

Characterization of biofilm-forming abilities of antibiotic-resistant *Salmonella typhimurium* DT104 on hydrophobic abiotic surfaces

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Background and Purpose: *Salmonella typhimurium* DT104 strain has emerged as a global human and veterinary public health concern because of its antibiotic resistance and extensive host range. Although it is thought to be more virulent, to date, factors relevant to its virulence have not been fully elucidated. Thus, understanding how this strain forms biofilms on hydrophobic surfaces will add to current knowledge on its possible virulence mechanism.

Methods: Biofilm-forming abilities of clinical isolates of *S. typhimurium* DT104 from human and animal sources on hydrophobic inanimate surfaces were assessed by absorbance at 600 nm of crystal violet-bound cells recovered from 96-well tissue culture plates after growth in a nutrient-rich growth medium and various adjusted media; and scanning electron microscopy based on standard procedures.

Results: In the nutrient-rich growth medium, Luria-Bertani (LB), biofilms were formed in small quantities, preferentially on polystyrene ($p < 0.05$), and followed different time courses. Significantly lower amounts of biofilms were formed on polystyrene when a nutrient-deficient growth medium (adherence test medium) was used. Inclusion of D-(+)-mannose in LB at a concentration of 100 mM significantly ($p < 0.05$) inhibited biofilm formation on polystyrene. D-(+)-glucose relatively enhanced biofilm formation but D-(-)-mannitol only insignificantly influenced the process. The action of mannose on polyvinyl chloride (PVC) was insignificant, suggesting that its action may be surface-dependent. Additionally, glucose significantly reduced biofilm growths of 2 of the isolates and only that of the PVC-loving strain T980021 on polystyrene and PVC, respectively. At the concentration tested, unlike xylose, both D-mannose and D-glucose significantly ($p < 0.05$) inhibited bacterial growth, providing a possible mechanism for their inhibitory action on biofilm formation by *S. typhimurium*. While stress of starvation resulted in significant reduction in biofilm formation on polystyrene in all but the PVC-loving strain T980021, high osmolarity had little effect on the quantity of biofilm formed on polystyrene. The extent of primary attachment to polystyrene as well as their capacity to form biofilm did not correlate with their cell surface hydrophobicity and exopolysaccharide production.

Conclusions: D-(+)-mannose inhibits biofilm formation by *S. typhimurium* DT104 on polystyrene but not on PVC. There was also a general lack of correlation between the ability of *S. typhimurium* DT104 to form biofilm and its physicochemical surface characteristics.

Key words: Biofilms, *Salmonella typhimurium* DT104, surface properties, two-dimensional gel electrophoresis

Introduction

Non-typhoid salmonellae are a common cause of bacterial gastroenteritis worldwide [1,2]. The serotype *Typhimurium*

remains the most frequently isolated in human, swine, avian, and bovine salmonellosis [3,4]. An emergent strain, *Salmonella typhimurium* DT104, is of particular concern because of its antibiotic resistance and wide host range [5-7]; the strain is also thought to be more virulent than other *Typhimurium* strains [8,9]. However, virulence assays based on stress response, tissue invasion, macrophage survival and mortality rate in animals, have indicated otherwise [10-12].

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The primary mode of existence of many bacteria in natural, clinical and industrial settings is in the form of biofilm, a sessile and highly structured community. Biofilm is defined as a 'matrix-enclosed bacterial population adherent to each other and/or to surfaces or interfaces' [13,14]. The formation of biofilm is an important strategy in microbes for survival in a variety of environmental stresses of physical, chemical and biological nature [15,16]. Since the colonization of mucosal surfaces by pathogenic bacteria is believed to proceed via the formation of biofilm [17], the process represents a source of persisting and relapsing infections, and thus contributes significantly to pathogenesis [16, 17]. Biofilm formation has even been correlated with virulence in *Salmonella enteritidis* [18,19].

In this study, we examined the biofilm-forming abilities of antibiotic-resistant clinical isolates of *S. typhimurium* DT104 in relation to their cell surface characteristics with the aim of understanding their mode of surface colonization.

Methods

Bacterial isolates and culture media

Eleven antibiotic-resistant clinical isolates of the *S. typhimurium* DT104 of human (306-98, T980018, T980021, T980042, and T980043) and animal (ST3, ST4, ST5, ST29, ST39, and ST41) origin were used in this study. The human strains were obtained from Centers for Disease Control and Prevention (USA) courtesy of the National Institute of Infectious Diseases of Japan; the animal strains were isolated in Japan. Relevant characteristics of the isolates are as shown in Table 1.

Bacteria were propagated in either trypticase soy broth (TSB; BBL, USA), Luria-Bertani broth (LB; Daigo, Inc., Japan) or as indicated in the text; solid media cultures were grown on trypticase soy agar (TSA; BBL, USA). Biofilm formation was assessed in the following media: LB starvation/adherence test medium (ATM; 60 mM sodium chloride [NaCl], 30 mM sodium bicarbonate, 20 mM potassium chloride, 111 mM D-glucose, distilled water to 1000 mL) [20], LB supplemented with 10% NaCl (LB-S), LB supplemented with 100 mM of one of D-(+)-glucose (LB-G), D-(+)-mannose (LB-M), D-(-)-mannitol (LB-ML), and D-(+)-xylose (LB-XY). Unless otherwise indicated, all chemicals used were purchased from Wako Chemical Company, Japan.

Biofilm formation assay

The microtitre plate assay used in this study was adapted from O'Toole and Kolter [21], with some modifications. Briefly, bacteria were grown in LB broth overnight at 37°C and 10 µL was inoculated into one of 1.5-mL microfuge tube (polypropylene), flat-bottom 24-well polystyrene tissue culture plate (FALCON®; Becton Dickinson & Co., USA), and 96-well polyvinyl chloride (PVC) microtiter plate containing 90 µL of LB broth. Plates were incubated at 37°C for 24 h.

Cultures were then removed and rinsed 3 times with sterile distilled water to remove unattached bacteria. The tubes or wells were either air-dried at room temperature or dried at 37°C, and adherent bacteria were stained at room temperature with 200 µL of 1% weight/volume (w/v) aqueous solution of crystal violet (CV; Merck, Germany) for 20 min. The tubes or wells were rinsed

Table 1. Sensitivity of test strains to common antibiotics

Strain	Source	Country of isolation	Minimal inhibitory concentration (µg/mL)										Resistance
			A	T	C	F	G	K	S	Su	TMP	NA	
306-98	Human	USA (CDC)	>512	128	256	64	1	2	512	>512	0.5	4	ACFSSuT
T980018	Human	USA (CDC)	>512	64	256	64	0.5	2	256	>512	0.5	4	ACFSSuT
T980021	Human	USA (CDC)	>512	256	256	128	1	2	>512	>512	1	4	ACFSSuT
T980042	Human	USA (CDC)	>512	64	256	64	1	2	128	>512	0.5	4	ACFSSuT
T980043	Human	USA (CDC)	>512	128	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST3	Cattle	Japan	1	2	8	8	0.5	2	128	>512	0.5	4	SSu
ST4	Cattle	Japan	>512	128	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST5	Cattle	Japan	>512	32	256	64	1	2	256	>512	0.25	4	ACFSSuT
ST29	Cattle	Japan	>512	128	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST39	Cattle	Japan	>512	32	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST41	Cattle	Japan	1	2	8	8	0.5	2	256	>512	0.5	4	SSu

Abbreviations: A = ampicillin; T = tetracycline; C = chloramphenicol; F = florfenicol; G = gentamicin; K = kanamycin; S = streptomycin; Su = sulfamethoxazole; TMP = trimethoprim; NA = nalidixic acid; CDC = Centers for Disease Control and Prevention

3 times with sterile distilled water after the dye was removed, and dried as before; stained adherent cells were detached from the walls of the tubes or plates using 300 μ L of dimethylsulfoxide (CV; Merck, Germany), and the absorbance at 600 nm (A_{600}) of the solubilized biofilm extracts measured using a UV-2200 Spectrophotometer (Shimadzu Corporation, Japan). Results are means of 2 experiments using 3 independent samples.

Time course assay of biofilm formation on polystyrene

Cultures of the isolates were grown in LB broth in polystyrene tissue culture plate over a period of 15 h at 37°C, during which biofilm was quantified by the same method as described above. Results are means of a single representative triplicate determination.

Effect of carbon sources on biofilm formation

Bacteria were grown in LB, LB-G, LB-M, LB-ML, or LB-XY broths, and biofilm formed in polystyrene tissue culture or PVC microtitre plates was quantified as described earlier. Results are means of at least 4 experiments. Our choice of 100 mM as test sugar concentration was arbitrary, and based on previous works in which glucose at concentrations up to 111 mM either did not affect biofilm formation or enhanced it in *Escherichia coli* and *S. enteritidis*, respectively [18,22].

Effect of carbon sources on bacterial growth

Parallel 2-mL samples of LB, LB-G, LB-M, LB-ML and LB-XY broths were inoculated with one colony each of a particular isolate, incubated at 37°C for 24 h with shaking (65 tps per min), and viable cells in each tube were determined after appropriate dilution in saline (0.9 g NaCl, distilled water to 100 mL) and overnight incubation at 37°C. Results are means of 4 independent experiments.

Effects of starvation and osmotic stresses on biofilm formation

Cells in a biofilm usually have increased tolerance to unfavorable external conditions, and in some bacterial ecosystems, the conversion of planktonic cells into a biofilm-producing community is triggered by environmental stress factors [23-26]. Consequently, we assessed the biofilm-forming abilities of the selected isolates cultured in a starvation medium and under high osmotic salt stress. Biofilm formation was quantified

after 24-h culture of bacteria in LB-S and ATM broths in 24-well polystyrene tissue culture plate using the same procedure as described above. Experiments were carried out in triplicate.

Scanning electron microscopy

Ten mL LB broth containing polystyrene chips (0.5 cm \times 0.5 cm; cut from plates made of this material and pre-sterilized with 100% ethanol) were inoculated with 1 colony of a particular isolate, and incubated at 37°C without shaking for 24 h. The chips were then removed, washed 3 times with 50 mM sodium phosphate buffer (pH 7.0) and fixed by immersion in 3% glutaraldehyde-50 mM sodium phosphate buffer (pH 7.0) for 2 h on ice. Subsequently, the chips were washed 3 times with buffer, post-fixed by immersion in 1% osmium tetroxide-50 mM sodium phosphate buffer (pH 7.0) for 1 h at room temperature, washed 3 times with buffer, and dehydrated through graded ethanol-water mixtures in the following order: 25% (15 min), 50% (15 min), 75% (15 min), 90% (15 min) and 100% (15 min \times 3). Dried chips were then immersed in 100% ethanol:tert-butyl alcohol (1:1) for 10 min twice, transferred to tert-butyl alcohol for another 10 min, later to 1 mL (just sufficient to cover the chip) tert-butyl alcohol and kept at -20°C to freeze for 1 h.

Frozen samples were then vacuum dried for 24 h in a vacuum freeze dryer (Eiko Engineering Co. Ltd., Japan) at 15 lb/in² pressure, mounted on scanning electron microscope (SEM) sample tube using double stick tape, sputter-coated with osmium gas discharging at 1.1-1.2 kV voltage and 2-4 mA current using JOS-100D Osmium Plasma Coater (Jeol Technics Co. Ltd., Japan) at 0.9 Pa pressure and viewed with a JSM-T300 SEM (Jeol Technics Co. Ltd., Japan) at a constant accelerating voltage of 10 kV. Photographs were taken on Fuji Polaroid film. Two repeat experiments were carried out to confirm observations.

Multicellular phenotypes

Multicellular behavior in bacteria is characterized by the expression of diverse phenotypes on plates. Colonial morphology of bacteria was studied on tryptone (T) medium (1.0 g Bacto T [Difco, USA], 1.5 g Bacto agar [Difco], distilled water to 100 mL), TSA, LB agar without salt (LB_w; 1.0 g T, 0.5 g yeast extract, 1.5 g agar, distilled water to 100 mL), T medium containing 0.015% w/v CV, TSA-0.015% w/v CV and LB_w containing 0.002% w/v Coomassie brilliant blue R (CBB; Sigma Chemicals, St Louis, MO, USA).

Motility assay

Agar motility was assayed based on Bloemberg et al [27]. LB medium, LB-2% NaCl and LB medium without salt containing 0.3% agar were separately prepared in plates and stab-inoculated with bacteria taken from deoxycholate hydrogen sulfide lactose agar culture. Inoculated plates were incubated at 30°C for 8 h, and the diameters of the migration of bacteria from the point of inoculation (observed as a turbid zone) were measured. Results are means of at least 3 independent experiments.

Cell surface hydrophobicity

Cell surface hydrophobicity was determined by microbial adhesion to hydrocarbon (MATH) as described originally by Rosenberg et al [28], with modifications. Briefly, bacteria were grown in TSB at 37°C for 18 to 24 h, harvested by centrifugation (6000 rpm, 5 min, 25°C), washed with and re-suspended in sterile distilled water to an initial A_{600} of 0.3 to 0.6, and measured using UV-2200 spectrophotometer (Shimadzu Corporation, Japan). Two mL each of the adjusted bacterial suspension and xylene were mixed for 2 min by vortex (Scientific Industries, Inc., Bohemia, USA) in IWAKI® borosilicate glass tubes (Asahi Techno Glass, Japan) at room temperature. The phases were allowed to separate for 1 h at ambient temperature, and the A_{600} of the lower aqueous phase was measured as before. Cell surface hydrophobicity was calculated from: $([A_{\text{initial}} - A_{\text{aqueous phase}}] / A_{\text{initial}}) \times 100$. Triplicate determinations were made for each of 2 separate cultures of all the isolates (i.e., $n = 6$). We used xylene as the hydrocarbon phase because it is more hydrophobic and can permit the detection of relatively less hydrophobic cells than aliphatic hydrocarbons such as hexadecane [29]. Xylene is also less negatively charged when in contact with aqueous solutions at pH 7.0, including phosphate-buffered saline (PBS), than aliphatic hydrocarbons such as hexadecane, n-octane, etc. [30].

Adherence assay

The in vitro adherence of the isolates to polystyrene was assayed based on the method of Onaolapo et al [31]. Briefly, each isolate was grown with shaking (65 tpm) for 24 h in 10 mL LB broth containing 2 pieces (1 cm × 1 cm) of polystyrene cut from culture plate made of this material (the cut pieces were pre-sterilized by soaking in 100% ethanol for 1 h and drying aseptically). The polystyrene chips were then removed, washed through two 10-mL rinses of normal saline (0.9 g NaCl, distilled water to 100 mL), and each piece transferred into separate

9.9 mL normal saline (approximate 1:100 dilution), vortex-mixed vigorously for 2 min, and plated out on TSA after appropriate dilution in normal saline. Plates were incubated at 37°C for 24 h, and colonies were counted. Results are means of 3 independent determinations.

Exopolysaccharide assay

Exopolysaccharide (EPS) produced by each of the isolates was extracted as previously described [32,33] with modifications. Briefly, bacteria were grown in 30 mL of TSB broth (37°C, 24 h) with shaking, and cells harvested by centrifugation (6000 rpm × 20 min × 4°C); the cells were washed once with 1 M NaCl-10 mM ethylenediamine tetra-acetic acid (EDTA) solution to release cell-bound EPS, and supernatant was added to culture supernatant, which contained soluble EPS. The supernatant mixtures were extracted with 2 volumes of cold (−20°C) isopropanol for at least 12 h at 4°C to precipitate EPS, which was collected by centrifugation (6000 rpm × 20 min × 4°C). Protein contaminants in the EPS were precipitated with 20% trichloroacetic acid on ice for at least 45 min and removed by centrifugation. EPS in the resulting filtrates were then re-precipitated with 2 volumes of cold isopropanol as described earlier and air-dried. The carbohydrate content of EPS was assayed by the phenol-sulfuric acid method of Dubois et al [34] as described by Hancock and Poxton [35] using D-glucose as standard. The experiment was done a total of 3 times.

Electrophoretic analysis of major surface components

Whole cell protein and lipopolysaccharide (LPS) were extracted as previously described by Hitchcock and Brown [36]; OMP was prepared by the sarkosyl method of Filip et al [37]; and EDTA-soluble proteins were extracted in PBS-10 mM EDTA at 45°C for 20 h, prepared as described by Poxton and Byrne [38]. One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was based on Laemmli [39]. LPS was stained with silver as described by Hardy et al [40]. Proteins were stained with 0.25% CBB in methanol:acetic acid:water (5:1:4) and destained with methanol:acetic acid:water (5:1:4). Broad range molecular weight marker (New England BioLabs, UK) was used as size reference.

Statistical analysis

Data were analyzed by the 1-way analysis of variance using WebStat 2.0 (Department of Statistics, University of

South Carolina, Columbia, SC, USA), and significance of results determined at the 5% probability level ($p=0.05$).

Results

Biofilm formation

All isolates formed biofilm after 24 h of growth preferentially on polystyrene (Table 2) but also on PVC and polypropylene (Table 3). One of the isolates (T980021), however, demonstrated a preference for PVC. The most adherent isolates on polypropylene, PVC and polystyrene were respectively strains T980018, T980021 and T980043.

Kinetics of biofilm formation on polystyrene

Biofilm growths by the isolates followed different kinetics, being optimal at different times over the entire experimental period (Table 4) depending on the isolate. The biofilms of some of the isolates progressively decreased after 200 min of incubation, but that of T980043 was optimal at 600 min of incubation.

Effects of carbon source on biofilm formation

Mannose significantly ($p<0.05$) inhibited biofilm formation of all the isolates (except ST3 and T980021) on polystyrene tissue culture plate (Table 2). The effect of D-glucose was however, mixed: while it significantly

inhibited the biofilm processes of some of the isolates (306-98, T980042 and T980043), it either enhanced or insignificantly altered the process in others (Table 3). On PVC microtiter plate (Table 3), mannose did not always significantly inhibit biofilm growths, its action being significant only on T980018 and T980021. Glucose significantly ($p<0.05$) enhanced biofilm formation of only 2 of the isolates (T980042 and T980043). Both mannose and glucose, however, significantly ($p<0.05$) inhibited the biofilm growths of T980021 (the most adherent on PVC). The effect of D-glucose appeared to depend on the surface being colonized.

Effects of carbon source on bacterial growth

Growths of all the strains were significantly ($p<0.05$) inhibited by all the carbon sources at the test concentration; the only exception was strain T980043, whose growth was not affected by xylose as shown in Fig. 1, providing evidence that microcolony formation by these isolates may be dependent on the presence of an efficient growth cycle.

Effects of starvation and osmotic stresses on biofilm formation on polystyrene

Culture of the isolates in starvation medium, instead of the nutrient-rich LB medium, resulted in significantly ($p<0.05$) lower amounts of biofilm formed on

Table 2. Biofilm formation on polystyrene by antibiotic-resistant *Salmonella typhimurium* DT104 stains in nutrient-rich and modified media

Strain	Biofilm (A_{600}) ^a						
	LB ^b	LB-M ^b	LB-G ^b	LB-ML ^b	LB-XY ^b	LB-S ^b	ATM ^b
306-98	0.28 (0.01)	0.17 (0.03)	0.18 (0.02)	0.16 (0.04)	0.17 (0.04)	0.22 (0.01)	0.15 (0.01)
T980018	0.22 (0.04)	0.19 (0.00)	0.24 (0.03)	0.20 (0.02)	0.23 (0.03)	0.22 (0.00)	0.16 (0.01)
T980021	0.19 (0.01)	0.18 (0.01)	0.20 (0.00)	0.14 (0.01)	0.14 (0.01)	0.24 (0.01)	0.18 (0.00)
T980042	0.24 (0.05)	0.16 (0.02)	0.22 (0.03)	0.17 (0.02)	0.15 (0.01)	0.27 (0.03)	0.18 (0.01)
T980043	0.33 (0.07)	0.19 (0.00)	0.24 (0.02)	0.18 (0.02)	0.18 (0.02)	0.24 (0.02)	0.16 (0.01)
ST3	0.21 (0.03)	0.21 (0.02)	0.25 (0.03)	0.27 (0.01)	0.26 (0.03)	ND	0.12 (0.03)
ST4	0.24 (0.02)	0.14 (0.01)	0.29 (0.01)	0.17 (0.02)	0.13 (0.02)	ND	0.09 (0.02)
ST5	0.25 (0.01)	0.19 (0.03)	0.34 (0.02)	0.27 (0.02)	0.16 (0.02)	ND	0.08 (0.01)
ST29	0.27 (0.03)	0.18 (0.02)	0.26 (0.01)	0.23 (0.03)	ND	ND	ND
ST39	0.26 (0.03)	0.17 (0.01)	0.29 (0.03)	0.20 (0.01)	ND	ND	ND
ST41	0.26 (0.02)	0.19 (0.01)	0.31 (0.02)	0.28 (0.02)	ND	ND	ND

Abbreviations: LB = Luria-Bertani broth; LB-M = LB broth containing 100 mM D-(+)-mannose; LB-G = LB broth containing 100 mM D-(+)-glucose; LB-ML = LB broth containing 100 mM D-(-)-mannitol; LB-XY = LB broth containing 100 mM D-(+)-xylose; LB-S = LB with 10% sodium chloride; ATM = adherence test medium (60 mM sodium chloride, 30 mM sodium bicarbonate, 20 mM KCl, 111 mM D-(+)-glucose, in distilled water; [20]); ND = not determined

^aBiofilm was quantified by absorbance measurements at 600 nm (A_{600}) of crystal violet-bound cells, based on the methods of O'Toole and Kolter [21].

^bValues in parenthesis are standard deviations of the means of 2 triplicate determinations. Statistical significance was determined by analysis of variance at $p=0.05$.

Table 3. Biofilm growth of selected antibiotic-resistant *Salmonella typhimurium* DT104 strains on polypropylene and polyvinyl chloride

Strain	Biofilm (A_{600}) ^a			
	Polypropylene ^b	Polyvinyl chloride ^c		
		LB	LB-M	LB-G
306-98 ^d	0.15 (0.05)	0.07 (0.02)	0.07 (0.05)	0.08 (0.03)
T980018 ^d	0.18 (0.05)	0.14 (0.06)	0.06 (0.01)	0.15 (0.05)
T980021 ^d	0.15 (0.04)	0.29 (0.15)	0.23 (0.01)	0.21 (0.02)
T980042 ^d	0.12 (0.01)	0.06 (0.01)	0.06 (0.00)	0.10 (0.03)
T980043 ^d	0.11 (0.05)	0.07 (0.03)	0.06 (0.01)	0.11 (0.04)

Abbreviations: LB = Luria-Bertani broth; LB-M = LB broth containing 100 mM D-(+) mannose; LB-G = LB broth containing 100 mM D-(+)-glucose

^aBiofilm was quantified by absorbance measurements at 600 nm (A_{600}) of crystal violet-bound cells based on the methods of O'Toole and Kolter [21].

^b1.5-mL microcentrifuge tube.

^c96-well microtiter plate.

^dValues in parenthesis are standard deviations of means of at least 3 determinations. Statistical significance was determined by analysis of variance at $p=0.05$.

polystyrene by all the test strains (Table 2). High salt concentration also resulted in reduced amount of biofilm formed by all the strains.

Scanning electron microscopy

All of the isolates formed biofilm in which bacterial cells aggregated together in microcolonies, attached to each other and to the polystyrene surface by an exopolymer substance (Fig. 2A, 2B, 2C, 2D and 2E). Except for the thickness of the biofilm, no other difference was observed amongst the isolates.

Multicellular phenotypes

All of the isolates grew on TSA, T, and LB_w-CBB media as rough-edged, non-mucoid colonies, without any apparent difference. They all bound CV efficiently within 24 h of growth on CV-containing media. On LB_w-CBB agar, the isolates bound CBB after 48 h of incubation.

Agar motility

The isolates migrate in normal LB medium in a manner related to their level of in vivo virulence (Fig. 3). The motility of all isolates in LB-S was significantly reduced by about half that in LB medium, with the more virulent strains still the most motile. In LB_w, motility was also reduced to different extent among the strains; the most virulent strain was the least motile under this condition.

Hydrophobicity, adherence to polystyrene and EPS production

The isolates were moderately hydrophobic (Table 5) and differed only relatively, with the more virulent strains being most hydrophobic. The least hydrophobic strain was the PVC-loving strain (T980021). Differences in hydrophobicity between the isolates were not sufficiently large to suggest any possible role in their virulence. The extent of primary attachment to polystyrene was

Table 4. Time course assay of biofilm growth on polystyrene by selected antibiotic-resistant *Salmonella typhimurium* DT104 strains

Strain	Biofilm (A_{600}) ^a			
	200 min ^b	400 min ^b	600 min ^b	800 min ^b
306-98	0.19 (0.02)	0.20 (0.01)	0.22 (0.03)	0.21 (0.04)
T980018	0.28 (0.04)	0.25 (0.01)	0.22 (0.02)	0.21 (0.01)
T980021	0.26 (0.06)	0.23 (0.01)	0.22 (0.02)	0.20 (0.01)
T980042	0.24 (0.01)	0.23 (0.01)	0.19 (0.01)	0.23 (0.01)
T980043	0.19 (0.01)	0.21 (0.01)	0.30 (0.11)	0.21 (0.01)

^aThe crystal violet (CV) binding assay [21], was used to quantify biofilm growth on 24-well polystyrene tissue culture plate. Bacteria were grown in wells containing Luria-Bertani broth for the indicated times and amounts of biofilm formed were quantified by absorbance measurements at 600 nm (A_{600}) of solubilized CV-bound adhered cells.

^bValues in parenthesis are standard deviations of means of at least 3 independent observations. Statistical significance was determined by analysis of variance at $p=0.05$.

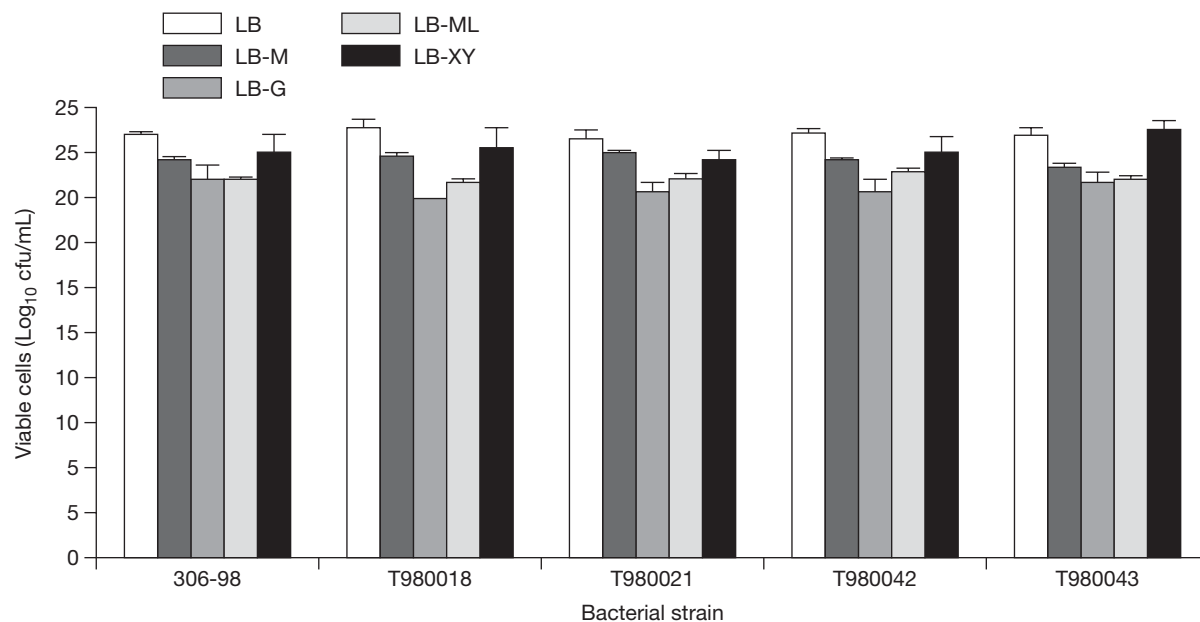


Fig. 1. Effect of carbon sources on the 24-h growth of *Salmonella typhimurium* DT104. Bacteria were grown at 37°C in Luria-Bertani (LB) medium and LB medium supplemented with 100 mM of a carbon source (D-(+)-mannose [LB-M], D-(+)-glucose [LB-G], D-(-)-mannitol [LB-ML], D-(+)-xylose [LB-XY]), and viable cells were counted after appropriate dilution in saline and overnight incubation at 37°C. Error bars are standard deviations of results from 4 experiments.

generally similar among the strains, except in one case (T980018). EPS production by the strains was not significantly different.

SDS-PAGE analysis of surface components

The isolates did not differ from each other in their major surface components (Fig. 4A, 4B, 4C and 4D). The extractable whole cell protein, OMP and EDTA-soluble protein were similar; LPS profiles were all ladder-like, characteristic of smooth strains.

Discussion

Salmonella have capacity for widespread environmental survival [41], which is related to both their non-requirement for strict growth conditions and ability to form biofilm, a contributing factor to their success as pathogens [4,42]. A relationship between the expression of antibiotic resistance and bacterial virulence has been suggested [43]. Resistant genes usually mediate changes in the cell surface physicochemical properties, such as hydrophobicity and charge, which are relevant to the initial stages of biofilm formation [44,45]. The formation of a mature biofilm on a surface is generally considered to consist of 4 sequential steps: (i) transport of the microorganisms; (ii) initial microbial adhesion; (iii) attachment by EPS production and through cell

surface structures; and (iv) colonization by growth of firmly attached organisms [46,47]. Physicochemical cell surface properties are known to be important in the initial interaction of bacteria with host tissues, with usually hydrophobicity and charge playing the major role. Consequently, we studied the biofilm-forming abilities of antibiotic-resistant clinical strains of *S. typhimurium* in relation to the surface properties relevant to surface colonization.

The results of this study indicate that although all the isolates formed biofilm on all the surfaces examined, they differ in surface preference. This variability may be the result of certain differences in bacterial cell surface appendages or physicochemical factors operating at the colonizing surface [48]. However, these differences were not sufficiently large as to reflect on the surface hydrophobicity and expression of surface components, judging from the similar MATH assay findings and protein and LPS profiles. The difference in their surface colonization pattern is further reaffirmed by the variations in the kinetics of biofilm formation on polystyrene, an indication of diversity amongst the strains.

Although the ability of salmonellae to form biofilm on diverse surfaces has already been reported [9,26, 49,50], the preferential colonization of polystyrene by the isolates has implications as a potential cause of

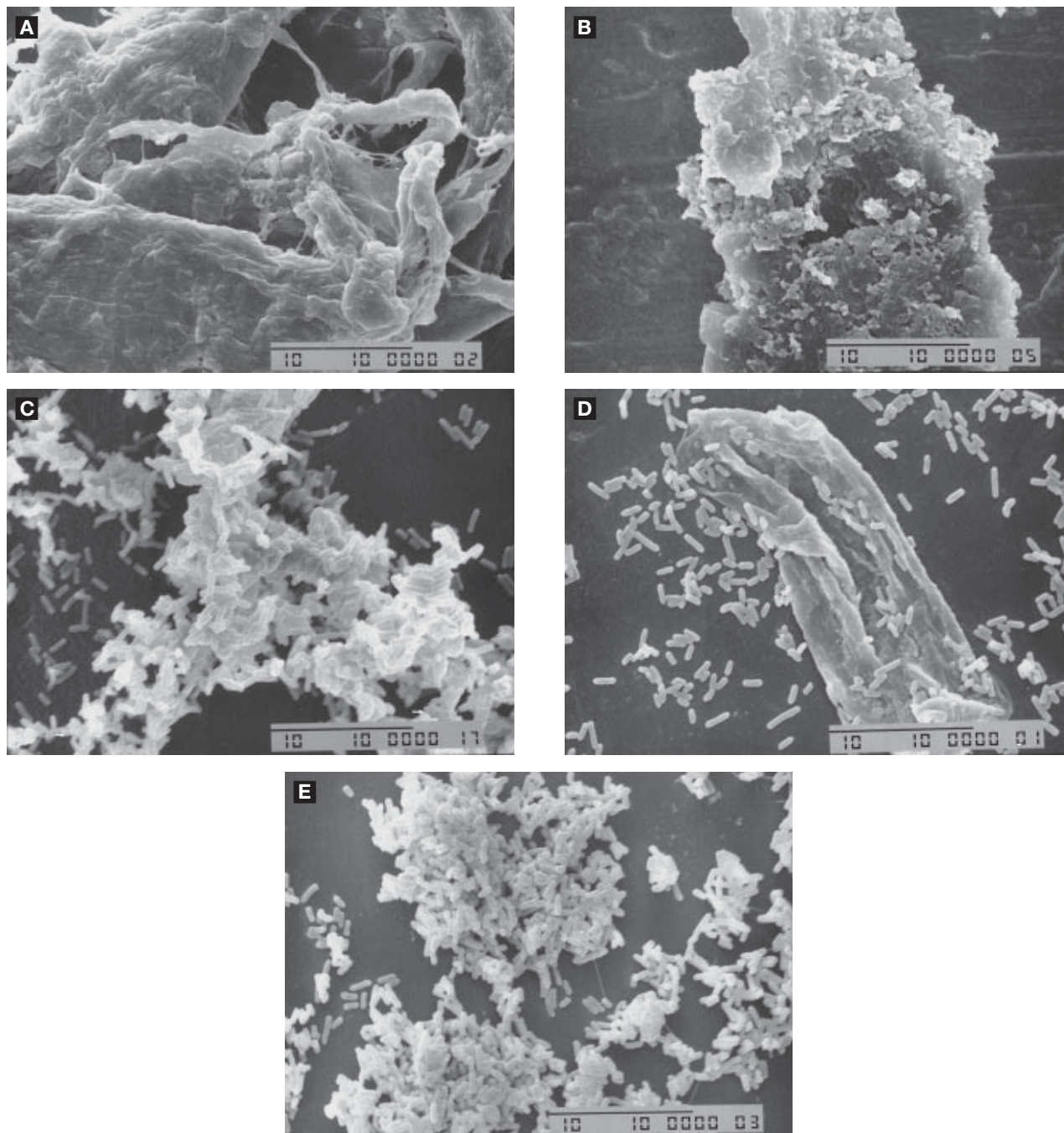


Fig. 2. Scanning electron micrographs of antibiotic-resistant *Salmonella typhimurium* demonstrating biofilm growths on polystyrene chips in Luria-Bertani broth after 24 h at 37°C. Chips were fixed in buffered 3% glutaraldehyde, post-fixed in buffered 1% osmium tetroxide, dried in graded ethanol concentrations, vacuum-dried, sputter-coated with fine platinum and viewed with scanning electron microscope. (A)-(E) are 306-98, T980018, T980021, T980042, and T980043, respectively. Scale bars = 10 µm.

contamination and persistent or relapsing infections in view of the use of polystyrene materials in industry and medicine. The observation that the isolates differ in ability to colonize the surfaces suggests a lack of definite pattern for microcolony formation by DT104s. This further suggests that different pathways may be utilized

for biofilm growth by salmonellae strains of a particular phage type within a given serotype.

It is necessary to note that differences in the growth rates of the isolates were insignificant (data not shown), and did not account for the differences in their biofilm-forming abilities. Formation of biofilm by salmonellae

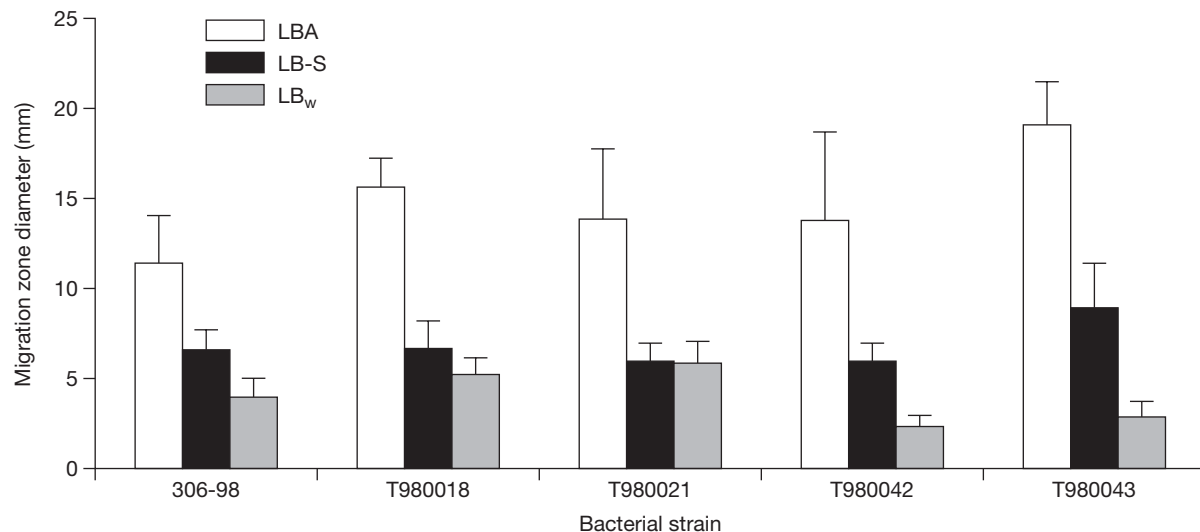


Fig. 3. Agar motility of *Salmonella typhimurium* DT104. Luria-Bertani (LB) medium containing 0.3% agar (LBA), LB-2% sodium chloride (LB-S) and LB medium without salt (1% tryptone, 0.5% yeast extract) [LB_w] containing 0.3% agar were prepared separately in plates and stab-inoculated with bacteria taken from deoxycholate hydrogen sulfide lactose agar culture. Inoculated plates were incubated at 30°C for 8 h, and the diameters of the migration of bacteria from the point of inoculation (observed as a turbid zone) were measured. Statistical analysis was done using 1-way analysis of variance, and significance level at $p=0.05$. Inserts on bars are mean values of at least 3 independent experiments.

is thought to be a strategy for survival in a variety of environmental stresses of physical, chemical and biological nature that they typically encounter during their life cycles [15,16]. In a related study, T980043 that formed more biofilm on polystyrene was interestingly the most virulent in the chick oral LD₅₀ (50% lethal dose) model [51], with the possible inference that the ability to form biofilm might be important in the virulence of some DT104 isolates. Accordingly, the virulence of the strain correlated with its EPS yield, motility, and

hydrophobicity, suggesting that physicochemical factors may be relevant. This fact remains to be further investigated using a large number of isolates. This view is further supported by the insignificant difference in the EPS contents of DT104s (except T980021, a reflection of within-strain variation) and compared with *S. typhimurium*.

EPS is important to microorganisms not only for initial attachment and firm anchorage of bacteria to solid surfaces [52,53], but also for the maintenance of optimum

Table 5. Hydrophobicity, adherence to polystyrene and exopolysaccharide (EPS) yields of selected antibiotic-resistant *Salmonella typhimurium* strains

Strains	Hydrophobicity ^a (%)	Adherence ^b ($\times 10^6$ cfu/cm ²)	EPS ^c (mg/mL)
306-98 ^d	69.08 (2.13)	6.37 (4.54)	0.21 (0.13)
T980018 ^d	64.05 (6.69)	15.62 (3.25)	0.25 (0.12)
T980021 ^d	46.84 (8.56)	8.42 (1.12)	0.21 (0.04)
T980042 ^d	67.81 (7.21)	4.96 (3.53)	0.24 (0.09)
T980043 ^d	70.39 (7.05)	6.48 (0.92)	0.26 (0.15)
ST L1388 ^d	62.86 (10.67)	7.65 (3.02)	0.12 (0.03)

^aHydrophobicity is defined as the percentage of cells that partition into the hydrocarbon phase in a microbial adhesion to hydrocarbon assay [28]. Phosphate-buffered saline washed and re-suspended (absorbance at 600 nm = 0.3-0.6) culture was mixed with xylene and the mixture left at room temperature for 1 h.

^bAdherence describes the number of bacterial cells adhering to 1 cm² surface of polystyrene chips after overnight growth in Luria-Bertani broth.

^cCarbohydrate content of crude EPS produced by bacteria after 24-h growth in trypticase soy broth determined by the phenol-sulfuric acid method.

^dValues in parenthesis are standard deviations of means of at least 3 independent observations. Statistical significance was determined by analysis of variance at $p=0.05$.

environmental conditions by trapping and retaining nutrients for growth of biofilms [54], the protection of bacteria from dehydration [28,55] and persistence and survival in hostile environments, including exposure to antimicrobial agents [56].

Adhesion of bacteria to polystyrene also did not correlate with EPS production in all the isolates, providing further evidence that EPS-mediated attachment may not be critical for DT104's colonization of surfaces. Since the organisms involved in initial adhesion (an important determinant of the strength of biofilm adhesion) represent only a small fraction of the number of organisms in a mature biofilm after growth [30], the lack of correlation of bacterial adhesion with biofilm in this study may suggest the relevance of other factors to the biofilm process of DT104s. The expression of multicellular phenotypes typical of *S. typhimurium* by all of the isolates rules out the possibility of influence on their surface colonization. Similarly, the insignificant difference in agar motility of the isolates raises doubt about any influence over biofilm growth. Bacterial motility is important in relation to the initial attachment of bacteria to a surface, and thus relevant to biofilm formation [54].

The significant inhibitory action of mannose on biofilm formation of all DT104s and *S. typhimurium* on polystyrene is an indication that *S. typhimurium* shares a common pathway with DT104s for its biofilm growth on polystyrene. Sensitivity of the biofilm process to mannose suggests a possible role for Type 1 pili in both DT104's and *S. typhimurium*'s biofilm growth. Type 1 pili contain mannose-specific adhesin, FimH, which facilitates pathogenesis through specific interactions with mannose oligosaccharides present on eukaryotic cell surfaces [6,57]. Pratt and Kolter [22] have reported a concentration-dependent mannose inhibition of biofilm formation via Type 1 pili in *E. coli*. Similarly, Austin et al [58] advocated a role for Type 1 pili in the adherence to stainless steel or Teflon in *S. enteritidis*. Our results have also indicated significant inhibition of the growth of *S. typhimurium* by mannose, in contrast with the report on *E. coli* [22].

Thus, we propose inhibition of bacterial growth as an additional mechanism for the inhibitory action of mannose on biofilm formation in *S. typhimurium*. Interestingly, on PVC, mannose did not inhibit the biofilm processes of some of the isolates whose biofilm growths on polystyrene were sensitive to it, with the indication that a particular isolate may colonize different surfaces by different mechanisms. The effect

of D-glucose on biofilm formation on polystyrene was variable; while it inhibited the process in some of the isolates, it enhanced the biofilm growths of others. Thus, glucose metabolism may be an important factor in the biofilm process of *S. typhimurium*. On PVC, glucose significantly inhibited biofilm formation of *S. typhimurium* and the most PVC-loving DT104 isolate (i.e., T980021), as opposed to its action on polystyrene, suggesting that the specific role of glucose metabolism in the biofilm process of these 2 isolates may be dependent on the colonizing surface. Earlier studies in *S. enteritidis* [18,50] have shown that glucose is capable of enhancing biofilm formation on glass through increased glycogen synthesis. In *E. coli*, glucose neither influenced biofilm formation on PVC nor inhibited bacterial growth [22].

This study, therefore, provides evidence that the biofilm growths of some strains of *S. typhimurium* on polystyrene and PVC can either be enhanced or inhibited by glucose, depending on the colonizing surface. Importantly, D-glucose, like D-mannose, significantly inhibited bacterial growth, providing a possible mechanism for its inhibitory action on biofilm formation.

Starvation is known to alter bacterial surface characteristics such as hydrophobicity, charge and irreversible attachment, which are essential factors in biofilm formation [29,59]. Consequently, it is expected to enhance biofilm formation. The starvation medium used in this study is deficient in several essential nutrients, such as nitrogen, phosphorus, calcium, magnesium, sulfur, and iron, but with glucose as a source of energy, it had been shown to encourage biofilm growth of *S. enteritidis* on glass surface [19]. In our study, however, starvation reduced biofilm growth on polystyrene for both bacterial phage types, suggesting a possibly common starvation response pathway of biofilm formation for DT104 and non-DT104 strains. It also appears that the effect of starvation on biofilm formation of salmonellae may depend on the surface being colonized.

Osmotic drift due to the high salt concentration resulted in a lower (than optimal) water-activity (a_w) environment. Salmonellae grow optimally at $a_w = 0.99$ [60], and a lower a_w is disruptive of normal cellular activities [61]. Our results indicate that biofilm formation by *S. typhimurium* was significantly enhanced by the salt stress but the responses of DT104s were differential, suggesting that the effect of osmotic drift on biofilm growth may be independent of multi-resistance and geographical and host sources of DT104s.

In conclusion, although both *S. typhimurium* DT104s and the non-multi-resistant strain *S. typhimurium* L1388 form biofilm on abiotic surfaces, they differ in the quantity formed, in the kinetics of biofilm formation, and in the susceptibility of the biofilm process to mannose and glucose. Also, our finding of growth inhibition by mannose may suggest an additional mechanism for the inhibitory effect of mannose on biofilm formation in *S. typhimurium*. Since colonization of mucosal surfaces by pathogenic bacteria is believed to proceed via biofilm growth [17], these observations should help in further enhancing our understanding of how antibiotic-resistant *S. typhimurium* colonizes its environment and causes disease.

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