

Antibiotics-2015
(Las Vegas, USA)
September 14, 2015

Characterization of integrons and antimicrobial resistance genes in Gram-negative bacteria isolated from seafood in Japan: identification of a novel β -lactamase-encoding gene, *bla*_{CMY-39}

Ashraf M. Ahmed, Akito Maruyama,

Toshi Shimamoto, ^o Tadashi Shimamoto

(Graduate School of Biosphere Science, Hiroshima University)

Development of Resistant Bacteria

Antimicrobial-Resistant Bacteria

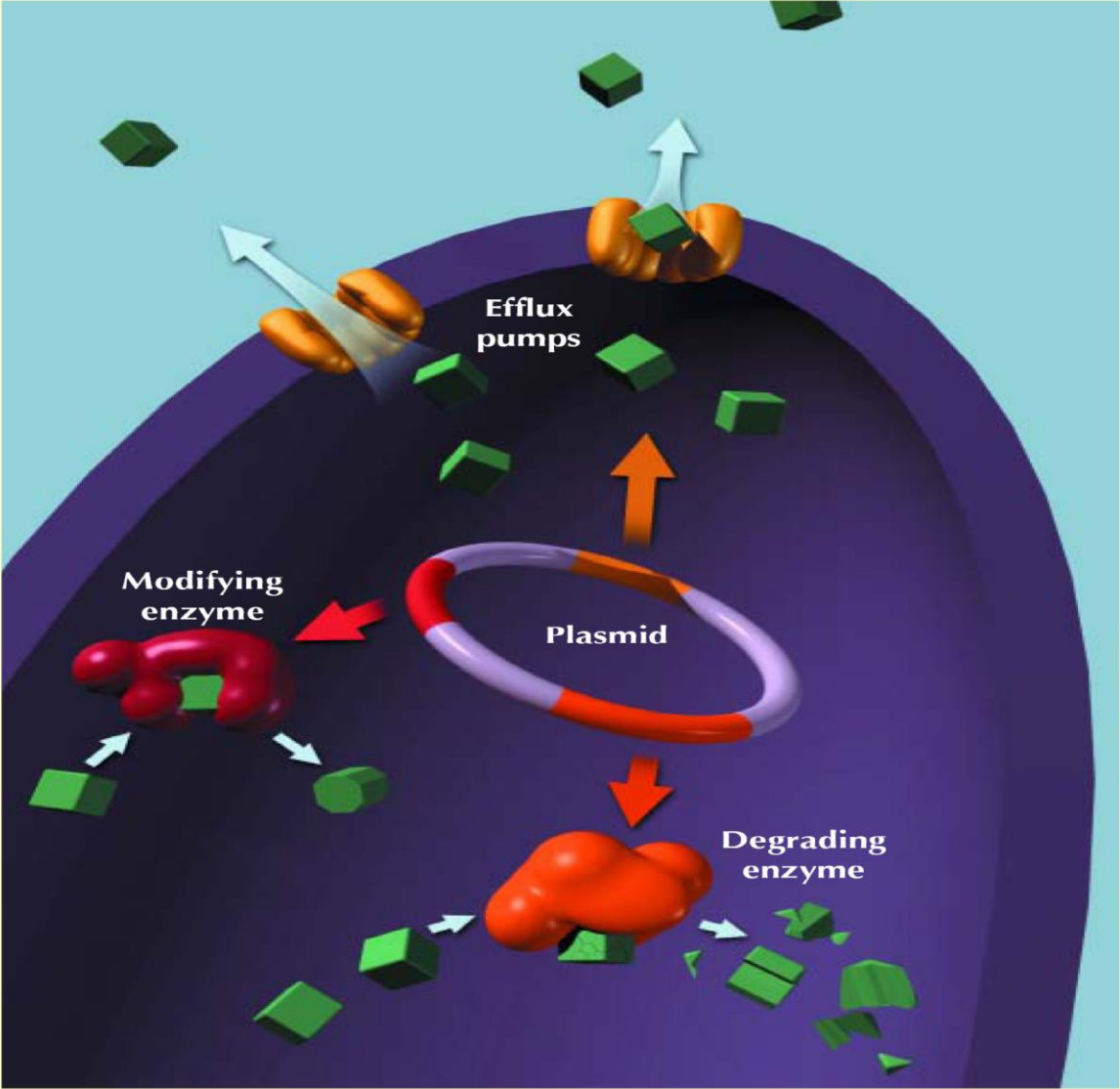
Antimicrobial
Resistance

Infection

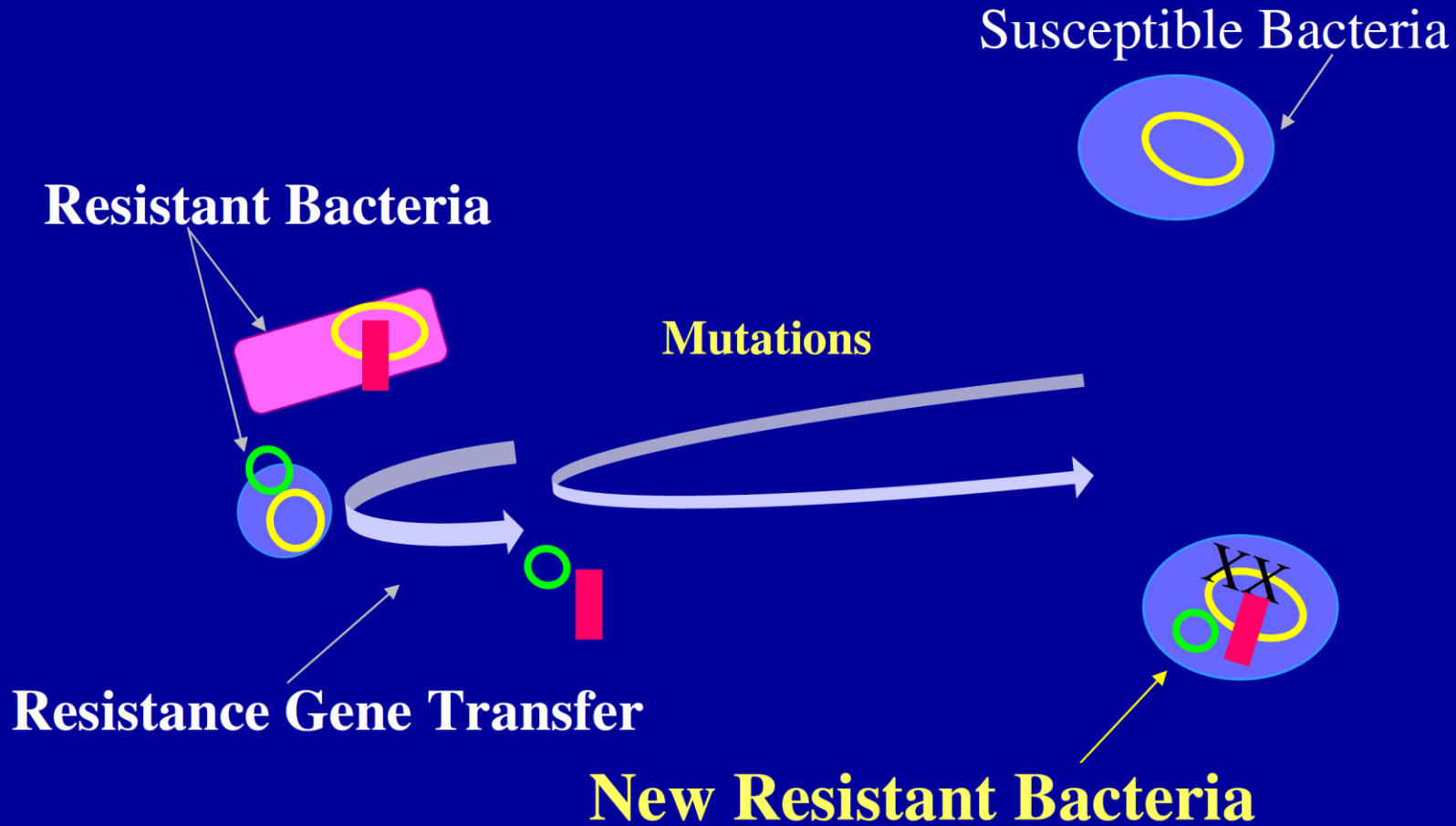
Antimicrobial Use



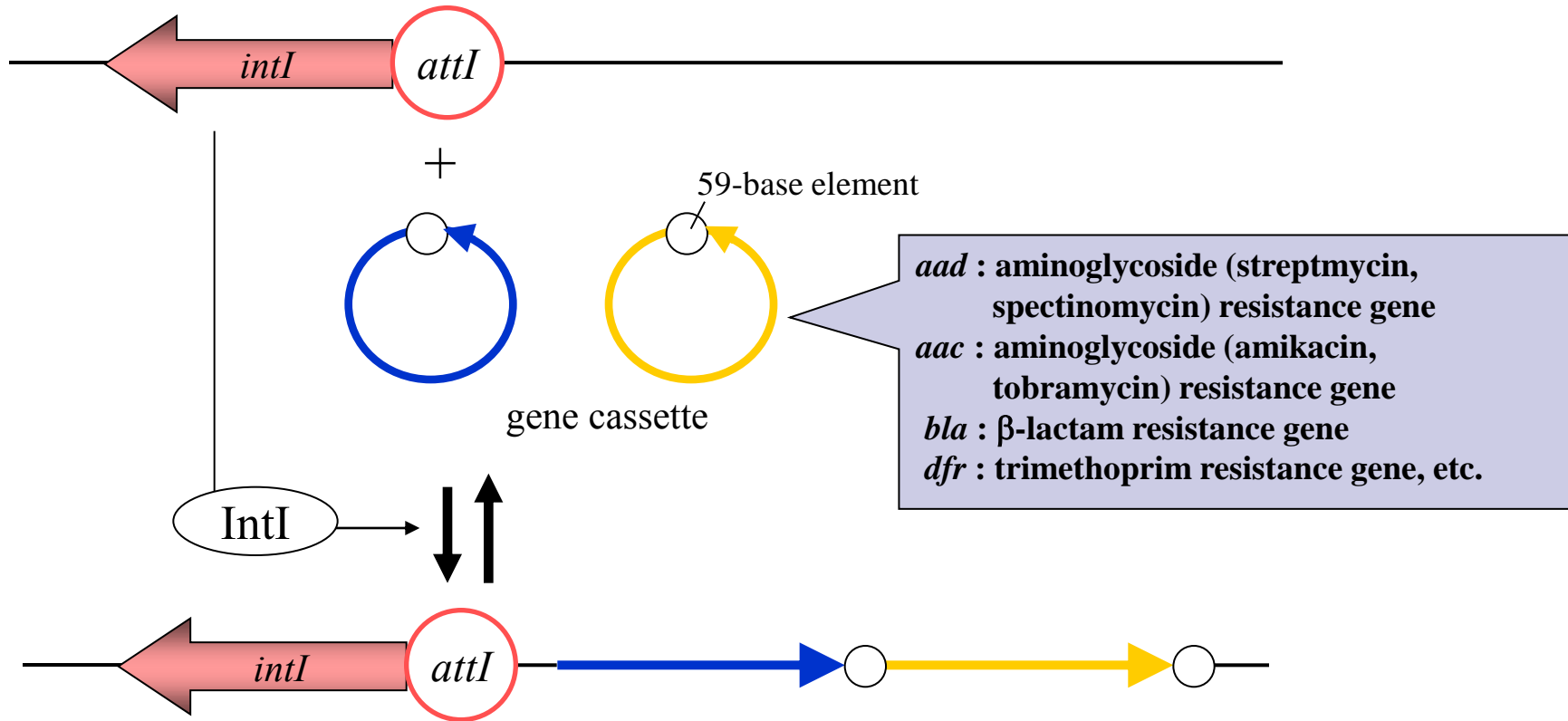
Role of Plasmids in Antimicrobial Resistance



Emergence of Antimicrobial Resistance



Integrations



- Class 1 integron ▪ ▪ ▪ 5'-CS(*intI1*, *attI1*),
resistance gene cassettes,
3'-CS(most cases: *qacEΔ1*, *sul 1*, *orf5*, *orf6*)
- Class 2 integron ▪ ▪ ▪ 5'-CS(*intI2*, *attI2*),
resistance gene cassettes (most cases: *dfrA1-sat1-aadA1*)
3'-CS

ESBLs (Extended-spectrum β -lactamases)

- β -lactamases hydrolyze the β -lactam ring and thus inactivate β -lactam antibiotics.
- The production of β -lactamases is the major mechanism of resistance to β -lactams, which are most frequently used for the treatment of various infectious diseases.
- ESBLs are enzymes that mediate resistance to extended-spectrum (third and fourth generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem).

Relationship between β -lactamase and β -lactam resistance

Enzyme	Penicillin	Cephalosporin				Cefamycine	Monobactam	Carbapenem
		1st	2nd	3rd	4th			
Class A								
TEM-1, TEM-2, SHV-1	R	R						
TEM-ESBLs, SHV-ESBLs	R	R	R	R	R		R	
CTX-M-ESBLs	R	R	R	R	R		R	
Class B MBL								
	R	R	R	R	R	R		R
Class C CMY								
	R	R	R	R		R	R	
Class D								
OXA-1	R	R						
OXA-ESBLs	R	R	R	R	R		R	

R: Resistant

Quinolone resistance genes

Traditional mechanism of quinolone resistance are...

- ① Mutation of DNA gyrase and topoisomerase IV
- ② Decrease of intracellular drug accumulation (efflux pumps, outer membrane porin change)

New quinolone resistance mechanisms are plasmid-mediated quinolone resistance.

③ *qnr* genes

qnr genes are plasmid-mediated quinolone resistance.

These genes encode pentapeptide repeat proteins that block the action of quinolones on bacterial DNA gyrase and topoisomerase IV.

In one report, low-levels of quinolone resistance result in an increase in the MIC of ciprofloxacin for some bacteria.

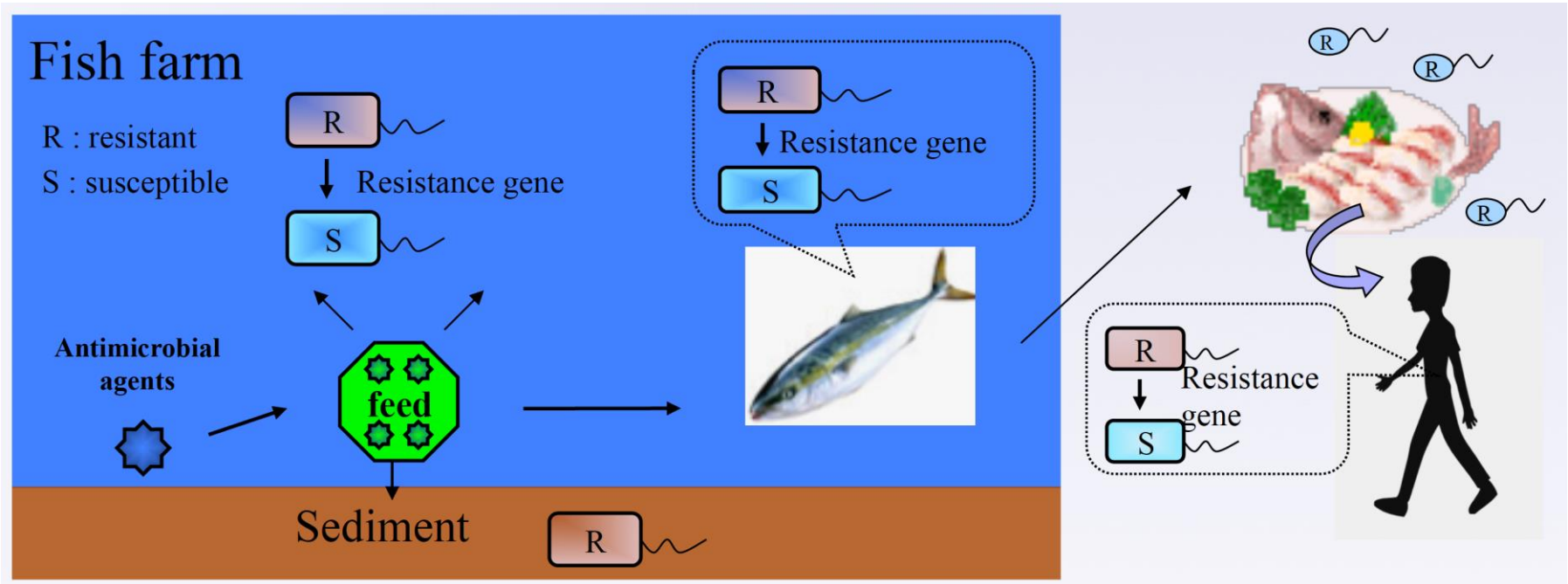
④ *aac(6')-Ib-cr* gene

- *aac(6')-Ib-cr* gene is *aac(6')-Ib* variant and aminoglycoside acetyltransferase resistance gene.
- *aac(6')-Ib-cr* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine.
- Its protein changed Trp102Arg and Asp179Tyr.
- When both *qnrA* and *aac(6')-Ib-cr* are present in the same cell, MIC is increased to four-fold than only *qnrA* gene.

⑤ *qep* gene (efflux pump)

Introduction

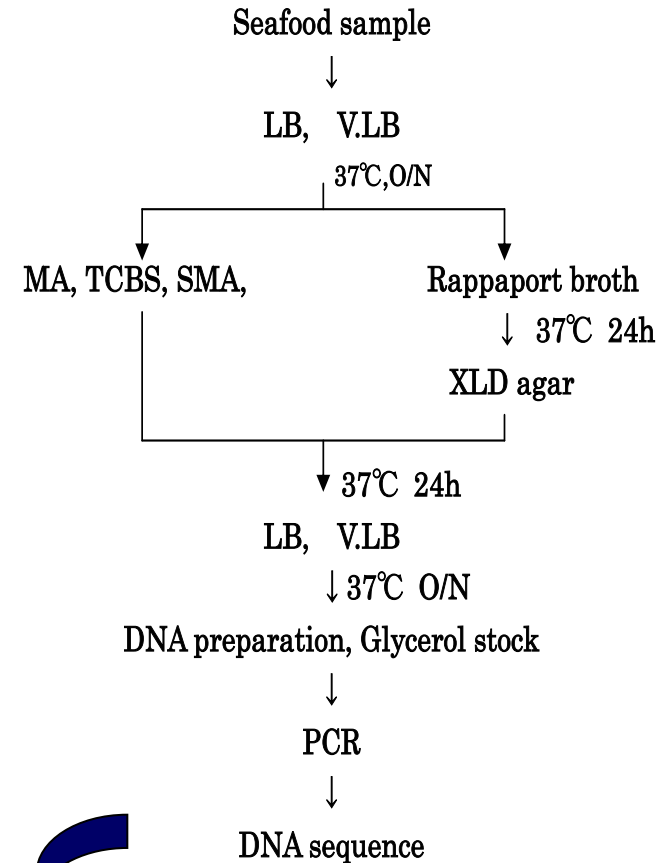
- In Japan, a variety of seafood has been traditionally consumed, and raw or lightly cooked seafood is favored. This eating habit seems to provide an explanation for many cases of foodborne diseases by *Vibrio*, *Aeromonas* and *Plesiomonas*, etc.
- Since the products of aquaculture are consumed by humans and since many antibiotic resistance determinants are encoded by transferable plasmids, cultured fish may serve as a vehicle for transmission of antibiotic resistance to bacteria that are commensal or pathogenic to humans.
- Until now, there have been few reports that focus on integrons and ESBLs, quinolone resistance in bacteria isolated from seafood.



Sampling and isolation of bacteria

No.	Sample	Country/Prefecture
1	Fish (<i>Pagrus major</i>)	Japan/Hiroshima
2	Clam	Japan/Hiroshima
3	Shrimp	Iceland
4	Seafood mix No. 1	Indonesia
5	Shrimp	Indonesia
6	Shijimi	Japan/Shimane
7	<i>Pagrus major</i>	Japan/Hiroshima
8	Shrimp	Greenland
9	Alfonsin	Japan/Kochi
10	<i>Pagrus major</i>	Japan/Ehime
11	Salmon	Chile
12	Flatfish	Alaska
13	Seafood mix No. 2	India, North Pacific, China
14	Seafood mix No. 3	Vietnam

【Protocol】



Total 215 isolates

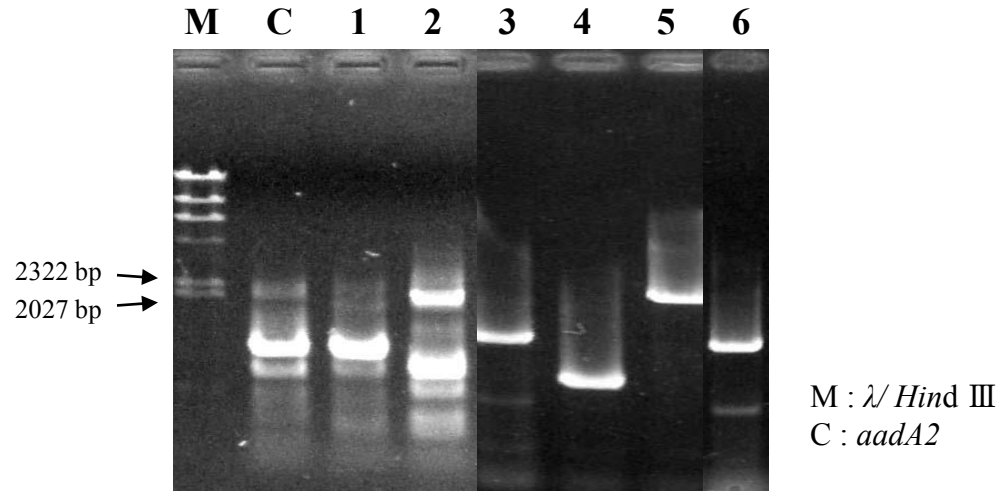
Isolated bacterial species

No.	Species	Number	%
1	<i>Citrobacter freundii</i>	53	23.3
2	<i>Enterobacter cloacae</i>	44	19.3
3	<i>Citrobacter koseri</i>	18	7.9
4	<i>Aeromonas hydrophila</i>	17	7.5
5	<i>Vibrio fluvialis</i>	10	4.4
6	<i>Klebsiella oxytoca</i>	9	4.0
7	<i>Yersinia enterocolitica</i>	9	4.0
8	<i>Vibrio alginolyticus</i>	8	3.5
9	<i>Pseudomonas aeruginosa</i>	8	3.5
10	<i>Escherichia coli</i>	7	3.1
11	<i>Proteus mirabilis</i>	7	3.1
12	<i>Pseudomonas spp</i>	5	2.2
13	<i>Vibrio parahaemolyticus</i>	4	1.8
14	<i>Vibrio mimicus</i>	3	1.3
15	<i>Vibrio cholerae</i>	2	0.9
16	<i>Klebsiella pneumoniae</i>	2	0.9
17	<i>Enterobacter aerogenes</i>	2	0.9
18	<i>Proteus vulgaris</i>	2	0.9
19	<i>Providencia rettgeri</i>	2	0.9
20	<i>Pantoea spp</i>	1	0.4
21	<i>Morganella morganii</i>	1	0.4
Total		215	100

Primers used in this study

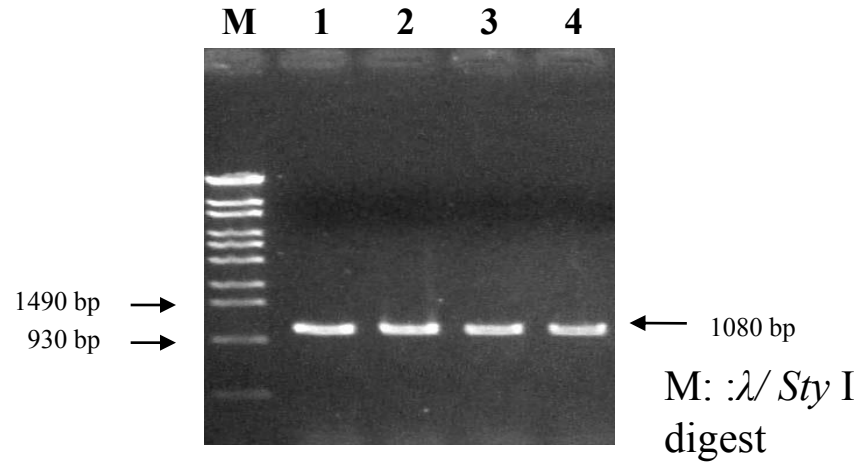
Primer	Sequence (5'–3')	Target	Reference
Integrans			
5'-CS	GGCATCCAAGCAGCAAG	Class 1 integron	Ahmed et al., 2007
3'-CS	AAGCAGACTTGACCTGA		
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron	Ahmed et al., 2007
hep51	GATGCCATCGCAAGTACGAG		
β-Lactamases			
TEM-F	ATAAAATTCTTGAAGACGAAA	<i>bla</i> _{TEM}	Ahmed et al., 2007
TEM-R	GACAGTTACCAATGCTTAATC		
SHV-F	TTATCTCCCTGTTAGCCACC	<i>bla</i> _{SHV}	Ahmed et al., 2007
SHV-R	GATTTGCTGATTTTCGCTCGG		
OXA-F	TCAACTTTCAAGATCGCA	<i>bla</i> _{OXA}	Ahmed et al., 2013
OXA-R	GTGTGTTTAGAATGGTGA		
CTX-M-F	CGCTTTGCGATGTGCAG	<i>bla</i> _{CTX-M}	Ahmed et al., 2007
CTX-M-R	ACCGCGATATCGTTGGT		
CMY-F	GACAGCCTCTTTCTCCACA	<i>bla</i> _{CMY}	Ahmed et al., 2007
CMY-R	TGGAACGAAGGCTACGTA		
CMY-F-2	ACGGAAGTATTTCATGATG	<i>bla</i> _{CMY} (whole gene)	Ahmed et al., 2007
CMY-R-2	GAAAGGAGGCCCAATATCCT		
Quinolone resistance genes			
qnrA-F	ATTTCTCACGCCAGGATTTG	<i>qnrA</i>	Ahmed et al., 2007
qnrA-R	GATCGGCAAAGGTTAGGTCA		
qnrB-F	GATCGTGAAAGCCAGAAAGG	<i>qnrB</i>	Ahmed et al., 2007
qnrB-R	ACGATGCCTGGTAGTTGTCC		
qnrS-F	ACGACATTCGTCAACTGCAA	<i>qnrS</i>	Ahmed et al., 2007
qnrS-R	TAAATTGGCACCCTGTAGGC		
aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	<i>aac(6')-Ib-cr</i>	Ahmed et al., 2007
aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		

Class 1 integron



Type	Lane	No.	Source	Species	Gene
I	1	2	Pagrus major	<i>Enterobacter cloacae</i>	<i>aadA2</i>
II	2	4	Pagrus major	<i>Aeromonas hydrophila</i>	<i>dfr12 - orf - aadA2</i> <i>dfr17</i>
III	3	89	Alfonsin	<i>Aeromonas hydrophila</i>	<i>bla</i> _{PSE-1}
	6	160	Shijimi (Water)	<i>Aeromonas hydrophila</i>	
IV	4	92	Pagrus major	<i>Klebsiella oxytoca</i>	<i>dfr17</i>
V	5	94	Pagrus major	<i>Citrobacter freundii</i>	<i>bla</i> _{PSE-1} - <i>aadA2</i>

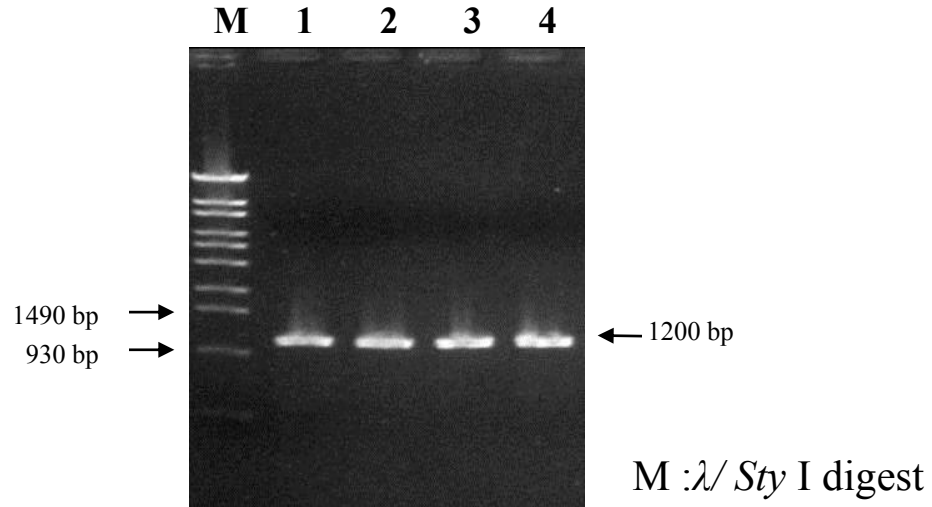
TEM (*bla*_{TEM})



Lane	No.	Source	Species	Type
1	2	SM1	<i>Enterobacter cloacae</i>	TEM-1
2	16	SM1	<i>Enterobacter cloacae</i>	TEM-1
3	21	SM1	<i>Enterobacter cloacae</i>	TEM-1
4	28	Pagrus major	<i>Citrobacter freundii</i>	TEM-1

SM : Seafood mix

CMY (*bla*_{CMY})



Lane	No.	Source	Species	Type
1	28	Pagrus major	<i>Citrobacter freundii</i>	CMY-13
2	94	Pagrus major	<i>Citrobacter freundii</i>	CMY-13
3	151	Salmon	<i>Citrobacter freundii</i>	CMY-2
4	176	SM3	<i>Citrobacter freundii</i>	CMY-39

CMY-39 (Wild strain, Transformant)

Strain	Gene													
		AMP	AMC	FOX	CEF	CTT	3rd			4th				
		2nd	1st	2nd			CAZ	CPD	CRO	ATM	CPM	CPR		
<i>C. freundii</i> 10a-1	<i>bla</i> _{CMY-39} , <i>qnrB2</i>	R	R	R	R	R	I	R	I	S	S	R		
<i>E. coli</i> TG1 (pBCMY-39)	<i>bla</i> _{CMY-39} , <i>amp</i> ^R	R	R	R	R	R	I	R	I	S	S	R		
<i>E. coli</i> TG1 (pBluescript)	<i>amp</i> ^R	R	S	S	R	S	S	S	S	S	S	S		

R, Resistant; I, Intermediate; S, Susceptible

AMP, ampicillin; AMC, amoxicillin–clavulanic acid; FOX, cefoxitin; CEF, cefalotin; CTT, cefotetan; CAZ, ceftazidime; CPD, cefpodoxime; CRO, ceftriaxone; ATM, aztreonam; CPM, cefepime; CPR, cefpirome

Amino acid sequence similarity of CMY-39

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CMY-39 1:MMKSLCCALLLTAPLSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMVAVAVIYQGKPYFPTWGKADI TNNHPVTQQTLELGSVSKTFNGVLGGDAIAR 100
Ci 29 1:-----FAAAKTEQQIADTVNRTITPLMQEQAIPGMVAVAVIYQGKPYFPTWGKADI TNNHPVTQQTLELGSVSKTFNGVLGGDAIAR 82
CMY-2 1:MMKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMVAVAVIYQGKPYFPTWGKADIANNHPVTQQTLELGSVSKTFNGVLGGDAIAR 100
GC3 1:MMKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMVAVAIYQGKPYFPTWGKADIANNRPVTQQTLELGSVSKTFNGVLGGDAIAR 100
..... *****. *****. *****. *****. *****. *****. *****. *****. *****. *****

CMY-39 101:GEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKALLRFYQNWQPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ 200
Ci 29 83:GEIKLSDPVTKYWPELTGKQWQGISLLHLATYTAGGLPLQIPDDVTDKALLRFYQNWQPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ 182
CMY-2 101:GEIKLSDPVTKYWPELTGKQWQGI RLLHLATYTAGGLPLQIPDDVRDKALLHFYQNWQPQWTPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ 200
GC3 101:GEIKLSDPVTQYWPELTGKQWQGISLLHLATYTAGGLPLQVDDVTDKALLRFYQNWQPQWAPGAKRLYANSSIGLFGALAVKPSGMSYEEAMTRRVLQ 200
*****. *****. *****. *****. *****. *****. *****. *****. *****. *****

CMY-39 201:PLKLAHTWITVPQSEKQDYAWGYREGKAVHVSPPGQLDAEAYGVKSSVIDMARWVQVNMDSARVQEKTLQGGIALAQSRYWRI GDMYQGLGWEMLNWPLKA 300
Ci 29 183:PLKLAHTWITVPQNEQKDYAWGYREGKAVHVSPPGQLDAEAYGVKSSVIDMARWVQVNMDSARVQEKTLQGGIALAQSRYWRI GDMYQGLGWEMLNWPLKA 282
CMY-2 201:PLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPPGQLDAEAYGVKSSVIDMARWVQANMDASHVQEKTLQGGIALAQSRYWRI GDMYQGLGWEMLNWPLKA 300
GC3 201:PLKLAHTWITVPQSEKQDYAWGYREGKPVHVSPPGQLDAEAYGVKSSVDMTRWVQANMDASQVQEKTLQGGIKLAQSRYWRI GDMYQGLGWEMLNWPLKA 300
*****. *** *****. *****. *****. *****. *****. *****. *****. *****. *****

CMY-39 301:DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKGTGGFGSYVAFVPEKNLGI VMLANKSYNPNRVEAAWRILEKLQ 360
Ci 29 283:DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKGTGGFGSYVAFVPEKKLGI VMLANKSY----- 342
CMY-2 301:DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKGTGGFGSYVAFVPEKNLGI VMLANKSYNPNRVEAAWRILEKLQ 360
GC3 301:DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKGTGGFGSYVAFVPEKNLGI VMLANKSYNPNRVEAAWRILEKLQ 360
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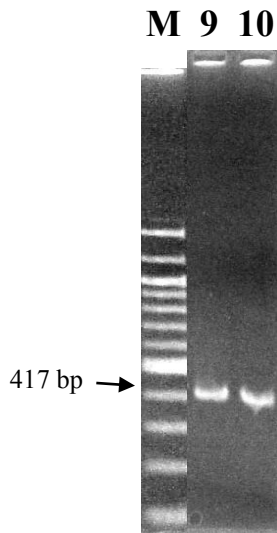
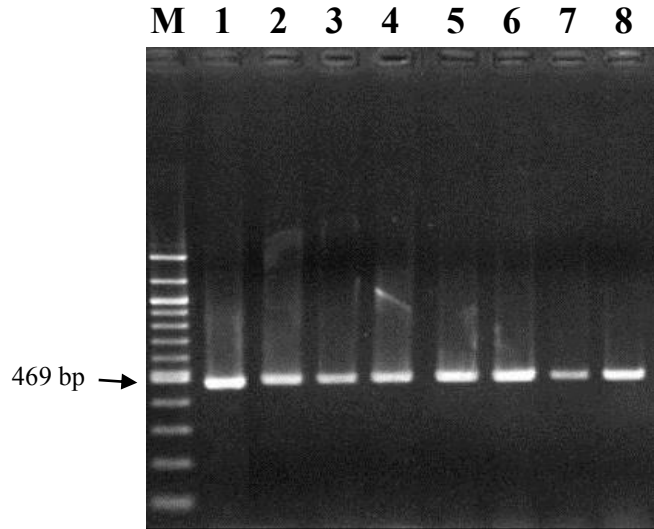
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CMY-39 : *Citrobacter freundii* 10a-1 AB372224, Ci 29 : *Citrobacter freundii* AmpC β -lactamase ABF56531,

CMY-2 : *Escherichia coli* β -lactamase AAZ99133, GC3 : *Citrobacter freundii* AmpC β -lactamase

Boxed motifs sequences of CMY-type β -lactamase
Blue arrow: omega loop region
Blue box: CMY-39 specific mtation

qnr



M : 100 bp ladder

Lane	No.	Source	Species	Type
1	28	Pagrus major	<i>Citrobacter freundii</i>	<i>qnrB2</i>
2	29	Pagrus major	<i>Citrobacter koseri</i>	<i>qnrB2</i>
3	94	Pagrus major	<i>Citrobacter freundii</i>	<i>qnrB2</i>
4	103	Salmon	<i>Enterobacter cloacae</i>	<i>qnrB6</i>
5	151	Salmon	<i>Citrobacter freundii</i>	<i>qnrB2</i>
6	176	SM3	<i>Citrobacter freundii</i>	<i>qnrB2</i>
7	205	SM3	<i>Citrobacter freundii</i>	<i>qnrB2</i>
8	211	Alfonson	<i>Citrobacter freundii</i>	<i>qnrB</i>
9	79	Shrimp	<i>Pantoea spp.</i>	<i>qnrS1</i>
10	153	Shrimp	<i>Enterobacter cloacae</i>	<i>qnrS1</i>

SM, seafood mix

Conclusion

No.	Species	Source	Country	Resistance phenotype	Resistance genes (integrons/ESBLs/ <i>qnr</i>)
2	<i>Enterobacter cloacae</i>	Pagrus major	Japan	AMP•CHL•SXT•STR	1) Class 1 integron (<i>aadA2</i>) 2) <i>bla</i> _{TEM-1}
4	<i>Aeromonas hydrophila</i>	Pagrus major	Japan	NAL•CHL•SXT•TET•KAN•STR•AMP•NOR	1) Class 1 integron (<i>dfr12 - orf - aadA2</i>) 2) Class 1 integron (<i>dfr17</i>)
16	<i>Enterobacter cloacae</i>	SM 1	Indonesia	SXT•STR•AMP•FOX	1) <i>bla</i> _{TEM-1}
21	<i>Enterobacter cloacae</i>	SM 1	Indonesia	CHL•SXT•TET•STR•AMP•FOX	1) <i>bla</i> _{TEM-1}
28	<i>Citrobacter freundii</i>	Pagrus major	Japan	CHL•TET•STR•AMP•FOX	1) <i>bla</i> _{TEM-1} 2) <i>bla</i> _{CMY-13} 3) <i>qnrB2</i>
29	<i>Citrobacter koseri</i>	Pagrus major	Japan	AMP	1) <i>qnrB2</i>
79	<i>Pantoea spp.</i>	Shrimp	Indonesia	AMP	1) <i>qnrS1</i>
89	<i>Aeromonas hydrophila</i>	Alfonsin	Japan	NAL•TET•STR•AMP•FOX	1) Class 1 integron (<i>bla</i> _{PSE-1})
92	<i>Klebsiella oxytoca</i>	Pagrus major	Japan	NAL•SXT•TET•STR•AMP	1) Class 1 integron (<i>dfr17</i>)
94	<i>Citrobacter freundii</i>	Pagrus major	Japan	CHL•TET•STR•AMP•FOX•LEX	1) Class 1 integron (<i>bla</i> _{PSE-1} - <i>aadA2</i>) 2) <i>bla</i> _{CMY-13} 3) <i>qnrB2</i>
103	<i>Enterobacter cloacae</i>	Salmon	Chile	SXT•TET•STR•FOX	1) <i>qnrB6</i>
151	<i>Citrobacter freundii</i>	Salmon	Chile	STR•AMP•FOX•CEF	1) <i>bla</i> _{CMY-2} 2) <i>qnrB2</i>
153	<i>Enterobacter cloacae</i>	Shrimp	Indonesia	AMP	1) <i>qnrS1</i>
160	<i>Aeromonas hydrophila</i>	Freshwater clam	Japan	STR•AMP	1) Class 1 integron (<i>bla</i> _{PSE-1})
176	<i>Citrobacter freundii</i>	SM3	India, China, North Pacific	STR•AMP•FOX•CEF•CTT•CPD•CPR	1) <i>bla</i> _{CMY-39} 2) <i>qnrB2</i>
205	<i>Citrobacter freundii</i>	SM3	India, China, North Pacific	STR	1) <i>qnrB2</i>
211	<i>Citrobacter freundii</i>	Alfonsin	Japan	AMP	1) <i>qnrB</i> variant

AMC, amoxicillin–clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CEF, cefalotin; CFP, cefoperazone; CHL, chloramphenicol; CPD, cefpodoxime; CRO, ceftriaxone; CTT, cefotetan; FOX, cefoxitin; LEX, cefalexin; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim, TET, tetracycline

SM, seafood mix

Conclusion

- 215 bacterial strains were isolated from seafood in Japan.
- Class 1 integrons were detected in six bacterial isolates and contained gene cassettes encoding resistance to trimethoprim (*dfrA12* and *dfrA17*), aminoglycosides (*aadA2*), and β -lactams (*bla*_{PSE-1}).
- All isolates were negative for class 2 integrons.
- *bla*_{TEM} and *bla*_{CMY} were detected, but SHV, OXA and CTX-M types were not detected.
- A novel type of CMY-type β -lactamase gene, *bla*_{CMY-39} was detected in *Citrobacter freundii*. The CMY-39 β -lactamase has substrate specificity to the fourth generation cephalosporin, cefpirome, in addition to the general CMY-type one.
- Plasmid-mediated quinolone resistance genes, including *qnrB2*, *qnrB6* and *qnrS1*, were also identified in 10 isolates.
- This study highlights the presence of integrons and antimicrobial resistance genes in seafood-associated bacteria in Japan, and indicates that seafood could be a reservoir and route of transmission of antibiotic-resistant bacteria to humans.