmid RP1 promotes adhesion of Gram-negative bacteria to medical prostheses and glass. *J. Med. Microbiol.* 24: 227-232.
- Onaolapo JA, Klemperer RMM (1986). Effects of R-plasmid RP1 on surface hydrophobicity of *Proteus mirabilis*. *J. Gen. Microbiol.* 132: 3303-3307.
- Pollack JR, Ames BN, Neilands JB (1970). Iron transport in *Salmonella typhimurium*: Mutants blocked in the biosynthesis of enterobactin. *J. Bacteriol.* 104: 635-639.
- Popoff MY, Le Minor L (1997). Antigenic formulae of the *Salmonella* serovars. WHO Collaborating Center for Reference and Research, Institut Pasteur, Paris, France.
- Poppe C, Smart N, Khakhria R, Jhonson W, Spika J, Prescott J (1998). *Salmonella typhimurium* DT104: A virulent and drug-resistant pathogen. *Can. Vet. J.* 39: 559-565.
- Poxton IR, Byrne MD (1981). Immunological analysis of the EDTA-soluble antigens of *Clostridium difficile* and related species. *J. Gen. Microbiol.* 122: 41-46.
- Sameshima T, Akiba M, Izumiya H, Terajima J, Tamura K, Watanabe H, Nakazawa, M (2000). *Salmonella typhimurium* DT104 from Livestock in Japan. *Jpn. J. Infect. Dis.* 53: 15-16.
- Schwartz KJ (1999). Salmonellosis. In: Straw DE, D'Allaire S, Mengeling WL, Taylor DJ (Eds) *Diseases of Swine*, 8th Ed., Iowa State University Press, Ames, Iowa. pp. 535-551.
- Szmolleny G, Kostya A, Kovacs S, Speed K, Jones Y, Laszlo VG, Gado I, Paszti J, Wray C, Nagy B (2000). Epidemiology and characterization of animal *Salmonella enterica* subspecies *Enterica* serotype Typhimurium DT104 in Hungary. *Acta Vet. Hung.* 48: 407-420.
- Threlfall EJ, Frost JA, Ward LR, Rowe B (1996). Increasing spectrum of resistance in multiresistant *Salmonella typhimurium*. *Lancet* 347: 1053-1054.
- Threlfall EJ, Ward LR, Rowe B (1998). Multiresistant *Salmonella typhimurium* DT104 and salmonella bacteraemia. *Lancet* 352: 287-288.
- Weinberg ED (1978). Iron and Infection. *Microbiol. Rev.* 42: 45-66.
- Williams P, Lambert PA, Haig HCC, Brown MRW (1986). The influence of the O and K antigens of *Klebsiella aerogenes* on surface hydrophobicity and susceptibility to phagocytosis and antimicrobial agents. *J. Med. Microbiol.* 21: 125-137.