ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2001, p. 723–726 Vol. 45, No. 3
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.3.723–726.2001

Incidence of Class 1 and 2 Integrases in Clinical and Commensal Bacteria from Livestock, Companion Animals, and Exotics

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Received 23 May 2000/Returned for modification 11 September 2000/Accepted 7 December 2000

Many pathogenic and commensal organisms are multidrug resistant due to exposure to various antibiotics. Often, this antimicrobial resistance is encoded by integrons that occur on plasmids or that are integrated into the bacterial chromosome. Integrons are commonly associated with bacterial genera in the family *Enterobacteriaceae*. We determined that class 1 integrases were present in approximately 46% of the isolates from the family *Enterobacteriaceae*; class 2 integrases were present only among *Escherichia coli* and *Salmonella* isolates. Seven percent of veterinary isolates were positive for class 3 integrase by DNA-DNA hybridization but could not be confirmed to be positive by PCR. None of the veterinary isolates possessed the class 4 integrase gene. The distribution of these integrase genes was variable within the members of the family *Enterobacteriaceae* when some or all integrase classes were absent from a particular genus. There was also considerable variability in the distribution of these integrases within a species, depending on the animal host. Unlike the class 1 integrases, the other integrase class, *intI2*, appears to be more restricted in its distribution among the members of the family *Enterobacteriaceae*. There is also considerable variability in the distribution of the class 1 integrases within *E. coli* strains isolated from different food animals. The class 1 integrases are the most widely disseminated of the four classes among the members of the family *Enterobacteriaceae* from both the clinical and normal flora of animals. This is the first report to closely examine the distribution of class 2 integrases in members of the family *Enterobacteriaceae* isolated in the United States.

Transfer of antibiotic resistance genes between different species of bacteria can be facilitated by mobile DNA elements, such as transposons and plasmids. Integrons have been identified on these mobile elements, and they often contain one or more genes that encode antibiotic resistance (29). Four classes of integrons have been described. The structure of the class 1 integron includes a 5′ and 3′ conserved segment (with respect to the antibiotic resistance cassette insertion point) and a variable region (15, 17, 21, 29). The 5′ conserved segment consists of the *intI* gene (integrase) and a promoter region expressing the inserted gene(s) (29). The 3′ conserved segment contains the defective quaternary ammonium resistance gene *qac*EΔ1 and the *sul1* gene, which encodes resistance to sulfonamides (21, 29). The variable region, located between the two conserved segments, is the site for the insertion of antibiotic resistance gene cassettes and includes *aatC* (also known as the 59-base element), which participates in the recombination mechanism (7, 10, 30). Class 1 integrons have also been identified on transposable elements such as mercury resistance transposon Tn*21* (8). Class 2 integrons are present on transposon Tn*7* and contain a dihydrofolate reductase gene cassette (6, 29). Class 3 integrons have recently been characterized in *Serratia marcescens*, isolated in Japan, by the identification of the bla_{IMP} gene, which encodes broad-spectrum β-lactam antibiotic resistance. The bla_{IMP} gene is flanked by a 59-base element and an integrase-like gene and the *aac(6′)-Ib* gene, which encodes aminoglycoside resistance (1). Class 4 integrons have been characterized only in *Vibrio cholerae* to date. This novel class contains the *intI4* gene, which encodes a new integrase which makes tandem arrays of *V. cholerae* repeated sequences similar to the arrays of antibiotic resistance gene cassettes found in class 1 integrons (18).

The family *Enterobacteriaceae* contains genera that inhabit the intestinal tracts of humans and animals. These include both nonpathogenic species (the commensals) and pathogenic species (11). Because many species of this family coexist in the intestinal tract and are frequently exposed to various antibiotics, there is potential for the dissemination of antibiotic resistance genes. Therefore, the presence of integrons in bacterial species that constitute the family *Enterobacteriaceae* is of interest with respect to the growing dilemma of antibiotic resistance. We examined the distribution of class 1 to 4 integrases among veterinary bacterial strains isolated from livestock, companion animals, and exotics.

MATERIALS AND METHODS

Bacterial strains. The bacterial isolates used in the present study are members of various species of the following genera: *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia* (the species *Escherichia coli* only), *Hafnia*, *Klebsiella*, *Proteus*, *Salmonella*, and *Yersinia*. Several isolates were obtained from archived diagnostic specimens cultured in the 1980s and 1990s. *Edwardsiella tarda*, *Edwardsiella*

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TABLE 1. PCR primers

Primer	Sequence[a]	Expected size (bp)	PCR $MgCl_2$ concn (mM)	PCR annealing temp (°C)	Reference
intI1	F: CCTCCCGCACGATGATC R: TCCACGCATCGTCAGGC	280	2	55	13
intI2	F: TTATTGCTGGGATTAGGC R: ACGGCTACCCTCTGTTATC	233	3	50	This study
intI3	F: AGTGGGTGGCGAATGAGTG R: TGTTCTTGTATCGGCAGGTG	600	3	50	This study
intI4	F: CGGTATGTCTAATTGCTCTTG R: TGGCCACAAAGACTCAATCAC	696	3	50	This study

[a] F, forward; R, reverse.

ictaluri, and *Yersinia ruckerii* were isolated from diseased fish. Avian *E. coli* isolates were isolated from the tracheas, lungs, air sacs, livers, and spleens of diseased chickens, quail, ostriches, and turkeys. Pathogenic *E. coli* strains were isolated from swine and cattle with clinical cases of infection; the strains had been submitted to the North Dakota State University diagnostic laboratory. Avian *Salmonella* strains were isolated from either carcass rinses or drag swabs of poultry houses in Georgia. Reptile *Salmonella* strains were acquired from captive reptiles at zoos in the southeastern United States.

Colony blots. Probes for the genes of interest were created by PCR with digoxigenin-labeled nucleotides by the procedure of Bass et al. (2, 16, 33). The Molecular Genetics Instrumentation Facility at the University of Georgia synthesized the oligonucleotides (Table 1). The following bacterial strains served as positive controls and as templates for the generation of PCR probes for DNA-DNA hybridization: *E. coli* SK1593(pDU202), (*intI1*$^+$) (32), *E. coli* J53.3::Tn*7* (*intI2*$^+$), *S. marcescens* AK9373 (*intI3*$^+$) (1), and *V. cholerae* (*intI4*$^+$) (18). The identity of the PCR product was confirmed by sequencing. All PCR amplification products had 99% identity to the target gene sequences that the primers were originally designed to amplify. DNA-DNA hybridizations were performed as described by Sambrook et al. (26), with hybridizations and washes done at 68°C for all probes with the exception of the *intI2* probe, for which a temperature of (60°C) was used. Hybridization was detected with the antidigoxigenin antibody-alkaline phosphatase conjugate provided with the Genius System (Boehringer Mannheim).

Antimicrobial susceptibility determination. The antimicrobial MICs for the bacterial isolates tested were determined with the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, Ohio), and the results were interpreted according to National Committee Clinical Laboratory Standards guidelines for broth microdilution methods (19, 20). Sensititre susceptibility testing was performed according to the manufacturer's instructions. The following antimicrobials were assayed: ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline.

RESULTS AND DISCUSSION

We examined the susceptibilities of veterinary isolates to a battery of antibiotics including ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline. Veterinary *E. coli* isolates were generally resistant to five or more antibiotics, while other gram-negative enteric organisms varied in their patterns of susceptibility to these antibiotics (Table 2). Isolates belonging to the genus *Hafnia* were susceptible to all seven antibiotics tested. We had previously determined that multiple-drug resistance exhibited by avian *E. coli* isolates correlated with the incidence of class 1 integrons in this population (2). Could the differences in antibiotic susceptibilities observed among our veterinary isolates be due to the prevalence of integrons among the members of the family *Enterobacteriaceae*?

Five hundred eighty-eight organisms (representing nine bacterial genera) were screened for the presence of class 1 through 4 integrases. Two hundred ninety-four (52%) of the 555 *Enterobacteriaceae* isolates contained either class 1 or class 2 integrases (Table 3). Class 1 integrases were the dominant class identified, with 271 (92%) of these 294 integrase-positive isolates having *intI1* (Table 3). Seventy-three and 61% of the *E. coli* and *Salmonella* isolates, respectively, contained class 1 integrases, while only 10 and 13% of *Hafnia* and *Enterobacter* isolates (two closely related commensal genera), respectively,

TABLE 2. Antibiotic susceptibility patterns of veterinary and commensal gram-negative bacterial isolates

Organism and origin	No. of resistant strains/total no. of strains (%)[a]							
	AMP	CHL	TET	STR	GEN	KAN	SUL	Five or more antibiotics
Escherichia coli								
Avian	30/100	11/100	87/100	97/100	65/100	27/100	97/100	64/100 (64)
Bovine	42/49	44/49	49/49	49/49	31/49	46/49	49/49	42/49 (86)
Porcine	5/28	16/28	25/28	27/28	4/28	26/28	26/28	20/28 (71)
Salmonella sp.								
Avian (domestic)	3/15	1/15	9/15	7/15	2/15	1/15	6/15	0/15 (0)
Avian (nondomestic)	4/22	2/22	4/22	4/22	0/22	2/22	7/22	3/22 (14)
Proteus sp.	5/19	2/19	5/19	0/19	0/19	0/19	7/19	0/19 (0)
Klebsiella sp.	5/7	2/7	2/7	3/7	1/7	2/7	3/7	2/7 (28)
Citrobacter sp.	2/11	0/11	4/11	1/11	0/11	0/11	3/11	0/11 (0)

[a] Abbreviations: AMP, ampicillin; CHL, chloramphenicol; TET, tetracycline; STR, streptomycin; GEN, gentamicin; KAN, kanamycin; SUL, sulfamethoxazole.

TABLE 3. Presence of class 1 and 2 integrases in clinical veterinary isolates belonging to the *Enterobacteriaceae* family

Genus and species (host)	No. of strains positive/total no. of strains (%)		
	intI1	*intI2*	*intI1*, *intI2*
Citrobacter (catfish, turtles[a])	6/12 (50)	0/12 (0)	0/12 (0)
Hafnia (exotics[a])	1/10 (10)	0/12 (0)	0/10 (0)
Klebsiella (horses, cattle[a])	4/7 (57)	0/7 (0)	0/7 (0)
Proteus (exotics[a])	4/23 (17)	0/12 (0)	0/12 (0)
Enterobacter (multiple species[a,b])	1/8 (12)	0/8 (0)	0/8 (0)
Escherichia coli			
Multiple species[a,b]	29/38 (76)	0/38 (0)	0/38 (0)
Poultry	66/100 (66)	14/100 (14)	11/100 (11)
Beef cattle	42/56 (75)	13/56 (23)	11/56 (20)
Swine	25/29 (86)	0/29 (0)	0/29 (0)
Total	162/223 (73)	27/223 (12)	22/223 (10)
Salmonella			
Wild or pet birds[c]	9/22 (41)		
Poultry[d]	79/100 (79)	2/100 (2)	2/100 (2)
Reptiles[d]	5/29 (17)	5/29 (17)	1/29 (3)
Total	93/151 (62)	7/129 (5)	3/129 (2)
Edwardsiella spp. (fish[a,e])			
Edwardsiella ictaluri	0/11 (0)	0/11 (0)	0/11 (0)
Edwardsiella tarda	0/48 (0)	0/48 (0)	0/48 (0)
Total	0/59 (0)	0/59 (0)	0/59 (0)
Yersinia ruckerii (fish[a,f])	0/95 (0)	0/95 (0)	0/95 (0)
Total for *Enterobacteriaceae*	271/588 (46)	34/555 (6)	25/555 (5)

[a] Isolates archived from 1980s.
[b] Isolates from companion animals and exotics.
[c] Isolates from birds with clinical cases of salmonellosis (8).
[d] *Salmonella* strains were isolated from the feces of either chickens or reptiles or they were acquired from drag swabs of poultry houses in north Georgia.
[e] Fish include Catfish, eel, trout, and salmon.
[f] Fish include trout and salmon.

were positive for *intI1*. Integrase genes were not identified in any of the aquatic pathogens (Table 3).

Forty (14%) of the 294 integrase-positive isolates of the family *Enterobacteriaceae* had the class 2 integrase. The class 2 integrases were restricted in their distributions, being identified only in *E. coli* and *Salmonella* isolates. Among the *Salmonella* isolates, the incidence of *intI1* ranged from 17% (reptile) to 79% (poultry), depending on the source of the isolate. Class 2 integrases were also variable in their distributions among the *Salmonella* isolates, with 2% of poultry isolates and 17% of reptile isolates being positive for *intI2*. As for the *Salmonella* isolates, the distribution of the class 2 integrases was variable in *E. coli*, depending on the animal source of the isolate. *E. coli* strains isolated from swine and companion animals were negative for *intI2*, but this gene was present in 14 and 23% of the *E. coli* strains from poultry and beef cattle, respectively. None of the veterinary isolates tested contained the class 4 integrase gene (*intI4*). The PCR amplification products originally used as DNA probes for Southern were confirmed through DNA sequencing of these PCR amplicons. The DNA sequences of the *intI2* PCR amplicons from three veterinary isolates, an avian *E. coli* isolate, a bovine *E. coli* isolate, and an avian *Salmonella* isolate, (Genbank accession nos. AF318070, AF318071, and AF318072, respectively), also had 99% identities to the sequence of the *intI2* integrase present in Tn*7*.

Thirty-four (12%) of the 294-integrase positive *Enterobacteriaceae* isolates were found to be positive for *intI3* by DNA-DNA hybridization. When several isolates, identified by DNA-DNA hybridization as having *intI3*, were screened by PCR with *intI3*-specific primers, only the control produced the expected 600-bp PCR amplicon. It is possible that a single mismatch involving the terminal 3′ nucleotide of either the forward or the reverse primer and its target might prohibit PCR detection of the *intI3* genes in these isolates (5). This is especially relevant with the recent report of a hybrid class 2 integrase in *Acinetobacter baumannii* (24). We may also be dealing with a variant hybrid class 3 integrase. We are in the process of cloning and sequencing DNA fragments that hybridize with the *intI3*-specific probe to resolve the discrepancy in our results between the DNA-DNA hybridizations and PCR.

Several isolates possessed multiple integrase classes. Class 1 and 2 integrases were present together in *E. coli* (10%) and *Salmonella* (2%) isolates. Interestingly, class 1 and 2 integrases were most prevalent among veterinary *E. coli* isolates that displayed resistance to five or more antibiotics.

Class 1 integrons and integrases have been identified in a number of different bacterial genera and appear to be prevalent in nature (2, 3, 22, 28, 31, 34). We found that the same bacterial genera or species associated with the normal flora in animals contained the same class 1 integrase gene as their human counterpart bacteria (4, 9, 23, 25). Our isolates of *Edwardsiella* and *Yersinia* were negative for the class 1 integrase gene. This is in contrast to a study by Petersen et al. (22), who reported class 1 integrases among bacteria isolated from trout. The lack of integrases in these two fish pathogens may be due to differences in bacterial species, host species, geography, or fish management practices with regard to antibiotic usage. The dissemination of the class 1 integrons, in many instances, has been attributed to the spread of an integron-containing transposon, Tn*21* (2, 34). We have recently found that class 1 integrons in avian *E. coli* strains contain a single antibiotic resistance gene, *aadA1* (streptomycin-spectinomycin resistance), and that this integron is part of a Tn*21*-like element (2). However, there are clearly cases in which class 1 integrons contain other resistance genes (9, 14, 23, 27) and map to plasmids (25, 31) or transposons other than Tn*21* (14). The nature of the class 1 integrons in many of these veterinary isolates is not known, but we do expect to find differences due to the dissimilarities in both the animal microflora and the therapeutic as well as prophylactic usage of antibiotics among these different animal species. It is interesting that several bacterial isolates from nondomestic, free-ranging animals contained integrases. We have observed similar results for *Salmonella* strains isolated from wild, nondomestic birds in the southeastern United States (12). Does the presence of these integrases and integrons represent the natural frequency of these mobile elements in nature, or does it signify human contamination of the environment?

The low incidence of *intI2* in select veterinary isolates (6%) was similar to the distribution of the class 2 integron (Tn*7*) in

human, urinary isolates of *E. coli*, *Enterobacter* spp., and *Klebsiella* spp. examined (3). The distribution of Tn*7* in clinical isolates correlates with the frequency of trimethoprim resistance (3, 13). Trimethoprim resistance is due to the dihydrofolate reductase enzyme encoded by the *dhfr* gene in Tn*7* (6). Differences in the frequency of *intI2* among our veterinary isolates might be attributed to differences in the prevalence of Tn*7* and usage of the antibiotic trimethoprim or the related veterinary analogue ormetoprim.

To the best of our knowledge this is the first study to look at the distributions of two new classes of integrases, class 3 and 4 integrases, in a large-scale study. None of the veterinary isolates assayed contained the class 4 integrase originally identified in *V. cholerae* (18). This integron therefore appears to be more limited in its distribution. The identification of three classes of integrases in multiple bacterial genera comprising both pathogenic and commensal organisms indicates that integrons have the capability to disseminate antimicrobial resistance genes within bacteria associated with diverse animal production environments. We are currently characterizing the integrons associated with both commensal and pathogenic bacteria such as *Salmonella* to determine whether the animal's microbiota can serve as a reservoir for antimicrobial resistance genes.

ACKNOWLEDGMENTS

The National Science Foundation's Prokaryotic Diversity Research Training Grant at the University of Georgia made this work possible. The Wallace Research Foundation and the International Academy of Oral Medicine and Toxicology support work in the laboratory of A.O.S. USDA NRICGP grant 99-35212-8680 supports work in the laboratory of J.J.M.

We thank Komanci Payne, Kathleen Keyes, and Amy Stallings for participation in the preparation of the PCR probes used in this study. We also thank Marie Maier for technical assistance.

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