Identification and Characterization of Class 1 Integron Resistance Gene Cassettes among Salmonella Strains Isolated from Imported Seafood

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Received 4 September 2008/Accepted 1 December 2008

A total of 210 Salmonella isolates, representing 64 different serovars, were isolated from imported seafood samples, and 55/210 isolates were found to be resistant to at least one antibiotic. Class 1 integrons from three multidrug-resistant Salmonella enterica strains (Salmonella enterica serovars Newport [strain 62], Typhimurium var. Copenhagen [strain 629], and Lansing [strain 803], originating from Hong Kong, the Philippines, and Taiwan, respectively) were characterized. Southern hybridization of plasmids isolated from these strains, using a class 1 integron probe, showed that trimethoprim-sulfamethoxazole and streptomycin resistance genes were located on a megaplasmid in strain 629. Our study indicates that imported seafood could be a reservoir for Salmonella isolates resistant to multiple antibiotics.

Salmonella spp. are recognized as major food-borne pathogens of humans worldwide. In the United States, there are an estimated 800,000 to 4 million Salmonella infections annually, and approximately 500 of the cases are fatal (8, 26). A variety of foods have been implicated as vehicles transmitting salmonellosis to humans, including poultry, beef, pork, eggs, milk, cheese, fish, shellfish, fruits, juice, and vegetables (1, 4, 9, 12, 23). Previous studies by field laboratories of the U.S. Food and Drug Administration have shown the prevalences of Salmonella isolates in imported and domestic seafood as 7.2% and 1.3%, respectively (6, 11, 27).

Mobile genetic elements, such as plasmids, transposons, and integrons, which disseminate antibiotic resistance genes by horizontal or vertical transfer, as part of either resistance plasmids or conjugative transposons, play an important role in the evolution and dissemination of multidrug resistance (2, 3, 10, 17). Salmonella genomic island 1 (SGI1), the first genomic island reported to contain an antibiotic resistance gene cluster, was identified in the multidrug-resistant Salmonella enterica serovar Typhimurium strain DT 104 (21).

Most studies of the prevalence and characterization of antimicrobial resistance genes and integrons in Salmonella spp. have focused on strains from clinical and veterinary sources. However, little is known about the occurrence of SGI1 and its variants in Salmonella spp. isolated from seafood. We have screened a set of drug-resistant S. enterica strains from seafood belonging to 64 different serovars for SGI1 and class 1 integron conserved sequences (CS). We report the presence of a class I variant integron carrying the dfrXII and aadA2 genes on a megaplasmid in serovar Typhimurium var. Copenhagen and on the chromosome in Salmonella enterica serovar Newport. We also found the variant class 1 integron carrying the dfrA1 and orfC genes in Salmonella enterica serovar Lansing strains from seafood.

A total of 210 Salmonella enterica strains isolated from seafood imported into the United States between 2000 and 2005 were identified and serotyped by the Pacific Regional Laboratory-Southwest of the FDA, Irvine, CA. The Salmonella strains represented 20 serogroups (Table 1) from various imported seafood items. The Salmonella strains were tested with 16 antibiotics (14) commonly used in either human or veterinary medicine on Mueller-Hinton agar (Difco Laboratories, Detroit, MI), using a disk diffusion method. The sensitivity and resistance were determined by the criteria of the Clinical and Laboratory Standards Institute (1999).

All Salmonella strains that were resistant to three or four antibiotics and trimethoprim were screened by PCR for the presence of class 1 integrons, using the CSL1 and CSR1 primers (Table 2) (14). To confirm other antibiotic resistance genes, we used primers and PCR methods described previously (13, 14, 16). To identify SGI1 in multidrug-resistant strains, PCR was performed by using primers U7-L12/LJ-R1 and 104-RJ/104-D (Table 2), corresponding to the left and right junctions of SGI1 in the Salmonella chromosome, respectively (16). For a positive control, serovar Typhimurium DT104 strain DT7 (13) was used. As a negative control, Escherichia coli cells or DNA was used. A reagent blank included in each PCR contained distilled water instead of template DNA. For sequencing, the PCR-amplified integrons were purified and cloned into plasmid vector pCR2.1 (Invitrogen Corp., Carlsbad, CA). The clones were investigated for the presence of inserts by isolating the recombinant plasmid, which was confirmed by digestion with the restriction enzyme EcoRI. Sequencing of both strands was performed. DNA sequences were analyzed with Lasergene...
VOL. 75, 2009 CLASS 1 INTEGRONS AMONG SALMONELLA SEROVARS IN SEAFOOD

INTRODUCTION

Salmonella spp. are common foodborne pathogens responsible for thousands of foodborne illnesses each year. This study aimed to detect and characterize class 1 integrons among Salmonella serovars isolated from seafood.

MATERIALS AND METHODS

The study involved 1193 isolates from imported seafood, which originated from 20 different countries. Serotyping was performed at the Pacific Regional Laboratory-Southwest of the FDA.

RESULTS

Salmonella serovars Weltevreden (17.6%), Saintpaul (5.1%), Newport (4.3%), and Senftenberg (4.3%) were the most common. Antibiotic resistance was high, with 34% of isolates resistant to at least one antibiotic.

Detection and characterization of the class 1 integron cassette and SGI1 in Salmonella strains

A 1.9-kb PCR product was amplified from 803 strain of S. enterica serovar Schwarzengrund. The integron cassette was identical to recent reports for S. enterica serovar Typhimurium var. Copenhagen.

DISCUSSION

The presence of class 1 integrons in Salmonella spp. indicates the potential for horizontal gene transfer, increasing the risk of antibiotic resistance. Future studies should focus on the prevalence and impact of integrons in different regions and food types.

TABLE 1. Salmonella serotypes isolated from imported foods

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>S. enterica serovar(s) or Salmonella group(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>Weltevreden</td>
</tr>
<tr>
<td>16</td>
<td>Newport</td>
</tr>
<tr>
<td>13</td>
<td>Saintpaul</td>
</tr>
<tr>
<td>10</td>
<td>Senftenberg</td>
</tr>
<tr>
<td>8</td>
<td>Lexington</td>
</tr>
<tr>
<td>7</td>
<td>Virchow</td>
</tr>
<tr>
<td>6</td>
<td>Enteritis, Bareily</td>
</tr>
<tr>
<td>5</td>
<td>Bovismorbidicans, Brunei, Java, Hvingtoss</td>
</tr>
<tr>
<td>4</td>
<td>Paratypi B var. Java, Thompson</td>
</tr>
<tr>
<td>3</td>
<td>Aberdeen, Cabana, Stanley, Derby, Lansing</td>
</tr>
<tr>
<td>2</td>
<td>Montevideo, Hadar, Agona, San Diego, Braenderup, Lanka, Salmonella enterica subsp. diarzonae, Oslo, Bareily variant, Salmonella monophasic group C2</td>
</tr>
</tbody>
</table>

TABLE 2. Primer pairs for integron PCR and sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Location</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSL1</td>
<td>GGC ATC CAA GCA GCA AGC</td>
<td>5’ CS</td>
<td></td>
</tr>
<tr>
<td>CSR1</td>
<td>AAG CAG ACT TGA CCT GAT</td>
<td>3’ CS</td>
<td></td>
</tr>
<tr>
<td>U7-L12</td>
<td>ACA CCT TGA GCA GCG GAA AG</td>
<td>thdF</td>
<td>500</td>
</tr>
<tr>
<td>LJ-R1</td>
<td>AGT TCT AAA GCG TTC TAG</td>
<td>TCG</td>
<td></td>
</tr>
<tr>
<td>104-RJ</td>
<td>TGA CGA GCT GAA GCG AAT TG</td>
<td>S044</td>
<td></td>
</tr>
<tr>
<td>104D</td>
<td>ACC AGG GCA AAA CTA CAC AG</td>
<td>ydY</td>
<td></td>
</tr>
<tr>
<td>aadA2F</td>
<td>TGT TGG TTA CTG TGG CCG TA</td>
<td>aadA2</td>
<td>380</td>
</tr>
<tr>
<td>aadA2R</td>
<td>GCT GCG AGT TCC ATA GTA TCT</td>
<td>aadA2</td>
<td></td>
</tr>
</tbody>
</table>

(NDASTAR, Inc., Madison, WI) software. Oligonucleotide primers and probes were purchased from MWG Biotech (High Point, NC).

Plasmid DNA was isolated using an alkaline lysis method with modifications described previously (19). Plasmids were separated by electrophoresis in 1× Tris-acetate-EDTA buffer at 64 V for 2 h on 1.0% agarose gels, stained with 40 μl of ethidium bromide (0.625 mg/ml) for visualization, and then transferred and cross-linked to positively charged nylon membranes (Roche, Indianapolis, IN). The resulting blots were hybridized at 65°C for 18 h with digoxigenin-labeled DNA probes (1.2-kb and 1.9-kb PCR-amplified products), using CSL1 and CSR1 primers specific for class 1 integrons (22).

Isolation and characterization of Salmonella spp. from seafood

The present study reports the presence of 20 different countries. The 210 Salmonella strains were characterized by serotyping at the Pacific Regional Laboratory-Southwest of the FDA, Irvine, CA, and found to have 64 different serovars, including Salmonella enterica serovars Weltevreden (17.6%), Newport (7.6%), Saintpaul (6.2%), Senftenberg (4.8%), Lexington (4.3%), Virchow (3.3%), Bareily (3.3%), Enteritis (2.9%), Paratypi B var. Java (2.4%), and Brunei (2.4%). Other serotype strains were less than 1%. Of the 210 Salmonella isolates, 49 (24%) were resistant to at least one antibiotic (tetracycline, streptomycin, or ampicillin). Six percent (12 strains) were resistant to more than two antibiotics. Three Salmonella strains (62, 629, and 803 [serovars Newport, Typhimurium var. Copenhagen, and Lansing, respectively]) were resistant to several antibiotics, including trimethoprim-sulfamethoxazole, sulfisoxazole, tetracycline, streptomycin, and spectinomycin (Table 3). Strain 62 was also resistant to ampicillin and chloramphenicol, and strain 803 was also resistant to four more antibiotics, kanamycin, gentamicin, ampicillin, and chloramphenicol (Table 3). Antibiotic resistance was not associated with particular Salmonella serovars.

Detection and characterization of the class 1 integron cassette and SGI1 in Salmonella strains

Salmonella enterica serovar Typhimurium var. Copenhagen strain 629 and serovar Lansing strain 803 amplified a 1.9-kb PCR product and serovar Newport strain 62 amplified a 1.2-kb PCR product when the primers CSL1 and CSR1 were used, suggesting the presence of a class 1 integrase (intI1). Salmonella enterica serovar Typhimurium var. Copenhagen strain 629 and serovar Lansing strain 803, resistant to trimethoprim-sulfamethoxazole, amplified a 500-bp region of the left junction of SGI1; however, serovar Newport strain 62 did not amplify any PCR products. All three strains were negative for the SGI1 right junction and retron sequences. To determine if the integron characteristic of SGI1 and its variant was present, primers which spanned the boundaries (Table 2) of In104 with the SGI1 backbone were used. There was no PCR amplification with these primers. Similar results were found in serovar Emek strain (16), Salmonella enterica serovars Oslo and Bareily (14), and several strains of S. enterica serovar Agona, in which the right junction was negative (8). The 1,242-bp sequence of serovar Newport strain 62 demonstrated that it contained a dihydrofolate reductase gene (dfrA) and an orfC cassette. The gene cassette showed 100% identity with the dfrA1 and orfC genes, encoding trimethoprim-sulfamethoxazole resistance and an unknown function, respectively (Fig. 1A). This cassette array is identical to recent reports for Salmonella enterica serovars Typhimurium var. Copenhagen strain 629 and the 1,917-bp sequence of serovar Lansing strain 803 demonstrated that they contained a dihydrofolate reductase gene (dfrXII), orfF, and an aadA2 cassette. The gene cassette showed 100% identity with dfrXII, orfF, and the aadA2 gene, encoding trimethoprim-sulfamethoxazole resistance, an unknown function, and streptomycin-spectinomycin resistance, respectively (Fig. 1B). This cassette array is identical to recent reports for Salmonella enterica serovar Schwarzengrund (GenBank accession no. DQ238105), a Salmonella enterica serovar Choleraesuis plasmid (AB366440), Staphylococcus spp. (AB297450, AB297448, AB297447), E. coli...
plasmids (EF450247, DQ390455, and DQ39054), Klebsiella pneumoniae (AY748453), Aeromonas hydrophila (DQ515960), and Vibrio cholerae O19 (DQ789997).

**Plasmid analysis and Southern hybridization.** Plasmid analysis of strains 62, 629, and 803 showed that only strains 629 and 803 have plasmids (Fig. 2B). Southern hybridization of a plasmid with class 1 integron probes showed homology with the strain 629 plasmid; however, it did not hybridize with the strain 803 plasmid (Fig. 2A). These data suggest that the integron is located only on a plasmid in strain 629, and in strain 803, the integrase is located on the chromosome. Class 1 integrons on plasmids are considered to be a main mechanism for the rapid spread of multidrug-resistant phenotypes among gram-negative bacteria (20).

Nontyphoidal *Salmonella* spp. are common food-associated pathogens, and *Salmonella* infections account for a large proportion of deaths associated with food-related illnesses (5, 25). Use of antimicrobials (for both therapeutic and nontherapeutic uses) in food animals is the dominant factor increasing the occurrence of drug-resistant isolates (1). An understanding of the molecular mechanism of resistance will provide useful markers to assist future studies investigating the evolution of multidrug resistance (2, 3, 16, 18).

To our knowledge, this is the first report of this class of variant integron in these three serovars (Lansing, Newport, and Typhimurium var. Copenhagen) of *Salmonella enterica* isolated from seafood from Hong Kong, the Philippines, and Taiwan. In serovar Typhimurium var. Copenhagen strain 629, the integron was located on a megaplasmid. The presence of class 1 integrons on plasmids is considered to be the main mechanism for the rapid spread of multidrug-resistant phenotypes among gram-negative bacteria (15, 20). *Salmonella enterica* serovar Emek, which has an integron cassette array (dfrA1-orfC) and also has SGI1 sequences on the left junction, has been isolated from humans (16). Previously, we isolated and characterized *S. enterica* serovars Oslo and Bareily variant class 1 integrons with a gene cassette array of dfrA1 and orfC (16). These two strains were resistant to trimethoprim-sulfamethoxazole, which represents the example in which gene replacement took place in one of the integron structures (14). In serovar Newport, the dfrA1 and orfC gene cassettes in the array were found instead of aadA2; they may have been introduced by homologous recombination with a class 1 integron containing the same array of gene cassettes from another bacterium. Another possibility involves an exchange between aadA2 and the two gene cassettes, which would imply excision of aadA2 and its replacement by the other gene cassettes (11).

SGI1 is the first genomic island containing an antibiotic resistance gene cluster identified in *S. enterica* serovar Typhimurium DT104. It is possibly important in the worldwide epidemic of this multidrug-resistant clone causing disease in animals as well as humans (16). The SGI1 variant antibiotic resistance gene clusters have been reported to occur in several *S. enterica* serovars with dfrA1 and dfrA10 (16), encoding trimethoprim resistance. These variant antibiotic resistance gene clusters were probably generated by recombational events, such as insertions or deletions. In our study, we found that two serovars, Lansing and Typhimurium var. Copenhagen, contained the left junction of genomic island SGI1; however, strain 62 serovar Newport did not. All three of these strains were negative for the SGI1 right junction and retron sequences.

The arrays of gene cassettes found in the integrons of serovars Newport, Typhimurium var. Copenhagen, and Lansing were the same as those reported to occur in integrons of *V. cholerae* isolated in Thailand and India (7, 24). Considering the origins of the serovars Newport, Typhimurium var. Copenhagen, and Lansing (seafood from Hong Kong, the Philippines, and Taiwan, respectively), a possible explanation could be the exchange of antibiotic resistance gene cassettes between epi-

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**TABLE 3. Properties of *Salmonella enterica* strains containing SGI1 or its variants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serovar</th>
<th>Antibiotic resistance profile</th>
<th>Presence of resistance gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ap</td>
<td>Cm</td>
</tr>
<tr>
<td>62</td>
<td>Newport</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>629</td>
<td>Typhimurium var.</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Copenhagen</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

* R, resistant; S, susceptible. Antibiotics: Ap, ampicillin (10 μg/ml); Cm, chloramphenicol (30 μg/ml); Sm, streptomycin (10 μg/ml); Sp, spectinomycin (50 μg/ml); Su, sulfamethoxazole (550 μg/ml); Te, tetracycline (30 μg/ml); Tp, trimethoprim.

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**FIG. 1.** Map of variant class 1 integrons of *Salmonella enterica* serovars Newport (62) (A), Typhimurium var. Copenhagen (629) (B), and Lansing (803). The 5′ CS (containing the intI1 gene) and the 3′ CS (containing the intI1 gene) are shown by open boxes. The individual gene cassettes are shown together with their recombination sites, with a 59-bp element indicated by a circle. The dfrA1 and dfrXII genes confer resistance to trimethoprim-sulfamethoxazole, and aadA2 confers resistance to streptomycin. ORF, open reading frame.
demid multidrug-resistant V. cholerae strains and Salmonella strains. In Asia, multidrug-resistant V. cholerae epidemics in humans might be responsible for the spread of antibiotic resistance genes. Human colonization by V. cholerae creates a hyperinfectious bacterial state, which is perpetuated even after bacteria are purged into natural aquatic reservoirs and may contribute to the epidemic spread of cholera. These aquatic reservoirs may be an ecological niche where antibiotic resistance gene exchange takes place between different pathogenic enterobacteriaceae.

These results suggest that the use of antimicrobials in aquaculture farming in Southeast Asia may be selecting for antimicrobial-resistant Salmonella species that can contaminate imported seafood coming into the United States. It is important that antimicrobials be appropriately used in aquaculture on a global basis to preserve the efficacy of the existing drug and to limit the risk of transfer of resistant food-borne pathogens to humans.

**Nucleotide sequence accession numbers.** The sequences of the Salmonella enterica serovar Typhimurium var. Copenhagen (629) and Lansing (803) dshfXII-orfA-aadA2 cassettes have been submitted to the GenBank database under accession no. EF555787 and EF550586. The accession number for the sequence of the Salmonella enterica serovar Newport (62) dshfA-orfC cassette is EF547513.

We thank Carl E. Cerniglia, John B. Sunderland, and Huizhong Chen for critical review of the manuscript.

This work was supported by the National Center for Toxicological Research, U.S. Food and Drug Administration.

The views presented here do not necessarily reflect those of the FDA.

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