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Characterization of *Salmonella enterica* Serovar Typhimurium from Marine Environments in Coastal Waters of Galicia (Spain)

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Twenty-three *Salmonella enterica* serovar Typhimurium isolates from marine environments were characterized by phage typing, pulsed-field gel electrophoresis (PFGE) analysis, plasmid analysis, and antibiotic resistance, and the distribution of the different types in the coastal waters were subsequently analyzed. Five phage types were identified among the isolates (PT41, PT135, PT99, DT104, and DT193). PT135 isolates were exclusively detected during the winter months from 1998 to 2000, whereas DT104 and PT41 isolates were detected exclusively in the summer months from 2000 to 2002. XbaI PFGE analysis revealed 9 PFGE types, and plasmid profiling identified 8 plasmid types (with 1 to 6 plasmids) among the isolates. Only three isolates presented multidrug resistance to antibiotics. Two DT104 isolates were resistant to 8 and 7 antibiotics (profiles ACCeFNaSSuT and ACeFNeSSuT), whereas a PT193 isolate presented resistance to 6 antibiotics (profile ACFSSu). In addition, four PT41 isolates were resistant to a single antibiotic. The detection of multidrug-resistant phage types DT104 and DT193 in shellfish emphasizes the importance of monitoring the presence of *Salmonella* in routine surveillance of live bivalve molluscs.

Salmonella is considered one of the most important causal agents of food-borne illness in developed countries (6). Raw food of animal origin and cross-contamination of ready-to-eat products are the main vehicles for infection (6, 15). The genus *Salmonella* comprises more than 2,400 serotypes, most of which are considered potential human pathogens, but only a reduced number of serotypes have been associated with human infections (4). *Salmonella enterica* serovar Typhimurium is a common cause of salmonellosis in many countries (13, 20, 26). It represents the most common serotype isolated from humans and animals in the United States (http://www.cdc.gov) (8) and the second most common cause of human salmonellosis in the United Kingdom (http://www.hpa.org.uk). In Spain, serovar Typhimurium was the second most frequently isolated serovar from humans and foods in 2000 (32, 33), with 1,403 (23%) and 113 (19%) isolates, respectively. Phage typing allows identification of more than 200 definitive phage types for serovar Typhimurium (2); however, a few of these tend to dominate within a geographical region during a given period of time (2). In the 1990s, the multidrug-resistant serovar Typhimurium DT104 emerged in cattle populations in the United Kingdom, and it rapidly spread to other countries (4) and to a wide range of animal species, including humans (9, 22, 31). In Spain, phage types DT104 and U302 were the most prevalent phage types identified in both human and food isolates in 2000 (32, 33).

Salmonella has been frequently identified in aquatic and marine environments (5, 11, 16, 17, 24, 35, 36, 37). In addition to sewage from human and industrial activities, coastal waters receive water directly from rivers, which can carry enteric bacteria originating from their natural reservoirs inland (5, 25, 28). The presence of enteric bacteria in marine environments represents a source of contamination for the organisms present in the coastal habitats. Many of these are consumed by humans without further processing, potentially representing a vehicle for pathogen transmission and therefore a risk to public health. The vast majority of studies looking at the presence of *Salmonella* in marine environments have evidenced two main observations: only a small but constant number of serovars have been found in these environments and, in most cases, these do not coincide with the main zoonotic serovars identified in the surrounding areas (11, 17, 25, 29, 35, 36). In spite of the variability in sampling size (n = 37 to 251), in most of these studies the maximum number of serotypes identified has been almost constant between 17 and 20 (11, 25, 35, 36). Serovar Typhimurium has normally been the main clinically significant serovar isolated (5, 11, 25, 29, 36). This characteristic indicates a good capacity of adaptation and survival of this serotype in marine environments (5).

The former studies on serovar Typhimurium in marine environments have been limited to the identification of the isolates to the serotype level, without any additional characterization. Molecular typing data are essential for epidemiological studies, and this information would facilitate the identification of the possible sources of contamination in coastal waters and related environments. This type of information is also valuable for assessing the potential risk to public health associated with the presence of *Salmonella* in live shellfish. In the present

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TABLE 1. Identification of the 23 isolates of *S. enterica* serovar Typhimurium included in this study

Origin[a]	Zone[a]	Isolate no.	Date of isolation (day-mo-yr)[b]	Source	Phage type	XbaI type	Plasmid profile	Antibiotic resistance[c]
Arousa	VII	UCM-97	10-July-00	Mussels	104	X08	P05	A, C, Ce, F, Na, S, Su, T
Pontevedra	III	UCM-229	17-Sep-02	Mussels	104	X08	P06	A, Ce, F, Ne, S, Su, T
Arousa	VIII	UCM-10	16-Feb-98	Oysters	135	X09	P07	Susceptible
Arousa	VII	UCM-77	15-Dec-99	Mussels	135	X09	P07	Susceptible
Arousa	VII	UCM-75	31-Jan-00	Mussels	135	X09	P07	Susceptible
Vigo	IV	UCM-74	31-Jan-00	Mussels	135	X09	P07	Susceptible
Arousa	II	UCM-78	14-Feb-00	Mussels	135	X09	P07	Susceptible
Arousa	IX	UCM-79	14-Feb-00	Mussels	135	X09	P07	Susceptible
Arousa	IX	UCM-80	14-Feb-00	Mussels	135	X09	P07	Susceptible
Arousa	VIII	UCM-73	24-Jan-00	Oysters	193	X07	P03	A, C, Ce, S, Su, T
Pontevedra	V	UCM-94	21-Aug-00	Clams	41	X02	P06	Susceptible
Pontevedra	II	UCM-95	11-Sep-00	Mussels	41	X01	P06	F
Arousa	VI	UCM-131	20-Aug-01	Mussels	41	X01	P06	Sf
Arousa	VIII	UCM-133	3-Sep-01	Oysters	41	X01	P06	Ne
Pontevedra	II	UCM-147	1-Oct-01	Mussels	41	X01	P06	Susceptible
Arousa	IX	UCM-182	4-Mar-02	Mussels	41	X01	P06	Susceptible
Arousa	VIII	UCM-228	16-Sep-02	Oysters	41	X01	P06	Susceptible
Pontevedra	II	UCM-234	23-Sep-02	Oysters	41	X01	P06	G
Arousa	VII	UCM-143	19-Sep-01	Mussels	99	X06	P04	Susceptible
Arousa	VII	UCM-146	24-Sep-01	Oysters	99	X06	P04	Susceptible
Vigo	V	UCM-196	14-May-02	Mussels	99	X05	P02	Susceptible
Pontevedra	II	UCM-96	11-Sep-00	Mussels	UNTY[d]	X03	P08	Susceptible
Arousa	VII	UCM-100	28-Sep-00	Mussels	UNTY	X04	P01	Susceptible

[a] Rias of Galicia and molluscan harvesting zones.
[b] Jan, January; Feb, February; Mar, March; Aug, August; Sep, September; Oct, October; Dec, December.
[c] Antibiotics: A, ampicillin; Ak, amikacin; Ap, apramycin; Ax, amoxicillin-clavulanic acid; C, chloranphenicol; Ce, cefoperazone; Cf, ceftazidime; Ct, colistin; F, furazolidone; G, gentamicin; Na, nalidixic acid; Ne, neomycin; S, streptomycin; Sf, sulfamethoxazole-trimethoprim; Su, compound sulfonamides; T, tetracycline.
[d] UNTY, untypeable.

study, we have characterized serovar Typhimurium isolates from marine environments by phage typing, pulsed-field gel electrophoresis (PFGE), plasmid analysis, and antibiotic sensitivity testing. In addition, we have analyzed the distribution of the different types in the coastal waters.

MATERIALS AND METHODS

Bacterial isolates. A total of 23 isolates of *Salmonella* serovar Typhimurium from marine environments were characterized (Table 1). These isolates represented all of the serotype Typhimurium isolates identified from a total of 133 *Salmonella* isolates obtained from the analysis of 6,317 samples of molluscs and seawater taken from 1998 to 2002 (1998, $n = 933$; 1999, $n = 1{,}134$; 2000, $n = 916$; 2001, $n = 1{,}043$; 2002, $n = 2{,}291$) during routine monitoring surveillance (24, 25). The samples were collected in the mollusc production areas located in the four most important rias (estuaries similar to small fjords which extend from East to West) of Galicia in Northwestern Spain (Fig. 1). Mollusc cultivation is extensive in the estuarine portions of these rias, with a production of mussels alone exceeding 200,000 tons a year. Isolates were phage typed at the Veterinary Laboratories Agency-Weybridge (United Kingdom) according to standard methods (2).

PFGE. PFGE was preformed according to the one-day (24 to 28 h) standardized laboratory protocol for molecular subtyping of nontyphoidal *Salmonella* by PFGE (Pulse-Net, Centers for Disease Control and Prevention, Atlanta, Ga.) (12). A single colony of each isolate was streaked on tryptic soy agar and incubated overnight at 37°C. Using a cotton swab, a portion of the growth on the agar plate was transferred to 2 ml of cell suspension buffer (100 mM Tris, 100 mM EDTA [pH 8.0]), and the cell concentration was adjusted to 0.48 to 0.52 in a Microscan turbidity meter (Dade Behring, West Sacramento, Calif.). Immediately, 400 μl of adjusted cell suspension was transferred to 1.5-ml microcentrifuge tubes with 20 μl of proteinase K (20 mg/ml), subsequently mixed with 400 μl of melted 1% SeaKem Gold (Cambrex, East Rutherford, N.J.)–1% sodium dodecyl sulfate agarose prepared with TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and pipetted into disposable plug molds. Three plugs were transferred to 50-ml polypropylene screw-cap tubes with 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosyl) and 25 μl of proteinase K (20 mg/ml) and incubated at 54°C in a shaker water bath for 2 h with agitation. Thereafter, the plugs were washed twice with 15 ml of sterile water and three more times with TE buffer at 50°C for 15 min. Chromosomal DNA was digested with 50 U of XbaI (Promega, Southampton, United Kingdom). PFGE was performed on a CHEF DRIII system (Bio-Rad, Hercules, Calif.) in 0.5× Tris-Borate-EDTA (TBE) extended-range buffer (Bio-Rad) with recirculation at 14°C. DNA macrorestriction fragments were resolved on 1% SeaKem Gold agarose (Cambrex) in 0.5× TBE buffer. DNA from *Salmonella* Braenderup H9812 restricted with XbaI was used as a size marker. Pulse times were ramped from 2.2 to 63.8 s during an 18-h run at 6.0 V/cm. Macrorestriction patterns were compared with the use of BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Different profiles were designated with the letter X (XbaI types) in accordance with the restriction patterns. A difference of at least one restriction fragment in the patterns was considered the criterion for discriminating between different clones.

Antimicrobial susceptibility testing. Isolates were screened for susceptibility to a panel of 16 antibiotics on Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, United Kingdom) by a disk diffusion method, as described by the NCCLS guidelines (27). The following disks (Oxoid) were used: amikacin (30 μg), amoxicillin-clavulanic acid (30 μg), ampicillin (10 μg), apramycin (15 μg), chloramphenicol (10 μg), cefoperazone (30 μg), ceftazidime (30 μg), colistin (25 μg), furazolidone (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), neomycin (10 μg), streptomycin (25 μg), sulfamethoxazole-trimethoprim (25 μg), tetracycline (10 μg), and compound sulfonamide (500 μg).

Plasmid analysis. Plasmid DNA was isolated by the alkaline lysis method as described previously (19). Samples were analyzed by electrophoresis in 1× TBE buffer at 150 V for 4.5 h on 0.8% agarose gels with recirculation at 20°C. Plasmid-containing *Escherichia coli* strain 39R861 and a supercoiled DNA ladder (Gibco-BRL, Paisley, United Kingdom) were used as size markers. Plasmids were compared by the use of BioNumerics software. The molecular weights of the plasmids were calculated by comparison with the external markers, and images were normalized accordingly.

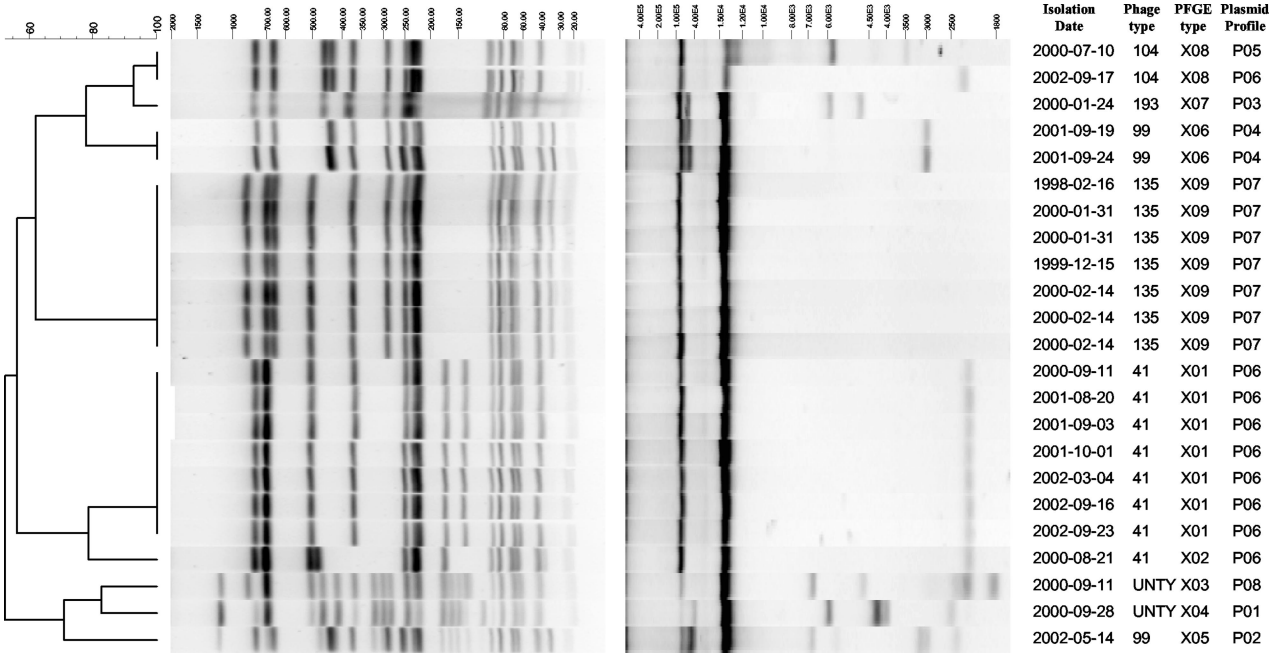
RESULTS

Phage typing of the 23 isolates identified 5 types (Table 1). The most prevalent types were PT41 and PT135, with 8 and 7

FIG. 1. Locations of the coastal areas and rias of Galicia included in the present study.

isolates, respectively. The other phage types identified were PT99, DT104, and DT193, with 3, 2, and 1 isolates, respectively; two isolates were untypeable. PT135 was first detected in 1998 and remained the prevalent phage type until 2000. PT135 isolates were found in samples from the rias of Arousa and Vigo. PT41 was the prevalent type from 2000 to 2002, and it was detected in the rias of Arousa and Pontevedra. The remaining phage types were only detected in association with sporadic contamination events. The most contaminated points were zones VII and VIII of the ria of Arousa, where all phage types were detected, and zones II and III of the ria de Pontevedra.

PFGE analysis of XbaI restriction of DNA from the 23 isolates generated 9 PFGE types (Table 1 and Fig. 2). Types X01 and X09 were the most prevalent and were present only among PT135 and PT41 isolates, respectively. PFGE type X12 was found exclusively among PT135 isolates, whereas the PT41 isolates were differentiated in two related types X01 and X02.



Isolation Date	Phage type	PFGE type	Plasmid Profile
2000-07-10	104	X08	P05
2002-09-17	104	X08	P06
2000-01-24	193	X07	P03
2001-09-19	99	X06	P04
2001-09-24	99	X06	P04
1998-02-16	135	X09	P07
2000-01-31	135	X09	P07
2000-01-31	135	X09	P07
1999-12-15	135	X09	P07
2000-02-14	135	X09	P07
2000-02-14	135	X09	P07
2000-02-14	135	X09	P07
2000-09-11	41	X01	P06
2001-08-20	41	X01	P06
2001-09-03	41	X01	P06
2001-10-01	41	X01	P06
2002-03-04	41	X01	P06
2002-09-16	41	X01	P06
2002-09-23	41	X01	P06
2000-08-21	41	X02	P06
2000-09-11	UNTY	X03	P08
2000-09-28	UNTY	X04	P01
2002-05-14	99	X05	P02

FIG. 2. Restriction patterns (X types) and plasmid profiles (P types) of 23 serovar Typhimurium isolates from marine environments. The dendrogram generated by Bionumerics software shows the relationships between PFGE types.

TABLE 2. Number of plasmids and plasmid sizes of each plasmid profile

Plasmid type	No. of plasmids	Size(s) of plasmids (kb)	No. of isolates
P01	6	90, 60, 6.0, 4.3, 4.0, 2.5	1
P02	5	90, 60, 7.0, 3.0, 2.5	1
P03	4	90, 60, 6.0, 4.5	1
P04	3	90, 60, 3.0	2
P05	4	90, 13, 6.0, 3.5	1
P06	2	90, 2.2	9
P07	1	90	7
P08	6	90, 7.0, 4.5, 2.5, 2.2, 1.8	1

The two DT104 isolates presented identical restriction patterns (PFGE type X08), very closely related with type X07 found in a DT193 isolate. PT99 isolates were discriminated in types X06 and X05. The two untypeable isolates presented closely related patterns (PFGE types X03 and X04).

Eight different plasmid profiles were identified among the isolates with 1 to 6 plasmids (Table 2 and Fig. 2). All of the isolates contained the serotype-specific plasmid (approximately 90 kb). The most prevalent plasmid profile was P06, containing the serotype-specific plasmid and a small plasmid of 2.2 kb. This plasmid profile comprised all of the PT41 isolates and one DT104 isolate. The second most prevalent plasmid profile was P10 with the serotype-specific plasmid only and including all of the PT135 isolates.

The presence of antibiotic resistance was confined to three phage types: DT104, DT139, and PT41 (Table 1). Three isolates were multiresistant to several antibiotics. The two DT104 isolates were resistant to 8 and 7 antibiotics (profiles ACCeFNaSSuT and ACeFNeSSuT), whereas the PT193 isolate presented resistance to 6 antibiotics (profile ACFSSu). Four PT41 isolates were resistant to a single antibiotic.

DISCUSSION

Several studies investigating the presence of *Salmonella* in marine environments have shown that serovar Typhimurium predominates over the rest of the clinically significant serovars (5, 11, 25, 29, 36). In a previous study carried out in the coastal waters of Galicia, our laboratory identified serovar Typhimurium as the second most prevalent serovar, representing 15% of the isolates (25). Serovar Typhimurium was most frequently isolated in the summer months and mainly localized in specific coastal areas in proximity to villages with a large summer tourist population (25). The results obtained in the present study indicate a specific pattern of detection of the different types. PT135/X09/P07 isolates were exclusively detected in winter months in the rias of Vigo and Arousa, and they were never isolated in the ria of Pontevedra. Isolates of this combined type were detected from 1998 to 2000. DT104/X08 and PT41/X01/P06 isolates were detected for the first time in 2000 and then detected until 2002; these combined types were isolated exclusively in summer months in coastal areas neighboring highly populated villages located in the rias of Pontevedra and Arousa. This pattern of detection evidences the existence of distinctive sources of contamination for each phage type. These sources contribute to contamination of coastal areas for long periods of time in different rias located many kilometers away. Unfortunately, the lack of information about the predominant animal and human serotypes and phage types present in this region makes it impossible to carry out further epidemiological studies.

Multidrug-resistant serovar Typhimurium DT193 has been responsible for outbreaks in humans in the late 1980s and early 1990s, mainly in Europe (14). Two major outbreaks were reported in Italy and the United Kingdom associated with contaminated pork products (23, 30). In Spain, DT193 was the third most prevalent phage type isolated from humans in 2000 (33). Multidrug-resistant DT104 is an important international human pathogen, and it is widespread in Western and Eastern Europe, North America, and the Middle East (18). Gastrointestinal infections with DT104 are mainly associated with raw food (1, 18). DT104 was the most prevalent serovar Typhimurium phage type in Spain during the last years. The detection of multidrug-resistant serovar Typhimurium DT104 and DT193 in molluscan shellfish (a ready-to-eat product that it is consumed raw without any culinary treatment) emphasizes the importance of monitoring the presence of *Salmonella* in routine surveillance in the heavily populated European coasts. This observation has a special relevance at this time when a new European regulation on microbiological criteria for foodstuffs (SANCO/4198/2001, revision 6) is under discussion. The draft 18.7.2003/PM of this document removes *Salmonella* (present in the applicable microbiological requirements for live molluscs included in the European Directive 91/492/EEC) (3) from the microbiological criteria for live bivalve molluscs. This has been based on the absence of reported cases of *Salmonella* infections associated with the consumption of shellfish. However, the investigations associated with human enteric infections are often restricted to identification of the etiological agent, without any further characterization of the isolates or identification of the food implicated. This lack of information could contribute to an underestimation of the real risk of *Salmonella* infections associated with shellfish consumption. As an example, according to data from the Spanish Weekly Epidemiological Bulletin (33), none of the 1,403 serovar Typhimurium isolates classified as non-outbreak related had an associated vehicle of the infection. Furthermore, 52 (78%) of the reported outbreaks did not include the identification of the food associated with the infection (34). This lack of information limits the use of the available epidemiological data in the assessment of the risk associated with the presence of *Salmonella* in live bivalve molluscs. In our opinion, risk assessments should be based on the detection and characterization of human pathogens present in shellfish more than on a lack of evidence of shellfish-borne infections.

Two of the main requirements for addressing surveillance of emerging food-borne diseases are the necessity for susceptible and rapid screening methods and the use of appropriate subtyping tools for characterization of the pathogens (1, 18). In the present study, phage typing has been shown to be an efficient tool for discriminating the serovar Typhimurium isolates from different sources and for studying the persistence over time in longitudinal investigations. PFGE results revealed a high degree of genetic homogeneity among isolates from the same phage type. This presence of a prevalent genomic clone in each of the phage types is consistent with the findings ob-

tained in other studies (4, 7, 10, 14, 21). A similar degree of homogeneity within each phage type was also revealed with the plasmid analysis. The results obtained in this work showed good agreement in the identification of types between the three typing techniques applied (PFGE, phage typing, and plasmid profiling). However, due to the high degree of clonality of serotype Typhimurium, the use of multiple typing techniques is considered the best approach for discriminating among isolates (21). The results of this study indicate the importance of surveillance of human pathogens for shellfish safety assurance programs and the role of the subtyping techniques for characterization of *Salmonella* populations and source-tracking studies.

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