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Abstract

Since the development of the first antibiotics in the 1940's, there has been widespread overuse in both clinical and agricultural applications. Antibiotic resistance has become a significant problem as a result of subsequent dissemination of antibiotics into the environment, and multiply-resistant strains of bacteria are now a major pathogenic threat. In this study eight separate strains of *Flavobacterium* responsible for recent disease outbreaks in fish hatcheries throughout Maine were collected and analyzed. All eight strains were found to be resistant to high levels of a number of different antibiotics, including those used for aquaculture as well as human chemotherapeutic applications. *Flavobacterium* isolates were also shown phenotypically to transfer antibiotic resistance determinants using a conjugation mating system in which *Flavobacterium* was the donor and *Escherichia coli* DH5-alpha was the recipient. This experiment suggests that it may be possible for *Flavobacterium* strains to transfer their multiple antibiotic resistance determinants to human pathogenic bacterial strains. Importantly, none of the hatcheries from which the *Flavobacterium* isolates were obtained had ever used antibiotics to treat their fish stock. It is possible that there is another selective agent responsible for the development of antibiotic resistance in the absence of antibiotic pressure. Mercury is one possible candidate, as all of the strains tested were resistant to mercuric chloride and it is known that genes encoding antibiotic resistance can be carried on the same mobile genetic elements that encode for mercury resistance. Preliminary data also suggest that the majority of the *Flavobacterium* isolates contain genes for mercuric ion reduction, which would confirm the mercury resistance genotype.

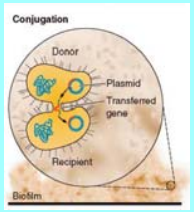
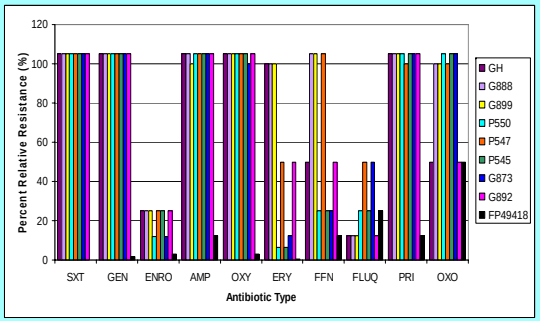


Image obtained from: Barkay, Tamar, Smets, Barth F. 2005. Horizontal gene flow in microbial communities. ASM News. 71:412-419.

The above diagram is a simple representation of a conjugation event. Conjugation is a common method by which bacteria acquire new genes for resistance. The conjugation mating system used in this study includes *Flavobacterium* donors and *E. coli* DH5-alpha as the recipient.

Figure 1



The Relative Aquaculture Specific Panel Showing Antibiotic Resistance in *Flavobacterium* Isolates. The relative resistance of all 8 *Flavobacterium* isolates (GH, G888, G899, P550, P547, P545, G873, G992) to the maximum aquaculture antibiotic concentration tested is shown. Resistance beyond the maximum concentration tested is represented by bars over 100. Also included is an ATCC reference strain of *Flavobacterium psychrophilum* (FP49418), which was isolated in the pre-antibiotic era.

Sensititre Aquaculture MIC plates were used and manufacturer's protocol was followed. Results were recorded 4 days after incubation at 20 ºC. Abbreviations: SXT= Trimethoprim/suflamethoxazole, GEN= Gentamicin, ENRO= Enrofloxacin, AMP= Ampicillin, OXY= Oxytetracycline, ERY= Erythromycin, FFN= Florfenicol, FLUQ= Flumequine, PRI= Sulphadimethoxine/ormetoprim, OXO= Oxolinic Acid

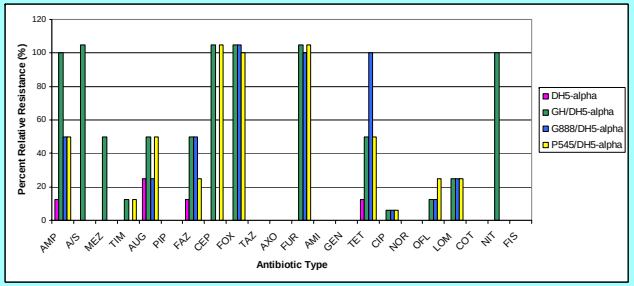
Figure 2

Isolate	AMP 0.5-16	A/S 8/4-16/8	MEZ 4-64	TIM 4/2-64/2	AUG 0.5/0.25-16/8	PIP 4-64	FAZ 1-16	CEP 8-16	FOX 2-16	TAZ 1-16	AXO 4-32	FUR 2-16	AMI 4-32	GEN 0.25-8	TET 0.25-8	CIP 0.06-2	NOR 4-8	OFL 0.25-4	LOM 0.5-4	COT 0.5/9.5-2/38	NIT 32-64	FIS 256
GH	16	16/8	16		4/2	32	>16	>16	>16	>16	>32	>16	>32	>8	8	>2	>8	>4	>4	>2/38	>64	>256
G888	16	>16/8	32		4/2	32	>16	>16	>16	>16	>32	>16	>32	>8	>8	>2	>8	>4	>4	>2/38	64	
G899	16	16/8	16		2/1	32	>16	>16	16	>16	>32	>16	>32	>8	8	>2	>8	>4	>4	>2/38		
P550	16		16		1/0.5	32	>16	>16	>16	>16	>32	>16	>32	>8	4	1		2	2	2/38	64	>256
P547	>16	>16/8	64	64/2	8/4	>64	>16	>16	>16	>16	>32	>16	>32	>8	2	0.5		2	4	>2/38		
P545	16	>16/8	16			32	>16	>16	>16	>16	>32	>16	>32	>8	2	0.5		1	2	>2/38	64	>256
G873	>16	>16/8	64	16/2	8/4	>64	>16	>16	>16	>16	>32	>16	>32	>8	2	0.5	8	2	4	>2/38		
G892	>16	>16/8	32	>64/2	16/8	64	>16	>16	>16	>16	>32	>16	>32	>8	>8	>2	>8	4	>4	>2/38	>64	>256

Clinical antibiotic MIC (minimum inhibitory concentration) values for *Flavobacterium* isolates. MIC data for all 8 *Flavobacterium* isolates is shown. Highlighted boxes indicate resistance to the maximum concentration of the antibiotic tested. Blank boxes represent susceptibility.

Sensititre MIC plates for gram-negative bacteria were used, and manufacturer's protocol was followed. Results were recorded 4 days after incubation at 23 ºC. Abbreviations: AMP= Ampicillin, A/S= Ampicillin/Sulbactam, MEZ= Mezlocillin, TIM= Ticarcillin/Clavulanic Acid, AUG= Amoxicillin/Clavulanic Acid, PIP= Piperacillin, FAZ= Cefazolin, CEP= Cephalothin, FOX= Cefoxitin, TAZ= Ceftazidime, AXO= Ceftriaxone, FUR= Cefuroxime (parenteral), AMI= Amikacin, GEN= Gentamicin, TET= Tetracycline, CIP= Ciprofloxacin, NOR= Norfloxacin, OFL= Ofloxacin, LOM= Lomefloxacin, COT= Trimethoprim/Sulfamethoxazole, NIT= Nitrofurantoin, FIS= Sulfisoxazole

Figure 3.



Conjugative transfer of antibiotic resistance using *Flavobacterium* donors and *E. coli* DH5-alpha as the recipient. Relative clinical antibiotic resistance of DH5-alpha and DH5-alpha transconjugants (GH/DH5-alpha, G888/DH5-alpha, P545/DH5-alpha) to the maximum antibiotic concentration tested is shown. Resistance beyond the maximum concentration tested is represented by bars over 100. The donors in this conjugation mating system, GH, G888, and P545, are all *Flavobacterium* isolates.

For the conjugation, one *Flavobacterium* isolate and DH5-alpha were mixed on BHI and incubated at 20ºC. Transconjugants were selected first on BHI with 8ug/ul CHL (chloramphenicol) at 37ºC, then on HEK (Hektoen agar) with 8ug/ul CHL at 37ºC, and finally on BHI + CHL at 37ºC. Inoculum from the final BHI + CHL plates was used for antibiotic MIC (incubated at 37ºC). Sensititre MIC plates for gram-negative bacteria were used, and manufacturer's protocol was followed. Results were recorded 48hrs after incubation. Abbreviations: AMP= Ampicillin, A/S= Ampicillin/Sulbactam, MEZ= Mezlocillin, TIM= Ticarcillin/Clavulanic Acid, AUG= Amoxicillin/Clavulanic Acid, PIP= Piperacillin, FAZ= Cefazolin, CEP= Cephalothin, FOX= Cefoxitin, TAZ= Ceftazidime, AXO= Ceftriaxone, FUR= Cefuroxime (parenteral), AMI= Amikacin, GEN= Gentamicin, TET= Tetracycline, CIP= Ciprofloxacin, NOR= Norfloxacin, OFL= Ofloxacin, LOM= Lomefloxacin, COT= Trimethoprim/Sulfamethoxazole, NIT= Nitrofurantoin, FIS= Sulfisoxazole

Results

- 8 pathogenic *Flavobacterium* isolates were obtained from fish hatcheries in Maine that had never used antibiotics to treat their fish stock
- All *Flavobacterium* isolates showed high levels of antibiotic resistance to agents used exclusively in aquaculture environments (Figure 1)
- All *Flavobacterium* isolates showed high levels of antibiotic resistance to antibiotics used in human chemotherapeutic applications (Figure 2)
- Conjugation between *Flavobacterium* isolate donors and *E. coli* DH5-alpha recipients resulted in transfer of antibiotic resistance determinants to DH5-alpha transconjugants (Figure 3)

Discussion

The eight *Flavobacterium* isolates obtained for this study were the cause of disease outbreaks in the Maine fish hatcheries from which they originated. All *Flavobacterium* isolates displayed strong resistance to both aquaculture and clinical antimicrobial compounds. The conjugation mating system employed in this study resulted in the transfer of resistance to select antibiotics from *Flavobacterium* donors to an *E. coli* recipient. This demonstrates that it may be possible for the *Flavobacterium* isolates characterized in this study to transfer their multiple antibiotic resistance determinants to human pathogenic bacteria.

Interestingly, the antibiotic resistance profile of the *Flavobacterium* isolates was not the result of antibiotic over-use, because the hatcheries from which they were obtained do not treat their fish stock with antibiotics. It is likely that there is another selective agent that has resulted in the accumulation of antibiotic resistance determinants in these isolates. Mercury, deposited atmospherically, is at toxic levels throughout Maine and other Northeastern States, making it one possible candidate. The presence of mercury could result in the transfer of antibiotic resistance determinants on the same mobile genetic elements as those for mercury resistance. All of the *Flavobacterium* isolates used in this study were mercury-resistant, and preliminary evidence suggests that many of the isolates carried genes for mercuric ion reduction, (data not shown). A mercury-resistant phenotype would support the hypothesis that the *Flavobacterium* isolates obtained their multiply-resistant antibiotic profile due to the presence of mercury in the environment.

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