4(2): 159-170 (2010) DOI: 10.3153/jfscom.2010016

Journal of FisheriesSciences.com

E-ISSN 1307-234X

RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

SHEWANELLA IN A TILAPIA FISH FARM

Shuxia Lu, Robert E. Levin*

Department of Food Science, Massachusetts Agricultural Experiment Station, University of Massachusetts, USA

Abstract: Twenty three strains of *Shewanella* were isolated from an inland fresh water tilapia fish farm and identified on the basis of biochemical tests. The mole% G+C for these isolates ranged from 45.5% to 48.9%. At one sampling of the fish tank water, 70% of the total aerobic CFU consisted of *Shewanella*. Random amplified polymorphic DNA (RAPD) analysis performed separately with three decamer primers designated LMPB4, LMPB1, and LMHLWL74 yielded 4, 5, and 4 different profile types respectively. Composite profiles derived from the 3 RAPD primers yielded 7 composite banding profiles from the 23 *Shewanella* isolates. The results of this study indicate that a heterogeneous population of *Shewanella* strains was frequently present in the fish tank water, and that on occasion, for as yet unknown reasons, a single clone may dominate the total aerobic bacterial population of the fish tank water.

Keywords: *Shewanella*, Tilapia, Fish, Farm, RAPD

Özet: **Tilapia Balık Çiftliklerinde *Shewanella***

Tatlı su tilapia balık üretim çiftliğinden biyokimyasal yöntemler kullanılarak yirmi üç shewanella suşu izole edilmiş ve tanımlanmıştır. Bu izolatların %G+C mol oranları % 45.5-48.9 aralığında değişmektedir. Tank suyunun bir örneklemesinde, total aerobik CFU' nun % 70'inin Shewanella olduğu tesbit edilmiştir. Rastgele Çoğaltılmış Polimorfik DNA (RAPD) analizi LMPB4, LMPB1 ve LMHLWL74 on bazlı primerleri ile yürütüldüğünde sırasıyla 4, 5 ve 4 farklı profil tipi elde edilmiştir. Bu 3 RAPD primerinden elde edilen kompozit profiller 23 *Shewanella* izolatından 7 farklı kompozit bant profilinin oluşturulmasını sağlamıştır. Bu çalışmanın sonuçları, su tanklarında genellikle heterojen olarak farklı *Shewanella* suşlarının bulunduğunu, sebebi henüz bilinmemekle birlikte, bazı durumlarda, bir suşun klonlarının aerobik bakteriyel populasyonunu domine edebileceğini göstermektedir.

Anahtar Kelimeler: *Shewanella*, Tilapia, Balık, Çiftlik, RAPD

* **Correspondence to:** Robert E. LEVIN, Department of Food Science, Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst, MA 01003-USA

E-mail: relevin@foodsci.umass.edu

Introduction

The organism now known as *Shewanella putrefaciens* has undergone an extensive taxonomic evolution during the past seven decades. The organism was first isolated from tainted butter by Derby and Hammer (1931) and classified as a member of the genus *Achromobacter* which is no longer extant. It was later transferred to the genus *Pseudomonas* by Long and Hammer (1941). It was then allocated to the genus *Alteromonas* by Lee *et al* (1977*)* on the basis of its much lower mols% G+C DNA content than the acceptable range of 58 to 65 mols% G+C for members of the genus *Pseudomonas***.** It was transferred by MacDonell and Colwell (1985) to the newly establish genus *Shewanella* under the family *Vibrionaceae* due to its perceived closer relationship with the genus *Vibrio* on the basis of 5S rRNA sequence data.

Bacterial isolates designated as *S. putrefaciens* are derived from a wide variety of sources including environmental samples: soil, oil fields, sea water, refrigerated foods such as fish, poultry, beef, and human clinical infections. Levin (1972) was the first to address the question as to whether the intense refrigerated food spoilage isolates of *P. putrefaciens* were identical to clinical isolates.

Castell *et al.* (1949) were the first to report that *P. putrefaciens* was involved in the spoilage of marine fish. Chai *et al.* (1968) found that the initial population of *S. putrefaciens* on fresh haddock fillets was consistently below 4% and that during refrigerated storage increased to 50 to 90% of the total bacterial population at the time of spoilage. Jørgensen and Huss (1989) concluded that *S. putrefaciens* constitutes the main spoilage organism on fish stored at 0^{o}C, irrespective of the origin of the fish and whether the fish is vacuum packed or stored in ice. Gram (1992; 1996) similarly stated *S. putrefaciens* to be the most important bacterium during spoilage of iced marine fish. The organism is also one of the predominant spoilage organisms of refrigerated meat (Borch *et al.* 1996).

Numerous reports have implicated *S. putrefaciens* as the cause of various human infections such as cellulitis (Chen *et al.* 1997), peritonitis (Dan *et al.* 1992), abscesses (Yohe *et al.* 1997; Pagani *et al.* 2003), bacteremia (Brink *et al.* 1995; Pagani *et al.* 2003), and ear infections (von Graevenitz and Simon, 1970; Holmes *et al.* 1975). Patients developing bacteremia have been found to have an underlying illness such as diabetes mellitus (Brink *et al.* 1995), burns (Reddi *et al.* 1985), liver or biliary disease (Brink *et al.* 1995), renal failure (Dan *et al.* 1992), or long-term catheterization (Bhandari *et al.* 2000).

There are several reports indicating infection due to *S. putrefaciens* following exposure to marine life and the marine environment such as shellfish contact (Heller *et al.* 1990), bathing in the sea (Papanaoum *et al.* 1998), traumatic spike injury from a fish (Chen, 1997) and living on a boat (Yohe *et al.* 1997).

S. putrefaciens is being increasingly recognized as an opportunistic pathogen and as a cause of human infection (Brink *et al*, 1995; Chen *et al*, 1997; Leong *et al*, 2000). Detecting and identifying marine food spoilage organisms and opportunistic pathogens in association with foods is therefore of importance.

Colonies of *S. putrefaciens* are characteristically salmon colored. The ability of *S. putrefaciens* to form H_2S was found to serve as a useful criterion of identity for the organism from marine fish with the use of pour plates of Peptone-Iron Agar (PIA; Levin, 1968). Subsurface colonies appear intensely black, whereas surface colonies usually have a black central area surrounded by a salmon colored peripheral area of growth. The ability of isolates to reduce trimethylamine oxide (TMA-O) to trimethylamine (TMA), to produce extracellular DNase and protease, to produce ornithine decarboxylase, and to possess a mols% G+C content of 43 to 48% are additional criteria of identity (Levin, 1972; Levin, 1975; Gilardi, 1985; Vogel *et al*. 1997).

For over three decades *S. putrefaciens* has been recognized as a genetically heterogeneous species. Owen et al. (1978) showed that the species comprised at least four clearly separate genomic groups, I to IV. One of the *S. putrefaciens* genomic groups (IV) has been reclassified as an additional species designated *S. alga* (Simidu et al (1990). Ziemke et al. (1998) designated Owen's genomic group II as a new species, *S. baltica*. Venkateswaran et al. (1999) designated Owen's group II as strains of a new species *S.*

oneidensis. Owen's group I strains are highly related to ATCC 8071 and should be considered as the true *S. purefaciens* according to Tryfinopoulou et al. (2007). On the basis of phenotypic properties, isolates of *S. putrefaciens* can be easily misidentified as *S. alga* (Vogel et al. 1997*)*.

An inland fresh water tilapia farm located in Amherst, Massachusetts, U.S.A. was used as the source for all samples. This tilapia farm has certain unique features including nitrifying tanks housing sand particles coated with nitrifying bacteria (*Nitrosomonas europa* and *Nitrobacter winogradskyi*) for conversion of ammonia sequentially to nitrite and then to nitrate. The resulting nitrate is then consumed by hydroponically grown basil plants. This allows the fish tank water to be constantly re-circulated with a loss of no more than about 2% of the total tank water daily.

In the present study isolates of *Shewanella* derived from various locations of this tilapia fish farm were characterized on the basis of a number of biochemical tests and the determination of the moles % G+C content of DNA. In addition, isolates were characterized with respect to RAPD analysis. The details regarding the operation of this tilapia plant and a flow diagram have previously been described (Lu and Levin, 2007).

Materials and Methods

Sources of Shewanella isolates

All samples for bacterial analysis were obtained and processed in 2003. Approximately 75 ml samples of tilapia fish tank water derived from 180,000 gallons of tilapia farm water in 250,000 gallon tanks maintained at 30^{o}C were collected in sterile 100 ml milk dilution bottles. All commercial culture media were Difco. Within 1 hr after collection the samples were decimally diluted in Tryptic Soy Broth (TSB) and 0.1 ml of each dilution surface smeared in triplicate onto Tryptic Soy Agar (TSA) plates for determination of total aerobic CFU. Plates were incubated at 32^{o}C for 24 hr. Salmon pigmented colonies regarded tentatively as *Shewanella* sp. were picked and streaked onto TSA plates. Isolated colonies were then picked and transferred to TSA slants for incubation and refrigerated storage.

Sand particles suspended in water from the nitrifying tank were collected in a 400 ml sterile beaker, covered with sterile aluminum foil, and then transported to the laboratory. The entire beaker of sand was poured onto sterile filter paper in a sterile Buchner funnel and then washed twice with 100 ml of sterile TSB to remove unattached bacteria using a vacuum filtration system. Wet sand (15.0 g) was then blended with 100 ml of TSB for 1 min to detach bacterial cells from the sand particles. Decimal dilutions were then prepared and plated as above.

50 g of basil roots were blended with 250 ml of TSB for 30 sec. Decimal dilutions were prepared and then cultured for CFU as described above.

Physiological and biochemical Characterization of the isolates

Characterization of all the *Shewanella* isolates was achieved as described below. Cell morphology, motility, gram stain, cytochrome oxidase, catalase, liquefaction of gelatin, proteolysis of casein, hydrolysis of DNA, reduction of nitrate, gas production from dextrose and were performed according to Burnett et al. (1957) and Pelczar and Reid (1958). Reduction of TMA-O to TMA was performed with 10 ml of TSB in tubes containing 0.01M TMA-O as previously described (Wood and Baird, 1943). TMA production after 2 days of incubation at 32^{o}C was examined by the method of Laycock and Reiger (1971).

Lecithinase production was determined by using nutrient agar supplemented with 10% (v/v) egg-yolk saline solution (50% v/v) (Stenstrom, 1990). Ornithine decarboxylase was detected by adding 2.0% DL-ornithine monohydrochloride to Decarboxylase Base Moeller (Difco) sealed with sterile mineral oil. Haemolytic activity was observed on plates of TSA supplemented with 5% defibrinated sheep blood. All strains were tested for the ability to grow at 4^{o}C by incubating inoculated tubes of TSB without dextrose for 12 days. Growth at 37^{o}C was assessed by inoculating PIA slants followed by incubation for 11 days. Growth at 42^{o}C was assessed by inoculating TSA plates followed by incubation for 1 day. H_2S production was determined by stab inoculating PIA deeps followed by incubation at 32^{o}C for 2 days. Growth on plates of Salmonella-Shigella (SS) Agar was determined after 7 days incubation at 37^{o}C. Tolerance to NaCl was determined in tubes holding 10 ml of TSB broth containing 6.0% and 10% NaCl incubated at 20^{o}C for up to 12 days (Levin, 1972; Gilardi *et al*. 1991; Nozue *et al*, 1992; Vogel *et al*. 1997; Venkateswaran *et al*. 1999).

Acid production from glucose was assessed using the medium of Hugh and Leifson (1953) with aerobic and anaerobic (tubes sealed with 0.5 cm of sterile mineral oil) incubation after 3 days at 32°C.

Resistance to O/129 (2,4-diamino-6,7-diisopropylpteridine, Sigma) was determined on inoculated smear plates of TSA agar using the disk diffusion method. Fifty µL of filter sterilized O/129 (150 mg/50 µL) were applied to 14 mm diam. filter paper discs placed onto the center of the plate followed by incubation at 32°C for 24 hr.

RAPD analysis

Cells from a single *Shewanella* colony were subcultured overnight onto a plate of TSA containing 0.5% dextrose at 32°C. One purified colony was picked and inoculated into 5 mL of TSB broth and incubated overnight at 32°C with rotary agitation (200 rpm). A portion of the broth culture (1.5 mL) was centrifuged at 12,000 rpm (16,000 g) for 5 min and the pellet washed twice with 1 ml of deionized water (dH_2O). The pellet was then resuspended in 250 µL of sterile dH_2O and adjusted to an absorbance of 1.6 at 600nm in 1 cm path length cuvets. This suspension was used directly for RAPD amplification or kept frozen at -20°C until used.

Three decamer primers LMPB4 5'-AAGGATCAGC, LMPB1 5'-GGAACTGCTA (Mazurier *et al*. 1992), and LMHLWL74 5'-ACGTATCTGC (Farber *et al*. 1994; Boerlin *et al*. 1995) synthesized by Sigma/Genesis were selected for RAPD typing of the *Shewanella* strains.

For the amplification procedure, 50 µL PCR reaction mixtures were prepared, each containing 1.25 U of Taq DNA polymerase (Qiagen, Fisher Scientific, cat. no. 201203), 2.5 mM $MgCl_2$, 0.2 mM each dNTPs (dATP, dTTP, dCTP dGTP) (Takara Shuzo, Fisher Scientific, cat. no. TAK4030). 0.4 µM of the primer and 5 µL of cell suspension a (A_{600} = 1.6). The reaction mixture was cycled through the following temperature profile: one cycle consisting of 94°C for 4 min, 35°C for 2 min and 72°C for 2 min followed by 43 cycles consisting of 94°C for 1 min, 35°C for 2 min and 72°C for 2 min. The final cycle was: 94°C for 1 min, 35°C for 2 min and 72°C for 10 min. All amplifications were performed in a DNA thermocycler (Techgene Ltd., Cambridge, UK).

For pattern analysis, 14 µL of the amplified DNA products were loaded and resolved on 1.6 % agarose gels by electrophoresis running at 5V/cm for 1.5 hr with 1 5 TBE running buffer (89 mM Tris-Base, 89 mM boric acid, 2 mM EDTA, pH8.4). The gels were stained with ethidium bromide (1 µg/mL) for 30 min. A DNA ladder ranging from 50 - bp to 2000 - bp (Sigma, cat. no. P9577) was included on each gel for molecular weight markers. Banding patterns were visualized with a UV transilluminator and photographed with a digital camera with orange filter.

DNA extraction and mols% G+C determination

Cells were harvested from 1L flasks containing 200 ml of TSB with 0.5% dextrose incubated overnight at 32°C with rotary agitation (200 rpm). DNA extraction was as previously described (Marmur, 1961; Levin, 1972).

The DNA melting point (Tm value) was determined as described by Marmur and Doty (1962) using an electrically heated cuvette and a calibrated thermistor temperature indicator and probe (Levin, 1969). The Mols% G+C content of DNA was calculated from the equation: %G+C = (Tm-53.9)/0.41 (Marmur and Doty, 1962). *Escherichia coli* K12 with a previously reported mols% G+C of 51.0% (Seidler *et al*. 1969) was used as a reference standard.

Results and Discussion

Incidence of *Shewanella* from Tilapia fish farm and characteristics of isolates

A total of 23 *Shewanella* strains were isolated from various sources of the tilapia fish farm on different dates (Table 1). The results in Table 1 indicate that strains of *Shewanella* were found to constitute 4.0%, 70%, and 9.9% of the bacterial flora in the water from the same tilapia tank at different sampling dates. A single sampling of the sand in the nitrifying tank yielded only one isolate of *Shewanella* (0.87%). The total aerobic bacterial counts in the water were found to be $2.0x10^4$ CFU/mL, $5.8x10^4$ CFU/mL, $8.8x10^4$ CFU/mL for March 5, Sept. 9, and Oct. 8 respectively (Table 1). The CFU counts of sampled water from the same fish tank appeared to increase with each sampling, presumably due to a significant increase in the size of the fish and amount of fecal load with time. *Shewanella* was not found on the roots of the hydroponically grown basil. This observation suggests that there

might be bacterial species on the roots that are either inhibitory or exclusionary to *Shewanella*. The observation that *Shewanella* from the fish tank water sample on Sept. 9 constituted 70% of the total aerobic bacterial population and thereby dominated the tilapia tank water may reflect an unknown nutritional dynamic.

The *Shewanella* isolates from March 5 were designated 26Ft, 46Ft, 65Ft and 93Ft, from Sept. 9 Ft1, Ft2, Ft3, Ft4, Ft5, Ft6, Ft7, and from Oct. 8 10ft, 11ft, 12ft, 21ft, 24ft, 42ft, 43ft, 44ft, 52ft, 69ft, 114ft. The single *Shewanella* isolate from sand in the nitrifying tank on July 9 was designated 60sp (Table 2, Table 3).

All presumptive *Shewanella* strains isolated were Gram-negative, and composed of rod-shaped motile cells. Young colonies grown on TSA plates containing 0.5% dextrose were salmon colored.

Growth occurred at 37^{o}C on PIA and at 42^{o}C on TSA containing 0.5% dextrose, but not at 37^{o}C on SS Agar with all 23 isolates (Table 2). All 23 *Shewanella* isolates produced hydrogen sulfide and grew at 4^{o}C both in TSB broth without dextrose and on PIA slants (Table 2). Among the 23 isolates, the only ones able to grow in 6% NaCl were six of the seven from tilapia tank water obtained on Sept. 9. None of the 23 isolates were able to grow in 10% NaCl (Table 2).

All the strains of *Shewanella* were positive for production of catalase, cytochrome oxidase, DNase, proteinase (casein proteolysis), gelatinase (gelatin liquefaction), and ornithine decarboxylase. They were all negative for production of lecithinase. They were non-fermentative and no acid was oxidatively produced from dextrose in the medium of Hugh Leifson (Hugh *et al*, 1953) after 2 to 7 days of incubation but they did reduce TMA-O to TMA, and also reduced nitrate to nitrite (Riley *et al*. 1972; Simidu *et al*. 1990; Reid *et al*. 1999; Venkateswaran *et al*. 1999). They were resistant to O/129 and exhibited β-haemolysis on sheep blood agar (Table 2). Although hemolysis was obscure on the first day of incubation, it became obvious on the following days at 32^{o}C.

Table 1. *Shewanella* from a tilapia fish farm.

Sampling date	Sampling location	*Shewanella*[a,b]	percent of isolates	Aerobic counts (CFU/ml or g[c])
March 5	Fish tank water	4 (100)	4.0%	2.0 x 10^4
July 9	Sand particles from nitrifying tank	1 (115)	0.9%	1.3 x 10^7
Sept. 9	Fish tank water	7 (10)	70%	5.8 x 10^4
	Basil root	0 (107)	0	2.2 x 10^6
Oct. 8	Fish tank water	11 (111)	9.9%	8.8 x 10^4

[a] Number inside parentheses indicates total number of colonies picked at random
[b] Number outside parentheses indicates number of *Shewanella* isolates randomly picked.
[c] CFU/g of wet sand or CFU/g of fresh basil root.

Table 2. Characteristics of *Shewanella* isolates

Character	Ft isolates 1, 2, 3, 4, 6, & 7	All Additional 17 isolates
Gram-negative stain	+	+
Motility	+	+
Pigmentation	+	+
Catalase	+	+
Cytochrome Oxidase	+	+
H_2S-production	+	+
Ornithine decarboxylase	+	+
Production of:		
DNase	+	+
Gelatinase	+	+
Proteinase	+	+
Lecithinase	-	-
β-haemolysis	+	+
Reduction TMA-O to TMA	+	+
Reduction of NO_3^- to NO_2^-	+	+
Gas produced from Glucose	-	-
Hugh-Leifson reaction:		
Aerobic (+O_2)	Alk	alk
Anaerobic (-O_2)	-	-
O/129 resistance	+	+
Growth at:		
37°C on S.S. Agar	-	-
37°C on Peptone-Fe agar	+	+
42°C on TSA[a]	+	+
4°C in TSB [B]	+	+
4°C on Peptone-Fe agar slant	+	+
20°C in TSB with 6% NaCl	(+) [C]	(-) [C]
20°C in TSB with 10% NaCl	-	-

[a] containing 0.5% dextrose
[b] without dextrose
[c] parentheses indicate a difference

RAPD banding profiles with three primers

Figure 1 presents the RAPD banding profiles for each of the seven isolates of *Shewanella* from fish tank water on Sept. 9 with the three random primers. Two RAPD profiles were collectively obtained. The isolate Ft5 was consistently distinguished from the other six strains (which yielded identical profiles) with each of the 3 primers.

The additional 16 *Shewanella* strains were also subjected to RAPD analysis with the three primers. The RAPD profiles obtained were given arbitrary numerical designations shown in Table 3. From the 23 strains examined, 4, 5 and 4 different RAPD profiles (Fig. 2) were observed with primers LMPB1, LMPB4, LMHLWL74 **r**espectively. There is a consensus band at about 800-bp with primer LMPB4 and at 900-bp with LMHLWL74 respectively. Based on RAPD analysis the 23 strains of *Shewanella* could be broken down into seven separate composite RAPD types with the use of the 3 primers (Table 3). This indicated that strains of *Shewanella* from the tilapia farm were genotypically heterogeneous but with same common clones predominantly.

The RAPD types of *Shewanella* were found to be different at different sampling dates except for the one isolate (60sp) from the sand particle of the nitrifying tank that corresponded to the dominant type 2 profiles of the fish tank water isolate of Oct. 8, 2003.

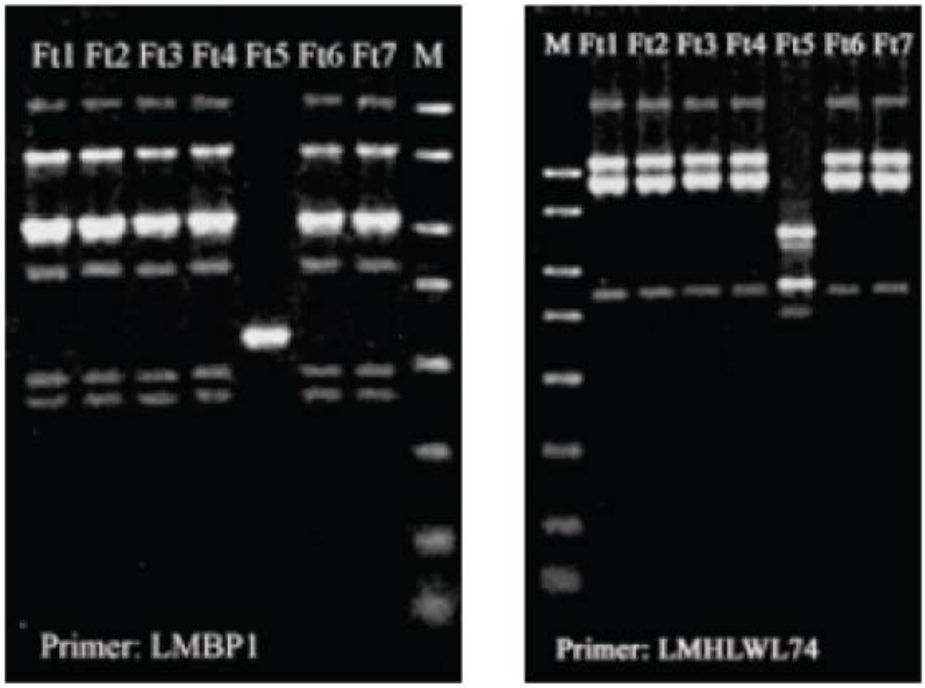

Figure 1. RAPD patterns determined with three primers for 7 isolates of S*hewanella*. Lanes 1 through 7, RAPD patterns of Ft1 to Ft7 respectively.

Table 3. S*hewanella isolates* and their RAPD banding profiles with three primers

Sample and date	Isolate no.	Banding profiles (P-LMPB 4) A	(P-LMPB 1) B	(P-LMHLWH 74) C	Composite profile	Profile no.
Fish tank water, March 5	26_{Ft}	1	1	3	$A_1B_1C_3$	1
	46_{Ft}	1	1	3	$A_1B_1C_3$	1
	65_{Ft}	1	1	3	$A_1B_1C_3$	1
	93_{Ft}	1	1	3	$A_1B_1C_3$	1
Sand, July 9	60sp	2	1	3	$A_2B_1C_3$	2
Fish tank water, October 8	10ft	2	1	3	$A_2B_1C_3$	2
	11ft	2	4	3	$A_2B_4C_3$	4
	12ft	2	1	3	$A_2B_1C_3$	2
	21ft	2	1	3	$A_2B_1C_3$	2
	24ft	2	1	3	$A_2B_1C_3$	2
	42ft	2	1	3	$A_2B_1C_3$	2
	43ft	2	1	3	$A_2B_1C_3$	2
	44ft	2	1	3	$A_2B_1C_3$	2
	52ft	2	5	3	$A_2B_5C_3$	5
	69ft	2	1	4	$A_2B_1C_4$	6
	114ft	2	1	3	$A_2B_1C_3$	3
Fish tank water, September 9	Ft5	3	2	1	$A_3B_2C_1$	7
	Ft1	4	3	2	$A_4B_3C_2$	3
	Ft2	4	3	2	$A_4B_3C_2$	3
	Ft3	4	3	2	$A_4B_3C_2$	3
	Ft4	4	3	2	$A_4B_3C_2$	3
	Ft6	4	3	2	$A_4B_3C_2$	3
	Ft7	4	3	2	$A_4B_3C_2$	3
		4 types	5 types	4 types		7 types

Determination of moles % G+C Content

The moles% G+C of the different RAPD types ranged from 45.5 ± 0.6 to 48.9 ± 0.6 respectively (Table 4). Levin (1972) previously differentiated intense fish spoilage isolates of *P. putrefaciens* and human clinical isolates into two groups: one group with a low moles % G+C content of 43.8 to 47.8% and the other group with a high moles % G+C content of 51.9 to 55.0%. He reported that the low G+C group was dominated by environmental and refrigerated food isolates, but did contain several human clinical isolates. Our results were similar to those he reported for fishery isolates and indicated that they were not *S. alga* isolates which have a high moles % G+C content of 52 to 54%, are able to grow on SS Agar, and in 10% NaCl but are unable to grow at 4 ^{o}C (Nozue *et al*, 1992; Vogel *et al*, 1997; Venkateswaran *et al*, 1999).

Table 4. G+C content of different RAPD types of *S. putrefaciens.*[a]

	RAPD type						
	1	2	3	4	5	6	7
Mols% G+C	47.3	48.9	48.4	47.9	47.6	45.4	48.3
Stand. Dev.	± 0.9	± 0.6	± 0.6	±0.4	±0.4	±0.6	±0.7

[a]Mols% G+C of *Escherichia coli* K12: 51.0 ± 0.6.

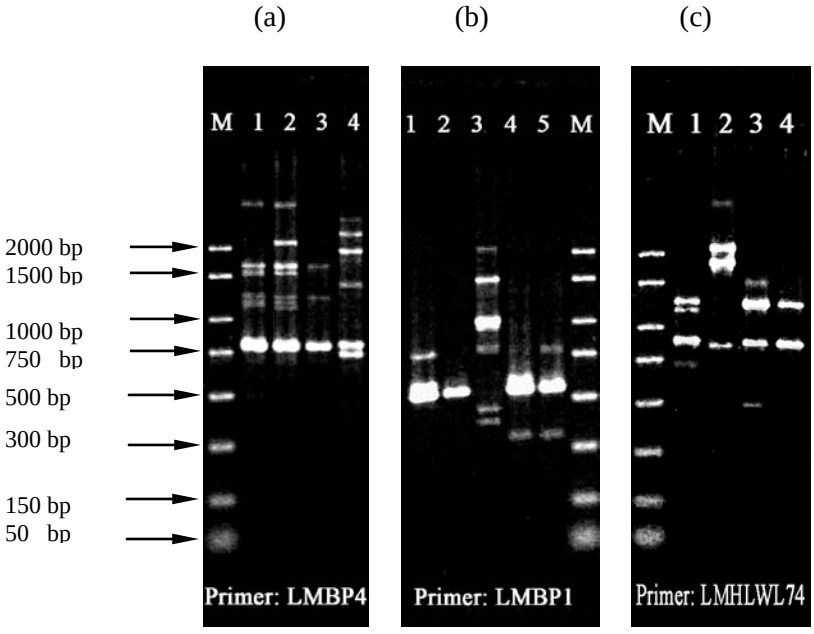

Figure 2.RAPD profiles of *Shewanella* with three primers. (a) Lanes 1 through 4, RAPD types 1 through 4 with primer LMPB4; lane M, DNA ladder. (b) Lanes 1 through 5, RAPD types 1 through 5 with primer LMPB1; lane M, DNA ladder. (c) Lane M, DNA ladder; lanes 1 through 4, RAPD types 1 through 4 with primer LMHLWL74.

Sakata *et al* (1984) reported that the predominant bacterial genera in the intestine of fresh water reared tilapia were *Vibrio*, *Aeromonas* and *Pseudomonas*. The present study is the first report that *Shewanella* can dominate the aerobic bacterial flora of a fresh water tilapia fish farm.

Basil roots failed to yield *Shewanella*. This might be due to some antagonistic activity. Previous studies have reported that isolates of some *Pseudomonas* are capable of inhibiting the growth of *S. putrefaciens* (Gram, 1993; Gram and Melchiorsen, 1996).

Nozue *et al* (1992) attempted to clarify the physiological differences between *S. alga* and *S. putrefaciens* which are very similar. Thirty six strains of *S. alga* and 41 strains of *S. putrefaciens* were phenotypically and molecularly characterized. The two species can presumably be differentiated on the basis that *S. alga* grows at 42°C (Nozue *et al*, 1992; Khashe *et al*, 1998), and in 10% NaCl (Vogel et al, 1997; Venkateswaran *et al*, 1999) (Nozue *et al*, 1992; Khashe *et al*, 1998) while *S. putrefaciens* does not. In addition, strains of *S. alga* have a moles% G+C content of 52 to 54% in contrast to strains of *S. putrefaciens* that have a range of 45 to 48% (Nozue *et al*, 1992).

Tryfinopoulou et al. (2007) found that 16S rRNA sequence similarities (>97%) and phenotypic similarities of *S. baltica*, *S. purefaciens*, and *S. oneidensis* made it difficult to allocate their *Shewanella* isolates from Aegean sea fish to a specific species .

In the present study the isolates of *Shewanella* from the tilapia farm had a G+C content ranging from 45.5 to 48.9%, were □ -hemolytic on sheep blood agar, grew at 4°C, 37°C, and 42°C, did not grow on SS agar, and did not produce lecithinase. Six of the seven *Shewanella* isolates from tilapia tank water obtained on Sept. 9. were able to grow in 6% NaCl but did not grow in 10% NaCl. An examination of the data in Table 5 makes it difficult to allocate these isolates to any one specific species of *Shewanella.*

Table 5. Physiological characteristics of groups I, II, III, and IV derived from the grouping *of S. putrefaciens* isolates by Owen et al. (1978) and tilapia farm isolates of this study.[a]

Characteristic	Group I *S. putrefaciens*	Group II *S. baltica*	Group III *S. oneidensis*	Group IV *S. algae*	Isolates from this study
Moles % G +C content	45-48[b], 43-47[e]	46[b]	45[b]	52-54[c], 52-55[e]	45.5-48.9
Hemolysis	0[b], 61.5[e] ,13[d]	0[b]	0[b]	75[b], 100[c], 100[d], 62[e]	100
H_2S production	100[b]	+[b]	100[b]	100[b]	100
Growth under conditions of:					
6% NaCl	100[b]		40[b]	100[b]	26
10% NaCl	0[b]		0[b]	100[b], 96[e]	0
SS Agar	52[d], 15[f]			100[d], 96[f], 15[f]	0
Growth at:					
4 °C	90[f]			0	100
42 °C	4[d]			96[f], 97[c]	100
Production of:					
Gelatinase	0[b], +[f] ,91[d]	-[e]	100[b], +[f], +[f]	96[d], 100[c], 50[b]	100

[a]Numerical values are the percentage of positive strains unless otherwise indicated.

[b]From Venkateswaran et al. (1999)

[c]From Nozue et al. (1992)

[d]From Khashe and Janda (1998)

[f]Vogel et al. (1997)

[f]Tryfinopoulou et al. (2007)

Conclusions

The percent of *Shewanella* in tilapia farm tank water was found to vary from 4.0% to 70% derived from total aerobic plate counts. Hydroponically grown basil plants associated with the fish farm tank water failed to yield *Shewanella isolates*. This is the first report of a single clone of *Shewanella* numerically dominating the aerobic bacterial flora of the tank water of a tilapia fish farm.

Acknowledgements

This research was supported by a Special Seafood Safety Grant from the USDA.

References

Bhandari, S., Pan, T.L.T., Horvath, J., Tiller, D. (2000). CAPD, swimming in *Shewanella*, *Nephrol Dial Transplant*, **15:** 1484-1485. **doi:10.1093/ndt/15.9.1484**

Boerlin, P., Bannerman, E., Ischer, F., Rocourt J., Bille, J. (1995). Typing *Listeria monocytogenes*: a comparison of random amplification of polymorphic DNA with 5 other methods, *Research in Microbiology***, 146**: 35-49. **doi:10.1016/0923-2508(96)80269-5**

Borch E., Kant-Muermans M.L., Blixt Y. (1996). Bacterial spoilage of meat and cured meat products, *International Journal of Food Microbiology*, **33**: 103-120. **doi:10.1016/0168-1605(96)01135-X**

Brink, A.J., van Staten, A., van Rensburg, A.J. (1995). *Shewanella* (*Pseudomonas*) *putrefaciens* bacteremia, *Clinical Infectious Diseases*, **20**: 1327-1332.

Burnett, G.W., Pelczar M.J., Conn, H.J. (1957). Preparation of media. In M.J Pelczar,. Jr., R.C. Bard, G.W. Burnett, H.J. Conn, R.D. DeMoss, E.E. Evans, M.W. Jennison, A.P. McKee, A.J. Riker, J.Warren, O.B. Weeks, F.A Weiss, eds. *Manual of Microbiological Methods*. pp. 37-63. McGraw-Hill, Inc., New York, Toronto, London:

Castell, C.H., Richards, J.F., Wilmot, I. (1949). *Pseudomonas putrefaciens* from cod fillets, *Journal of the Fisheries Research Board of Canada*, **7**: 430-431.

Chai, T., Chen, C., Rosen, A., Levin, R.E. (1968). Detection and Incidence of Specific Species of Spoilage Bacteria on Fish, *Applied Microbiology*, **16**: 1738-1741.

Chen, Y.S., Liu, Y.C., Yen, M.Y., Wang, J.H., Wang, J.H., Wann S.R., Cheng, D.L. (1997). Skin and soft-tissue manifestations of *Shewanella putrefaciens* infection, *Clinical Infectious Diseases*, **25**: 225-229.

Dan, M., Gutman R., Biro, A. (1992). Peritonitis caused by *Pseudomonas putrefaciens* in patients undergoing continuous ambulatory peritoneal dialysis. *Clinical Infectious Diseases*, **14**: 359-360.

Derby, H.A., Hammer, B.W. (1931). Bacteriology of butter. IV. Bacteriological studies on surface taint butter. *Iowa Agric. Exp. Stat. Res. Bull.* **145**: 387-416.

Farber, J.M., Addison, C.J. (1994). RAPD typing for distinguishing species and strains in the genus *Listeria*, *Journal of Applied Bacteriology*, **77**: 242-250.

Gilardi, G.L. In Lennette, E.H., Balows, A., Hausler, W.J., Jr., and Shadomy, H.J. (1985). *Pseudomonas*. *Manual of Clinical Microbiology*, 4th ed. pp. 350-372. Washington, DC: American Society for Microbiology.

Gilardi, G.L., In: Balows, A., Hausler, W.J. Jr, Herrmann, K.L., Isenberg, H.D., Shadomy, H.J. (1991). *Pseudomonas* and related genera. *Manual of Clinical Microbiology*. 5th ed. pp. 429-441. Washington, DC: American Society for Microbiology.

Gram, L. (1992). Evaluation of the bacteriological quality of seafood, *International Journal of Food Microbiology*, **16**: 25-39. **doi:10.1016/0168-1605(92)90123-K**

Gram, L., Melchiorsen, J. (1996). Interaction between fish spoilage bacteria *Pseudomonas* sp. and *Shewanella putrefaciens* in fish extracts and on fish tissue, *Journal of Applied Bacteriology*, **80**: 589-595.

Gram, L. (1993). Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish, *Applied and Environmental Microbiology*, **59**: 2197-2203.

Gram, L, Huss, H.H. (1996). Microbiological spoilage of fish and fish products, *International Journal of Food Microbiology*, **33**: 121-137. **doi:10.1016/0168-1605(96)01134-8**

Heller, H., Tortora, G., Burger, H. (1990). *Pseudomonas putrefaciens* bacteremia associated with shellfish contact, *The American Journal of Medicine,* **88**: 85-86.

Holmes, B., Lapage, S.P., Malnick, H. (1975). Strains of *Pseudomonas putrefaciens* from clinical material, *Journal of Clinical Pathology,* **28**: 149-155.

Hugh, R., Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria, *The* Journal *of Bacteriology*, **66**: 24-26

Jørgensen, B.R., Huss, H.H. (1989). Growth and activity of *Shewanella putrefaciens* isolated from spoiling fish, *International Journal of Food Microbiology*, **9**: 51-62.

Khashe, S., Janda, J.M., (1998). Biochemical and Pathogenic Properties of *Shewanella alga* and *Shewanella putrefaciens*, *Journal of Clinical Microbiology,* **36**: 783-787.

Laycock, R.A., Reiger, L.W. (1971). Trimethylamine-producing bacteria on haddock (*Melanogrmmus aeglefinus*) fillets during refrigerated storage, *Journal of the Fisheries Research Board of Canada,* **28**: 305-309.

Lee, J.V., Gibson, D.M., Shewan, J.M. (1977). A numerical taxonomic study of *Pseudomonas*-like marine bacteria, *Journal of General Microbiology,* **98**: 439-451.

Leong J., Mirkazemi M., Kimble F. (2000). *Shewanella putrefaciens* hand infection, *Australian and New Zealand Journal of Surgery,* **70**: 816-817. **doi:10.1046/j.1440-1622.2000.01962.x**

Levin, R.E. (1968). Detection and incidence of specific species of spoilage bacteria on fish I. Methodology, *Applied Microbiology,* **16**: 1734-1737.

Levin R. E. (1969). Electrically Heated Cuvette Chamber for deoxyribonucleic acid melting point determinations. *Applied Microbiology,* **18**: 528-530.

Levin, R.E. (1975). Characteristics of weak-H_2S-producing isolates of *Pseudomonas putrefaciens* from human infections *Ant. van Leeuwen, J. Microbiol. Serol.* **41**: 569-574.

Levin, R.E. (1972) Correlation of DNA base composition and metabolism of *Pseudomonas putrefaciens* isolates from food, human clinical specimens, and other sources. *Ant. van Leeuwen, J. Microbiol. Serol.* **38**: 121-127.

Long, H.F., Hammer B.W. (1941). Classification of organisms important in dairy products. III. *Pseudomonas putrefaciens*, *Iowa Agric. Exp. Stat. Res. Bull,* **285**: 176-195.

Lu, S., Levin, R. (2008). Dominant bacterial Genera of a tilapia fish farm and RAPD typing of *Vibrio* isolates. *Journal of FisheresSciences.com*, **2:** 183-195. **doi:10.3153/jfscom.2008020**

MacDonell, M.T., Colwell, R.R. (1985). Phylogeny of the *Vibrionaceae* and recommendation for two new genera, *Listonella* and *Shewanella*, *Systematic and Applied Microbiology***, 6**: 171-182.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms, *Journal of Molecular Biology,* **3:** 208-218.

Marmur, J., Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature, *Journal of Molecular Biology* ,**5**: 109-118.

Mazurier, S., Wernars, K. (1992). Typing of *Listeria* strains by random amplification of polymorphic DNA (RAPD) analysis, *Journal of Medical Microbiology,* **38**: 322-327.

Mazurier, S.-I., Wernars, K. (1992). Typing of *Listeria* strains by random amplification of polymorphic DNA, *Research in Microbiology,* **143**: 499-505. **doi:10.1016/0923-2508(92)90096-7**

Nozue, H., Hayashi, T., Hashimoto, Y., Ezaki, T., Hamasaki, K. Ohwada, K. and Terawaki, Y. (1992). Isolation and characterization of *Shewanella alga* from human clinical specimens and emendation of the description of *S. alga* Simidu et al. 1990. 335: *International Journal of Systematic Bacteriology,* **42**: 628-634.

Pagani, L., Lang, A., Vedovelli, C., Moling, O., Rimenti, G., Pristera, R. and Mian, P. (2003). Soft tissue infection and bacteremia caused by *Shewanella putrefaciens*, *Journal of Clinical Microbiology*,**41**: 2240-2241. **doi:10.1128/JCM.41.5.2240-2241.2003**

Papanaoum, K., Marshmann, G., Gordon, L.A., Lumb, R., Gordon, D.L. (1998). Concur-

rent infection due to *Shewanella putrefaciens* and *Mycobacterium marinum* acquired at the beach, *Australian Journal of Dermatology*, **24**: 798-800.

Pelczar, M.J., Jr, Reid, R.D. (1958). *Laboratory Exercises in Microbiology*. pp. 71-74. McGraw-Hill, Inc. New York, Toronto, London.

Reddi, G.S., Shukl, N.P., Singh, K.V. (1985). *Pseudomonas putrefaciens* as a cause of infection in burn, *Indian Journal of Pathology & Microbiology*, **28**: 303-308.

Riley, P.S., Tatum, H.W., Weaver, R.E. (1972). *Pseudomonas putrefaciens* isolates from clinical specimens, *Applied Microbiology*, **24**: 798-800.

Seidler, R.J., Starr, M.P., Mandel, M. (1969). Deoxyribonucleic acid characterization of *Bdellovibrios*, *Journal of Bacteriology*, **100**: 786-790.

Stenstrom, I.-M., Molin, G. (1990). Classification of the spoilage flora of fish, with special reference to *Shewanella putrefaciens*, *Journal of Applied Bacteriology*, **68**: 601-618.

Tryfinopoulou, P., Tsakalidou, E., Vancanneyt, M., Hoste, B., Swings, J., Nychas, G. (2007). Diversity of *Shewanella* population in fish *Sparus aurata* harvested in the Aegean Sea, *Journal of Applied Bacteriology*, **103**: 711-721. **doi:10.1111/j.1365-2672.2007.03355.x**

Venkateswaran, K., Moser, D.P., Dollhopf, M.E., Lies, D.P., Saffarini, D.A., MacGregor, B.J., Ringelberg, D.B., White, D.C., Nishijima, M., Sano, H., Burghardt, J., Stackebrandt, E., Nealson. K.H. (1999). Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. Nov, *International Journal of Systematic Bacteriology*, **49**: 705-724.

Von Graevenitz, A., Simon, G. (1970). Potentially pathogenic, nonfermentative, H_2S-producing gram-negative rod (1b), *Applied Microbiology*, **19**: 176.

Vogel, B.F., Jørgensen, K., Christensen, H., Olsen, J.E., Gram, L. (1997). Differentiation of *Shewanella putrefaciens* and *Shewanella alga* on the basis of whole-cell protein profiles, ribotyping, phenotypic characterization, and 16S rRNA gene sequence analysis, *Applied and Environmental Microbiology*, **63**: 2189-2199.

Wood, A.J., Baird, E.A. (1943). Reduction of trimethylamine oxide by bacteria. 1. The *Enterobacteriaceae*, *Journal of the Fisheries Research Board of Canada*, **6**: 194-201.

Yohe, S., Fishbain, J.T., Andrew, M. (1997). *Shewanella putrefaciens* abscess of the lower extremity. *Journal of Clinical Microbiology*, **35**: 33-36.