AN ABSTRACT OF THE THESIS OF

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Haejung An

Bacterial histamine formation in mackerel and albacore was studied by inducing histamine in the muscles under controlled storage conditions. The optimum temperature for histamine formation was 25°C. The highest level of histamine detected was 283 mg/100 g in the 2-day stored mackerel; and 67.1 mg/100 g in the 6-day stored albacore. To identify the bacteria crucial to histamine formation, histamine formers were isolated using the conventional culture method. Enteric bacteria were most frequently isolated from the fish. Weak histamine formers were found in the gill and skin of fresh fish, and they required the enrichment step. Prolific histamine formers were mostly isolated from the decomposed muscles during storage at 25°C. *Morganella morganii* was the most prolific histamine former, producing >3,000 ppm in culture broth. *M. morganii* was the most prevalent histamine former in mackerel. In albacore, however, the most prevalent species was *Hafnia alvei*, a weak histamine former, resulting in less histamine accumulation than mackerel. Weak histamine formers, identified as natural bacteria in the marine environment, were found in mackerel during storage at 4°C after fish became

unsuitable for human consumption. At 0°C, neither histamine-forming bacteria nor histamine was detected in fish.

M. morganii formed significant amounts of histamine (>200 mg/100 g) in artificially contaminated fresh and frozen mackerel, albacore, and mahi-mahi when the fish were improperly stored at ambient temperatures (25°C). Growth of *M. morganii* was controlled by storage of fish at 4°C or below, but histamine formation was controlled only during frozen storage. For rapid detection of *M. morganii*, a PCR assay was developed by designing 16S rDNA targeted primers. Unique primers found for *M. morganii* were: the forward primer, 5'-CTCGCACCATCAGATGAACCCATAT-3'; and the reverse primer, 5'-CAAAGCATCTCTGCTAAGTTCTCTGGATG-3'. Nine CFU/ml of *M. morganii* inoculated in albacore homogenate were detected with a 6 h-enrichment of samples in TSB at 37°C.

It would be necessary to monitor the presence of *M. morganii* in fish during handling and storage due to its high histamine-producing capability and prevent its contamination and proliferation after capture. The PCR assay developed in this study would be helpful to routinely monitor its presence in fish.

Identification of Bacteria Crucial to Histamine Formation and Monitoring their Occurrence and Histamine Accumulation in Scombroid Fish

by

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I understand that my thesis will be become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Shin-Hee Kim, Author

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CONTRIBUTION OF AUTHORS

Dr. Haejung An was involved in the design, analysis, and writing of each manuscript. Dr. Katharine G. Field, Dr. Michael T. Morrissey, Dr. Robert J. Price, Dr. Cheng-i Wei, and Dr. Dong-Suck Chang assisted in writing manuscripts. Dr. Thomas P. Pitta assisted in performing the PCR assay.

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Chapter 1

Introduction and Literature Review

Histamine: the main causative agent of scombroid poisoning

In December, 1995, FDA issued seafood regulations based on the principles of Hazard Analysis and Critical Control Point (HACCP) (FDA, 1998). The aim of this regulation is to produce wholesome products and to prevent hazards that could cause foodborne illnesses (FDA, 2000). A HACCP system is designed to identify hazards, establish critical control points and critical limits, monitor procedures, and keep record for verification of procedures. It is involved in collecting fish samples to monitor their quality and inspecting fish processing facilities. Since December, 1997, HACCP program has been implemented by about 3,600 seafood processors, most of which are small businesses (FDA, 2001). All seafood processors are required to conduct a hazard analysis of each product and control each identified hazard under a HACCP plan developed. The important hazards identified in seafoods are pathogens, parasites, ciguatera, histamine, environmental chemicals, aquaculture drugs, unapproved food additives, and physical hazards (FDA, 2000). Among them, histamine is one of the major causes of seafood-related illnesses in the U.S. (FDA, 1998; CDC, 2000). The industry is having some difficulty in meeting time and emperature controls outlined in the Hazards Guide.

Histamine regulation

Monitoring histamine at each handling and processing step in accordance to HACCP regulation is a critical task in fishery industry. Histamine is a chemical hazard and the main causative agent of scombroid poisoning, a food borne chemical intoxication (FDA, 1998). Histamine is extremely stable; thus it cannot be easily removed or destroyed by cooking, smoking, canning, retorting, curing, or freezing (Bremer, 1998). Common sensory examination by consumers cannot ensure the absence or presence of the toxin, since histamine does not have odor or color (López-Sabater et al., 1996a). Chemical testing is the only reliable test for detection of histamine in products, but it is expensive and may not be available to most seafood processors (Price and Melvin, 1994). Histamine is generally not evenly distributed in a fish, and differences across sections can be as high as ten-fold (Baranowski et al., 1990). Therefore, the FDA has established 5 mg/100 g, 10 fold lower than the previous action level, as a new guideline for histamine in fish species susceptible to histamine formation (FDA, 1998). Any fish containing histamine above this level must be discarded and cannot be used as human food.

Outbreaks of scombroid poisoning

Scombroid, ciguatera, and paralytic shellfish poisoning were responsible for 62.5% of all seafoods related incidents in 1988-1997 in the U.S. (CDC, 1996; 2000a). Scombroid poisoning was reported in 145 outbreaks involving 811 cases (Table 1.1). It showed an wider geographic occurrence than any other seafood poisoning, with incidents reported from 45 states. Fish has been implicated in most cases of scombroid poisoning, and the outbreaks are specifically associated with the consumption of certain fish species,

such as, tuna and mahi-mahi. In 1988, tuna was implicated in 11 out of the 16 scombrotoxin outbreaks (CDC, 1996). An incident caused by the consumption of mahi-mahi resulted in 148 cases of scombroid poisoning in 1990. Tuna was implicated in 9 of the scombroid poisoning outbreaks in 1992. Tuna imported from Ecuador caused 74 cases of the incidents in 20 different states. In general, fish served in restaurants were involved in most outbreaks of scombroid poisoning, mainly resulting from improper holding temperature during distribution and storage of fish.

Table 1.1. Numbers of scombroid poisoning reported in the U.S., 1988-1997 (CDC, 1996; 2000a)

Year	Outbreaks	Cases	Deaths
1988	16	65	1
1989	17	80	0
1990	11	194	0
1991	17	40	0
1992	15	135	0
1993	5	21	0
1994	21	83	0
1995	16	91	0
1996	12	37	0
1997	15	65	0

During the surveillance period 1998-1999, 427 food-borne disease outbreaks involving 7,987 patients were reported in the surveillance area (CDC, 2000b). Of reported outbreaks, 297 (70%) had no identified etiologic organisms; 48 (11%) were due to *Salmonella*; 33 (8%) to caliciviruses; 9 (2%) to *Shigella*; 6 (1%) to scombroid poisoning; 5 (1%) to *Clostridium perfringens*; 4 (1%) to *Escherichia coli* O157:H7; and

25 to other etiologies. Although the incident of scombroid poisoning is often underreported due to mild and transient symptoms, it has been consistently reported through the surveillance. The FDA has intensified its inspection efforts to focus particularly on controlling histamine and pathogens after two years (1998-1999) of inspection implementation of HACCP in the seafood industry (FDA, 2000).

Histamine toxicity

Scombroid poisoning is a typically mild illness with a variety of symptoms involving the skin, gastrointestinal tract, and nervous system (Stratton and Taylor, 1991). Initial symptoms may include a tingling or burning sensation in the mouth, a rash on the upper body, and a drop in the blood pressure (Russell and Maretic, 1986). The most frequently encountered symptoms are rash, nausea, diarrhea, flushing, sweating, and headache. Symptoms can also be similar to those of coronary heart disease, increasing the possibility of an invasive medical intervention if misdiagnosed (Becker et al., 2001).

The severity of the symptoms vary considerably with the amount of histamine consumed and individual sensitivity to ingested histamine (FDA, 1998). However, induction period and duration of the illness are typically short. The onset of intoxication symptoms is rapid, ranging from immediate to 30 minutes. The duration of the illness is usually three hours but may last several days. The toxic dose threshold for histamine in foods is not precisely known because of varying human sensitivity. Although symptoms are self-limiting in most persons, scombroid poisoning can be life-threatening in persons with conditions such as asthma or heart conditions. Antihistamine medication such as diphenhydramine and cimetidine often can relieve symptoms; however, severe cases of

toxicity require the same aggressive management as acute anaphylaxis (Becker et al., 2001).

In many toxic studies, histamine is not the sole compound responsible for scombroid poisoning, because it does not trigger the toxic responses typical for scombroid poisoning when given orally (Taylor and Lieber, 1979; Rodríguez-Jerez et al., 1994). Scombroid poisoning is caused by histamine acting synergistically with other diamines present in fish, primarily putrescine and cadaverine. Its toxicity is increased in the presence of these amines by inhibiting the histamine detoxifying enzymes, diamine oxidase and histamine N-methyltransferase (Stratton and Taylor, 1991). Other biogenic amines, such as tyramine and phenylethylamine may act as a potentiator by inhibiting diamine oxidase (Rawles et al., 1996).

The histamine contents detected in the muscles of fish implicated in scombroid poisoning were: 168 mg/100 g of sailfish muscle (Hwang et al., 1995); 257-430 mg/100 g of amberjack (CDC, 1986); 919 mg/100 g of tuna sashimi (Russell and Maretic, 1986); 93.5 and 276 mg/100 g of marlin (Morrow et al., 1991); 50-160 mg/100 g of mahi-mahi (CDC, 1989); 728 mg/100 g of yellow-fin tuna (CDC, 1989); and 116 mg/100 g of canned tuna (Kim and Bjeldanes, 1979). Thus, based on the cases of scombroid poisoning, a toxicological level of histamine resulted in a health hazard is identified at levels higher than 50 mg/100 g of fish muscle (FDA, 1998). It is also observed that a histamine level, 20 mg/100 g, can be sufficient to cause symptoms of scombroid poisoning (CDC, 1988).

Fish species susceptible to histamine formation

Most natural toxins such as ciguatera toxin and saxitoxin are present in the fish or shellfish at the time of capture (FDA, 1998). However, histamine is formed postmortem in fish muscles with the proliferation of bacteria synthesizing histidine decarboxylase. This enzyme is responsible to convert free histidine in the muscle to histamine (Price and Melvin, 1994). Fish rich in free histidine are susceptible to histamine formation. Free histidine contents in fish muscles vary considerably due to the differences in feeding, season, sex, and stage of maturity (Antonine et al., 1999). In general, free histidine contents reported in these fish species ranges from 200 mg/100 g to 1,000 mg/100 g (Chen et al., 1989).

Fish species associated with scombroid poisoning

Scombroid fish are commonly implicated in incidents of scombroid poisoning (CDC, 2000a). Scombroid fish include various species of tuna (yellowfin, big-eye, bluefin, skipjack, and albacore), mackerel, bonito, and saury. Tuna (yellowfin and skipjack) and mackerel are frequently involved in scombroid poisoning, due to their widedistribution and consumption throughout the world (Stratton and Taylor, 1991). Non-scombroid fish such as mahi-mahi, bluefish, herring, sardine, and anchovy have also been reported to contain high levels of histamine (Rawles et al., 1996). In the U.S., mahi-mahi, bluefish, and tuna are the most commonly implicated fish species in scombroid poisoning (CDC, 1989). Pink salmon, redfish, yellowtail, marlin, and amberjack have also been involved in scombroid poisoning. Outside the U.S., pilchards, herring, anchovies, bluefish, and sardines have been involved in a number of outbreaks (Stratton

and Taylor, 1991). Mackerel, sardines, and pilchards are the major sources of scombroid poisoning in the U.K. Japan had an outbreak associated with the consumption of black marlin and anchovies. Fresh and hot-smoked kahawai (*Arripis trutta*) have frequently been involved in scombroid poisoning in New Zealand (Bremer, 1998).

Mishandling of fish resulting in histamine formation

Fresh fish immediately after capture contain negligible amounts of histamine. Histamine formation mostly results from time and temperature abuse of fish during handling, processing, and distribution (FDA, 1998). Fish held at 32°C can become toxic within 6 hr, and fish held at 21°C can become toxic within 24 hr (Price and Melvin, 1994). In most incidents of scombroid poisoning, the period between the fish harvest and chilling to 0°C was prolonged (>12 hr), or the temperature of fish muscles never reached below 20°C (Russell and Maretic, 1986).

One common source of mishandling fish is in the long line fishery. Yellowfin tuna, caught by long line in a commercial fishing boat, was retained on lines up to 20 hr in the water temperature of 25.8°C in the Gulf of Mexico. Although the tuna was shipped in iced vats from the fishing boat to a processor, the fish caused scombroid poisoning in Pennsylvania in 1998 (CDC, 2000b). Fish served in a restaurant have been frequently implicated in outbreaks of scombroid poisoning due to mishandling (CDC, 2000a). Tuna burgers, a relatively new item, resulted in increasing scombroid poisoning incidents in North Carolina (Becker et al., 2001). A total of 22 incidents of scombroid poisoning were reported between July 1998 and February 1999. Tuna implicated in the outbreaks contained 213 to 3,245 ppm histamine, resulting from the repeated freezing and

thawing of fish during storage. Fish imported to the U.S. from tropical areas, particularly mahi-mahi, have been implicated as a common source of scombroid poisoning (FDA, 1995). It is mainly due to the high ambient water and air temperatures in the originated area, mishandling conditions on boats, and market sanitary conditions (Ahmed, 1991).

Although the FDA has regulated seafood for decades, its jurisdiction is limited to international and interstate products (Becker et al., 2001). Local wholesale seafood businesses are subjected to state government inspection, which includes implementation of the HACCP principles. To reduce the risk of scombroid poisoning, it is required to enforce an inspection system to improve food safety in recreational and commercial fishing vessels and restaurants. The time from hooking the fish to unloading the fish on the dock is not covered by HACCP (CDC, 1998). Some investigators recommend that regulations be developed and enforced concerning how long a fish can remain on a fishing line.

Histamine accumulation in fish under controlled storage conditions

To ensure product safety, histamine production in various fish species was monitored under controlled storage conditions. Histamine formation is accelerated when fish are exposed to ambient temperature (20-25°C). During storage at refrigeration temperature, histamine is generally found when fish is unsuitable for human consumption by extended storage (Kim et al., 2001b). Histamine formation is effectively controlled at 0°C (Kim et al., 2001b, Price et al., 1991). Although temperature is a critical factor for histamine formation in fish, the levels of histamine detected are variable depending on

intrinsic factors of fish, such as, species, size, and free histidine concentration as described below.

Handling fresh mackerel is a problem due to its soft flesh, high lipid content, and delicate skin (Jhaveri et al., 1982). Average size of mackerel ranges from 400 to 500 g. Improper storage of mackerel resulted in more substantial deterioration of quality in a short period of time than larger size of fish, such as albacore and bluefin tuna (Kim et al., 2001b). Several species, i.e., chub, Spanish, and Atlantic mackerels, are implicated in the incidents of scombroid poisoning. The high levels of histamine, i.e., 270 mg/100 g (Clifford et al., 1989) and 500 mg/100 g (Russell and Maretic, 1986), were found in fish during storage at ambient temperature. However, the histamine formation was negligible in mackerel stored on ice. Histamine contents reported were 2 mg/100 g in 6 days of storage (Bennour et al., 1991); and 2 mg/100 g in 10 days (Jhaveri et al., 1982). Sardine is also a small pelagic and oily fish and characterized by its high proteolytic activity (Pacheco-Aguilar et al., 2000). In general, the quality changes progress rapidly by temperature abuse of fish immediately after catch. However, the amount of histamine detected in fish muscles at the time of rejection was 11.7 mg/100 g in 12 days of storage.

Mahi-mahi (*Coryphaena hippurus*) is mainly harvested off the Hawaii Coast in the U.S.; thus, the supply of locally-caught mahi-mahi is limited and seasonal due to the high demand for this species (Baranowski et al., 1990). Frozen fillets are imported from Latin America, Taiwan, and Japan. In the U.S., the highest risk are imported fresh and frozen fish from tropical areas (Ahmed, 1991). In general, fresh mahi-mahi has a shelf life of 10 days (FDA, 1998). When quality changes and histamine formation were studied in fresh mahi-mahi, the histamine content increased rapidly and reached 292

mg/100 g in 24 h at 32°C (Baranowski et al., 1990). At 0°C, decomposition was slow and moderate. The histamine level detected was 12 mg/100 g after 3 weeks of storage. When previously frozen fish was thawed and restored at 32°C, histamine level found was greatly reduced in fish muscles, presumably by microbial destruction.

Albacore (*Thunnus alalunga*) is a highly migratory species (Pérez-Villarreal and Pozo, 1990). In the Pacific, they school off the West Coast and in the waters of Hawaii. The majority of the fish are harvested off the Northwest Coast. Albacore are marketed at sizes between 5 and 12 kg, and the size is smaller than other tuna species (Craven et al., 1995). It has a longer shelf life than other scombroid fish species and has been involved in only a few incidents of scombroid poisoning despite considerable consumption of the fish (Pérez-Villarreal and Pozo, 1990; Stratton and Taylor, 1991). Optimum temperature for histamine formation in albacore is 25°C. At this temperature, the highest histamine level, 60.4 mg/100 g was found in whole fish stored for 7 days (Kim et al., 1999). Whole fish were more susceptible to histamine formation than dressed fish under those storage conditions. When previously frozen albacore were placed at 25°C, reduced amount of histamine was found at 7.14 mg/100 g in 7 days of storage. No histamine was found in fish stored in ice for 18 days. Another tuna species, bluefin tuna (*Thunnus thynnus*), are distributed in temperate and subtropical waters, and the average weight of fish is 18 kg. Histamine formation is less susceptible during storage in ice (López-Sabater et al., 1996a). Histamine was not detected until day 12 in the muscles stored at 0°C, as observed in albacore. Histamine content began to increase rapidly in fish during storage at 20°C. In 48 h of storage, much higher level of histamine, 400 mg/100 g, was detected in bluefin than albacore.

Fish handling to prevent histamine formation

Acceptable commercial fish usually contain less than 5 ppm histamine and rarely over 20 ppm histamine (FDA, 1995). The average histamine levels in commercial raw frozen fish found are 2 ppm for mahi-mahi; 4 ppm for albacore tuna; 2 ppm for yellowfin tuna; and 2 ppm for skipjack tuna. Fishermen usually catch tuna 20 to 200 miles offshore near the surface of the ocean, and it often takes 7-14 days to transfer fish through the distribution process to the customer (Price and Melvin, 1994). Thus, trips lasting over five days result in inferior fish reaching the consumer.

Recommendations for on-board handling

Handling on fishing vessel is a critical control point for the production of a safe and wholesome product particularly in fisheries for tuna and other fish species susceptible to histamine formation (Price and Melvin, 1994). Currently, FDA recommends that primary processors examine harvest vessel records that show chilling began as soon as possible after the fish were landed on the harvest vessel; fish were chilled to an internal temperature of 10°C or less within 6 hours of death; chilling fish to an internal temperature of 4.4°C or less within an additional 18 hours; and if unfrozen fish, the fish were maintained at 4.4°C or below (FDA, 1998). If these criteria are not met or these records are absent, the lot should be subjected to histamine analysis for at least sixty fish from a lot.

Albacore and other tuna species can be even more susceptible to histamine formation due to their high body temperature and large body size (Craven et al., 1995). Their body temperatures are 26-30°C, which are 12 to 14°C warmer than the ambient

temperature. The time required to lower the internal temperature of fish after capture can be dependent upon a number of factors, such as, the harvest method, the size of fish, and the chilling method (FDA, 1998). Several chilling methods (immediate icing, delayed icing, and chilling in refrigerated seawater before icing) have been used in the commercial albacore fishery industry (Jacoby, 1987). To ensure safety and quality of fish, handling techniques such as stunning, spiking, and bleeding of fish immediately after landing are recommended (Price and Melvin, 1994).

Temperature control during storage and distribution

Once chilled rapidly, the fish should be maintained as close as possible to the freezing point or frozen until it is consumed. The amount of ice required to chill fresh fish varies with several factors, i.e., the length of the trip, catch rate, the extend of insulation in the fish hold and the slush ice tank (Price et al., 1991). In general, proper chilling will require about two pounds of ice per pound of fish to ensure freshness of fish. The shelf life of tuna for high quality is 11 to 14 days in ice. Special handling is required to deliver fresh, unfrozen fish. The internal temperature of fish muscles should be maintained in a proper chilling system and measured by periodically throughout the fishing trips (Price and Melvin, 1994).

Freezing, such as, air blast freezing, brine immersion/dry storage freezing, and spray brine freezing, is the most commonly used method to prevent histamine formation in tuna industry (Jacoby, 1987). Freezer system must maintain a constant temperature at -30°C or below. Fish destined to be canned are frequently preserved by frozen storage prior to delivery to the canneries (Price et al., 1991). These fish are thawed before

processing and are subjected to additional handling. It may result in higher histamine content in canned fish than those observed in raw, freshly caught fish. Once the histidine decarboxylase is formed, it can continue to produce histamine in the muscles (Baranowski et al., 1985). The enzyme is likely to be more stable than the bacteria in the frozen state and may be reactivated very rapidly in the muscles after thawing. Due to its heat resistance, histamine can remain in sterilized cans or other products (López-Sabater et al., 1994). Therefore, it has been recommended that temperature in fish muscles be consistently controlled from the time they are caught until they are processed (CDC, 2000c).

Bacteria contributing to histamine formation in fish

Many different species of histamine-producing bacteria have been isolated from a variety of food sources. Frequently isolated species are distinguished by their isolation source. *Lactobacillus* spp., *Streptococcus* spp., *Bacillus* spp., and *Clostridium* spp. have been intensively isolated from cheese, fermented sausage, and wine but rarely from fish (Stratton et al., 1991). Histamine formers isolated from fish and fishery product are several species of enteric bacteria and natural bacteria in the marine environment.

Types and levels of histamine-producing bacteria

Histamine production typically follows by bacterial growth and histidine decarboxylase synthesis (Price and Melvin, 1994). Most frequently isolated histamine-producing bacteria in fish are mesophilic enteric bacteria; thus temperature is the most important factor to bacterial histamine formation among many environmental parameters.

Many different species of histamine formers have been isolated from temperature-abused fish under controlled storage conditions (Table 1.2). Among them, the species isolated from fish incriminated in scombroid poisoning are *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* (Rawles et al., 1996).

Histamine formers are divided by their histamine-producing capability according to the criteria described by Behling and Taylor (1982). Only a few of them were identified as prolific histamine formers. *Morganella morganii*, *Klebsiella pneumoniae*, and *Proteus vulgaris* are prolific histamine formers, producing >1,000 ppm histamine in the culture broth (López-Sabater et al., 1996a; Kim et al., 2001b). *Hafnia alvei*, *Citrobacter freundii*, *Enterobacter* spp., and *Serratia* spp. are weak histamine formers (<1,000 ppm). Other types of histamine formers isolated from fish are psychrotrophic and/or psychrophilic bacteria. Natural bacteria in the marine environment, i.e., *Photobacterium* spp., *Pseudomonas* spp., *Vibrio alginolyticus*, and *Aeromonas* spp., are isolated from fresh fish as well as abused fish at refrigerated temperatures (Kim et al., 2001b; Middlebrook et al., 1988; Morii et al., 1988). In general, the identified bacteria are weak histamine formers, producing <500 ppm histamine in culture broth (Frank et al., 1985). It has also been reported that not all the isolates of natural bacteria can produce histamine in culture broth (Ryser et al., 1984; Frank et al., 1985). Although *Pseudomonas fluorescens/putida* and *Pseudomonas putrefaciens* isolated from Spanish mackerel were detected as weak histamine formers (Middlebrooks et al., 1988), the strains isolated from ripened Spanish semi-preserved anchovies did not produce histamine (Rodríguez-Jerez et al., 1994).

Table 1.2. Histamine-producing bacteria identified from fish during storage

Histamine former	Fish species isolated
Morganella morganii	Bluefin (1), mackerel (2), sardine (3), skipjack (4), mahi-mahi (5), anchovy (6), mackerel (14)
Klebsiella pneumoniae	Bluefin (1), skipjack (4), anchovy (6), bonito (7), skipjack (8), albacore (13)
Klebsiella oxytoca	Bluefin (1), anchovy (6), albacore (13)
Hafnia alvei	Skipjack (4), bluefin (9), mackerel (12, 13), albacore (14)
Proteus vulgaris	Bluefin (1), mackerel (2, 14), sardine (3)
Proteus mirabilis	Bluefin (1), sardine (3), mahi-mahi (5), anchovy (6), skipjack (8), mackerel (12)
Citrobacter freundii	Bluefin (1), mackerel (2), albacore (13)
Enterobacter aerogenes	Bluefin (1), anchovy (6), bonito (7), skipjack (8), mackerel (12, 14), albacore (13)
Enterobacter cloacae	Bluefin (1), Anchovy (6), bonito (7), albacore (13)
Serratia fonticola	Bluefin (9), mackerel (10), albacore (13)
Serratia liquefaciens	Bluefin (1), anchovy (6), albacore (13)
Escherichia coli	Bluefin (1)
Pseudomonas spp.	Bluefin (11), bonito (10), mackerel (12, 14), albacore (13)
Clostridium perfringens	Skipjack (8), mackerel (12)
Photobacterium spp.	Mackerel (11), mackerel (14)
Vibrio alginolyticus	Sardine (3), mahi-mahi (5), skipjack (9), mackerel (14)
Plesiomonas shigelloides	Bluefin (1)
Acinetobacter lowffi	Bluefin (1), mackerel (12), albacore (13)
Aeromonas spp.	Mackerel (2), bluefin (10), mackerel (14)

1. López-Sabater et al., 1996; 2. Okuzumi et al., 1984; 3. Ababouch et al., 1991; 4. Omura et al., 1978; 5. Frank et al., 1985; 6. Rodriguez et al., 1994; 7. López-Sabater et al., 1994; 8. Yoshinaga and Frank, 1982; 9. López-Sabater et al., 1996; 10. Ryser et al., 1984; 11. Morii et al., 1988; 12. Middlebrooks et al., 1988; 13. Kim et al., 2001a; 14. Kim et al., 2001b.

Histidine decarboxylase

Two classes of histidine decarboxylase have been known: those that contain pyridoxal-5'-phosphate as the essential coenzyme (PLP-dependent), and those that require bound pyruvoyl residue as a coenzyme (Pvr-dependent) (van Poelje and Snell, 1990). The Pvr-dependent enzymes are found in gram-positive bacteria, whereas the PLP-dependent enzymes are more widespread in gram-negative bacteria and eukaryotes. The PLP-dependent enzyme has been isolated from *Morganella morganii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Vibrio anguillarum* (Barancin et al., 1998). The Pvr-dependent group includes *Lactobacillus* 30a, *Clostridium perfringens*, *Lactobacillus buchneri*, and *Micrococcus* sp. (van Poelje and Snell, 1990).

The characterization of PLP-dependent enzymes differs from that of Pvr-dependent enzymes. The amino acid sequences of the PLP-dependent enzymes show no homology with those of the Pvr-dependent enzymes (van Poelje and Snell, 1990). This is consistent with the dissimilar molecular structure, prosthetic groups, and substrate specificity (Kamath et al., 1991). Histamine is a competitive inhibitor of the Pvr-dependent enzymes but not of the PLP-dependent enzymes, thus making it possible to accumulate histamine in the cells and the muscle without inhibiting histidine decarboxylase activity (Vaaler et al., 1986).

Bacteria possessing PLP-dependent enzyme play an important role in histamine formation in fish muscle, since more gram-negative bacteria are isolated from fish than gram-positive bacteria. The PLP-dependent enzyme isolated from *M. morganii* has an identical subunit of *Mr* 43,000. The gene contains 1,131 nucleotides and encodes for 377 amino acids (Tanase et al., 1985). The PLP-dependent enzymes from *Klebsiella*

planticola and *Enterobacter aerogenes* have the same length of amino acid residues, 377, as *M. morganii* (Kamath et al., 1991). Three PLP-dependent enzymes identified show high sequence identity in both nucleic acid (75%) and amino acid (80%) homology. Therefore, it has been speculated that there has been no addition or deletion of nucleotides during evolution from the parental gene of three PLP-dependent enzymes. The amino acid sequence of *Vibrio anguillarum* has extensive homology with those of three bacteria (Tolmasky, 1995). The *V. anguillarum* gene sequence has 65.1%, 63.5%, and 61.4% identities with those of *K. planticola*, *M. morganii*, and *E. aerogenes*, respectively. The *hdc* has been found in the plasmid pJM1, which is an essential component for the biosynthesis of a sidrophore, anguibactin.

Origin of histamine formers

It has been reported that histamine formers are present in the salt-water environment (FDA, 1998). The bacteria are initially found in the skin, intestine, and gill of fresh fish, but rarely in the muscle (Kim et al., 2001b). Mishandling and storage of fish allow the bacteria to spread into the muscles and proliferate. In addition, bacterial contamination occurs during handling of fish on board, at the processing plants, in the distribution system, and at the level of consumer (Taylor, 1986). However, it is not clear that prolific histamine formers have been originated from the marine environment or mainly contaminated during handling, distribution, and processing. Prolific histamine formers have frequently been isolated from spoiled fish by temperature abuse in many studies.

Many researchers have suspected the presence of histamine formers in the intestine and gill as a part of bacterial flora in fresh fish. Histamine was usually detected in fish at higher levels in tissues adjacent to the gills or intestines (Taylor and Speckhard, 1983; Stratton and Taylor, 1991; López-Sabater et al., 1996a; Kim et al., 2001a). Histamine content of ungutted mackerel was 10-times more than that of gutted fish after storage at ambient temperature (Lehane and Olley, 2000). Tuna burgers and salads prepared from the belly meat were extensively implicated in the recent outbreaks of scombroid poisoning in North Carolina (Becker et al., 2001). When histamine formers were isolated from fresh mackerel, they were minor constituents of the flora and could be isolated only with the enrichment (Kim et al., 2001b). However, prolific histamine former, *Morganella morganii*, was isolated from fish abused at ambient temperature (25°C) in 1 day of storage. Thus, it has been suspected that the initial population of prolific histamine-producing bacteria in fresh fish may be too low to be isolated by the culture method ($<10^2$ CFU/g).

Changes of bacterial flora during storage of fish

The initial bacterial flora in fish reflects the marine environment of fish harvested, such as seasonal variation and geographical location (Gram et al., 1987; Kvenberg, 1991). However, dominant bacterial flora in fish changes depending on handling and storage conditions (Kim et al., 2001b). Histamine-producing bacterial species vary considerably in their optimal temperature and lower temperature limit for their growth and histamine formation. Exposure of fish to ambient temperature favors to growth of mesophilic histamine formers, even if they are minor bacterial species in fresh fish. On

the contrary, the spoilage patterns of fish during iced storage are usually similar, regardless of the differences in the initial microflora (Gennari et al., 1999). Enteric bacteria constitute a minor bacterial flora in fish during storage at 0°C (Gennari et al., 1999; Gram et al., 1987). Prevalent species during storage of fish under the condition are *Pseudomonas* spp., *Altermonas putrefaciens*, *Flavobacterium* spp., *Shewanella* spp., and *Acinetobacter* spp. These bacteria are rarely confirmed as histamine formers. It reflects that growth of prolific histamine formers and histamine formation in fish are effectively controlled under those storage conditions (Kim et al., 2001b).

The main contributor to histamine formation

Among prolific histamine formers identified, all *Morganella morganii* isolates have consistently shown to form high levels of histamine in culture broth (>2,000 ppm histamine) (Kim et al., 2001b). Other species such as *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Proteus vulgaris* varied greatly in their ability to produce histamine. Only a few isolates are identified as prolific histamine formers (Kim et al., 2001a; Lehane and Olley, 2000). Thus, the prevalence of *M. morganii* may lead to a significant level of histamine accumulation in fish muscles during storage.

M. morganii shows the typical characteristics of mesophilic bacteria in many studies. Generally, the temperature range of 25-37°C is optimal for histamine production in culture broth with this bacterium (Chen et al., 1989; Kim et al., 2000). During storage at 25°C, the species was also produced other biogenic amines such as cadaverine, putrescine, and phenyletylamine, which can potentiate histamine toxicity (Kim et al., 2000). The minimum temperature to produce toxicological levels of histamine in culture broth is 15°C (Klausen and Huss, 1987; Kim et al., 2000). Bacterial growth as well as

histamine formation were inhibited by storage below 4°C (Klausen and Huss, 1987). Coincidently, *M. morganii* has been isolated during storage at >25°C of various fish species, i.e., skipjack tuna (Omura et al., 1978), tuna (*Thunnus thynnus*) (López-Sabater et al., 1996b), albacore (Kim et al., 2000), mahi-mahi (Frank et al., 1985), mackerel (Kim et al., 2001b), sardine (Ababouch et al., 1991), and Spanish salted semi-preserved anchovies (Rodríguez-Jerez et al., 1994).

The isolation rates of *M. morganii* vary greatly depending on the fish species tested during storage. A total of 7 strains of *M. morganii* were isolated from sardine stored at ambient temperature (25°C) (Ababouch et al., 1991). The most frequently isolated histamine-producing bacteria were *Proteus* spp. Twenty-three strains out of 28 *Proteus* strains isolated were prolific histamine formers. Only 9% of histamine formers were *M. morganii* in mahi-mahi stored at 32°C (Frank et al., 1985). Eight strains of *M. morganii* and one strain of *P. mirabilis* were identified as prolific histamine producers (>1,000 ppm). *Morganella morganii* was isolated from bluefin tuna stored at 20°C along with other enteric bacteria, i.e., *Klebsiella oxytoca*, *Citrobacter freundii*, and *Enterobacter agglomerance* (López-Sabater et al., 1996a). *K. oxytoca* was also isolated as prolific histamine former, producing >2,000 ppm histamine in culture broth. In Pacific mackerel, *M. morganii* was the most prevalent histamine former and frequently isolated with the progression of fish spoilage during storage at 25°C (Kim et al., 2001b). Ten strains were isolated on day 2 and seventeen strains on day 3. *M. morganii* was identified as the main contributor to histamine accumulation in fish left at ambient temperature.

Histamine formers during storage of fish at refrigeration temperature

In general, with fish stored at 2-8°C, a hazardous level of histamine (>50 mg/100 g) in fish muscle is only found after fish become unsuitable for human consumption (Kim et al., 2001b). At these temperatures, histamine formation is assumed to be formed due to the growth of psychrotrophic or psychrophilic bacteria for extended storage periods (Bennour et al., 1991; El Marrakchi et al., 1990; Ryser et al., 1984).

Psychrotrophic enteric bacteria such as *Hafnia alvei*, *Serratia liquefaciens*, *Cedecae lapagei*, *Enterobacter intermedium*, and *E. cloacae* have been isolated from bluefin tuna during storage at 8°C (López-Sabater et al., 1996). A few isolates of *E. cloacae* and *H. alvei* produced 1,000 ppm histamine in culture broth. The remaining isolates were weak histamine formers (<500 ppm). *Photobacterium phosphoreum* (N-group bacteria), a natural bacterium in the marine environment, is frequently isolated from mackerel stored in ice (Okuzumi et al., 1984; Morii et al., 1988). The researchers reported that the bacterium may be primarily responsible for histamine production in fish muscles during storage at refrigeration temperature. This species produces 898 ppm histamine in Moller's basal medium with 1% histidine in ice for 16 days of storage (Morii et al., 1988). Other species reported are weak histamine formers. *Vibrio alginolyticus* constituted about 90% of histamine formers in mahi-mahi during storage in ice, but most isolates do not produce detectable amounts of histamine in culture broth (Frank et al., 1985). A few isolates are weak histamine formers (<100 ppm). *Plesiomonas shigelloides*, frequently isolated from the marine environment, produces 8 to 340 ppm histamine in culture broth (López-Sabater et al., 1996a). Thus, their role in the overall

histamine accumulation in fish would be insignificant compared to mesophilic prolific histamine formers.

Intrinsic factors of fish affecting bacterial histamine formation

In general, histamine production coincides with the microbial growth when fish are exposed to elevated temperatures (>15°C). However, histamine accumulation in fish is affected not only by the extrinsic parameters, such as, handling and storage temperatures, but also by the intrinsic factors, such as, pH, salt, and histidine concentrations of fish muscles.

Slightly acidic pH ranges (pH 5.5-6.5) close to the physiological pH of fish muscle enhance the production of histamine by histamine-forming bacteria (Eitenmiller et al., 1981). Maximum enzyme activity is found at pH levels that tend to inhibit bacterial growth. The optimal pH for enzyme induction in most bacterial species is between 5.0 and 5.5 (Chen et al., 1989). *Morganella morganii* showed the maximum histidine decarboxylase activity at pH 5, although the bacterial growth was limited (Eitenmiller et al., 1981; Ababouch et al., 1991). The optimum pH for histamine formation by *Photobacterium phosphoreum* was between 5 and 6, but the histidine decarboxylase activity of the bacterium was limited at pH 8.5 (Okuzumi et al., 1984).

When *M. morganii* was incubated at different NaCl concentrations, the optimum concentration for bacterial growth was 1%; and 2 to 3% for histamine production (Arnold and Brown, 1978). *M. morganii* A361 produced a high level of histamine even in the presence of 8% NaCl (Ababouch et al., 1991). These indicate that *M. morganii* can form histamine in a variety of seafood products containing a wide concentrations of salt.

Histamine formation by *Klebsiella pneumoniae* T2 in trypticase soy broth-histidine medium was optimum at 0.5% NaCl (Taylor and Woychik, 1982). The optimum concentration of NaCl for histamine formation by *P. phosphorium* was 1 to 3%, corresponding to the optimum concentration for their growth (Okuzumi et al., 1984).

Histidine can act either as a substrate or an inducer of histidine decarboxylase. Free histidine content in fish muscles vary considerably due to the differences in feeding, season, sex, and stage of maturity (Antonine et al., 1999). In general, fish species susceptible to histamine formation contains 200 to 1,000 mg free histidine/100 g (Fletcher et al., 1995; Yoshinaga and Frank, 1982). The minimum histidine concentration for histamine production of *M. morganii* was 100 to 200 mg/100 g of fish homogenate (Arnold and Brown, 1980). The level of histamine produced by *K. pneumoniae* T2 increased as the histidine concentration was increased to 2% in the culture. However, histamine content decreased somewhat at 3% histidine (Taylor and Woychik, 1982). *K. pneumoniae* 111-5 and *M. morganii* 110SC-2 produced higher levels of histamine in culture broth with 2.7% histidine than in the broth with 1% histidine (Chen et al., 1989).

Detection of histamine formers

Detection of histamine formers has relied on the conventional culture methods. Differential media have been used for rapid differentiation of many different species of histamine formers from non-histamine formers in fish. Niven's differential medium was developed for quantitative detection of histamine formers based on the color change of bromocresol purple due to the change in pH, when histidine in the medium is

decarboxylated (Niven et al., 1981). Although the Niven's medium has been widely used (Ababouch et al., 1991; Fletcher et al., 1995), it has a tendency to produce false results, presumably due to the outgrowth of non-histamine formers and the formation of alkaline compound in mixed culture (Rodríguez-Jerez et al., 1994).

The medium has been modified to develop other differential media, such as, modified Niven's, Joosten/Northolt, and Maijara, to enhance detection of histamine-forming lactic acid bacteria in cheese, meat, fish and poultry products (Joosten and Northolt, 1989; Maijara, 1993) and to screen histamine-producing bacteria from ripened sausages (Roig-Sagués et al., 1997). However, detection of histamine formers using the modified media alone is still susceptible to false reactions due to the formation of different alkaline compounds and fermentative activity of bacteria. When a prescreening step on selective media was included prior to screening on differential media for histidine decarboxylase, the isolation rate of true histamine-forming bacteria was improved (Roig-Sagués et al., 1997; Kim et al., 2001a).

Many researchers indicated the limitation of conventional culture method, i.e., lengthy assay time, labor requirement, and low sensitivity, for bacterial identification (Wolcott, 1991). Oligonucleotides have widely been used as hybridization probes or amplification primers for microbial detection (Gendel, 1996). The PCR assay has been widely used for rapid detection of pathogen in food. However, molecular-based techniques have not been widely applied for detection of histamine formers in food. Only universal primers were developed for detection of gram-positive histamine-producing bacteria, such as *Lactobacillus* 30A, *Clostridium perfringens*, *Leuconostoc œnos*, *Lactobacillus buchneri*, and *Micrococcus* spp., in cheese and dairy products (Jeune

et al., 1995). Since alignment of the *hdc* (histidine decarboxylase) gene sequences of these bacteria showed a high similarity, the primers were derived from conserved sequences in the *hdc* gene. Gram-negative histamine formers are the main histamine formers in fish, but the *hdc* gene sequences are known only for *M. morganii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Vibrio anguillarum* (Kamath et al., 1991). The molecular-based techniques or specific primers have rarely been developed for their detection. It is necessary to develop molecular-based techniques for sensitive detection of prolific histamine formers and apply it to monitor their origin and contamination route during fish harvest and handling.

Research objectives

The overall objective of this research is to assist the establishment of appropriate guidelines and the implement of HACCP in the fishery industry for a better control of histamine formation in fish products. Since the main histamine formers have not been intensively investigated in a variety of fish species, much more rigid guidelines have been established for all types of fish regardless of their harvest area and handling conditions. Monitoring the source, distribution, and prevalence of histamine-forming bacteria in fish can help establish more appropriate and effective guidance to prevent bacterial histamine formation during handling and storage of fish. Identification of the most crucial bacteria to histamine formation and development of molecular-based technique for its rapid detection would be beneficial to routinely monitor their presence in fish.

Specific objectives of this study were to:

1) study albacore and mackerel for product safety and identify the main histamine formers under various storage conditions.
2) isolate prolific histamine-producing bacteria from albacore and determine effect of temperature on the formation of histamine and other biogenic amines.
3) evaluate one-step and two-step isolation procedures for histamine formers and elucidate the source of histamine formers in fresh and temperature-abused albacore.
4) study distribution of histamine formers in fresh albacore and monitor prevalent histamine formers and histamine accumulation during storage at ambient temperature.
5) characterize histamine formation and proliferation of *M. morganii* in muscles of various fish species under controlled storage conditions.
6) develop a PCR assay for rapid and sensitive detection of *M. morganii* based on 16S rDNA targeted primers.

References

Ababouch, L., Afilal, M.E., Benabdeljelil, H., and Busta, F.F. 1991. Quantitative changes in bacteria, amino acids and biogenic amines in sardine (*Sardina pilchardus*) stored at ambient temperature (25-28°C) and in ice. Int. J. Food Sci. Technol. 26: 297-306.

Ahmed, F.E (ed.). 1991. Ch. 4. Naturally occurring fish and shellfish poisons. p. 87-110. *In* Seafood Safety. National Academy Press, Washington, D.C.

Antoine, F.R., Wei, C.I., Littell, R.C., and Marshall, M.R. 1999. HPLC method for analysis of free amino acids in fish using *o*-phthaldialdehyde precolumn derivatization. J. Agric. Food Chem. 47: 5100-5107.

Arnold, S.H. and Brown, W. 1978. Histamine (?) toxicity from fish products. Adv. Food Res. 24: 113-154.

Arnold, S.H., R.J. Price, and W.D. Brown. 1980. Histamine formation by bacteria from skipjack tuna, *Katsuwonus pelamis*. Bull. Jpn. Soc. Sci. Fish. 46: 991-995.

Barancin, C.E., Smoot, J.C., Findlay, R.H., and Actis, L.A. 1998. Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen *Vibrio anguillarum*. Plasmid. 39: 235-244.

Baranowski, J.D., Brust, P.A., and Frank, H.A. 1985. Growth of *Klebsiella pneumoniae* UH-2 and properties of its histidine decarboxylase system in resting cells. J. Food Biochem. 9: 349-360.

Baranowski, J.D., Frank, H.A., Brust, P.A., Chongsiriwatana, M., and Premaratne, R.J. 1990. Decomposition and histamine content in mahimahi (*Coryphaena hippurus*). J. Food Prot. 53: 217-222.

Becker, K., Southwick, K., Reardon, J., Berg, R., MacCormack, J.N. 2001. Histamine poisoning associated with eating tuna burgers. JAMA. 285: 1327-1330.

Behling, A.R. and S.L. Taylor. 1982. Bacterial histamine production as a function of temperature and time of incubation. J. Food Sci. 47: 1311-1317.

Bennour, M., El Marrakchi, A., Bouchriti, N., Hamama, A., and El Ouadaa, M. 1991. Chemical and microbiological assessments of mackerel (*Scomber scombrus*) stored in ice. J. Food Prot. 54: 784, 789-792.

Bremer, P.J. Osborne, C.M., Kemp, R.A., van Veghel, P., and Fletcher, G.C. 1998. Thermal death times of *Hafnia alvei* in a model suspension and in artificially contaminated hot-smoked kahawai (*Arripis trutta*). J. Food Prot. 61: 1047-1051.

Centers for Disease Control and Prevention. 1986. Epidemiologic notes and reports restaurant-associated scombroid poisoning fish poisoning-Alabama, Tennessee. MMWR 35: 264-265.

Centers for Disease Control and Prevention. 1989. Epidemiologic notes and reports scombroid fish poisoning-Illinois, South Carolina. MMWR 38: 140-142, 147.

Centers for Disease Control and Prevention. 1996. Surveillance for foodborne-disease outbreaks-United States, 1988-1992. *In* CDC surveillance summaries, MMWR 45: 1-72.

Centers for Disease Control and Prevention. 2000a. Surveillance for foodborne-disease outbreaks-United States, 1993-1997. *In* CDC surveillance summaries, MMWR 49: 1-72.

Centers for Disease Control and Prevention. 2000b. Limitations of field investigations: Etiology and food vehicle not determined for a substantial proportion of Foodborne Outbreaks in Food Net, 1998-1999.

Centers for Disease Control and Prevention. 2000c. Scombroid fish poisoning-Pennsylvania, 1998. MMWR 49: 398-400.

Chen, C.M., Wei, C.I., Koburger, J.A., and Marshall, M.R. 1989. Comparison of four agar media for detection of histamine-producing bacteria in tuna. J. Food Prot. 52: 808-813.

Clifford, M.N., Walker, R., and Wright, J. 1989. Studies with volunteers on the role of histamine in suspected scombrotoxicosis. J. Sci. Food Agric. 47: 365-375.

Craven, C., Hilderbrand, K., Kolbe, E., Sylvia, G., Daeschel, M., Gloria, B., and An, H. 1995. Understanding and controlling histamine formation in troll-caught albacore tuna: A review of preliminary findings from the 1994 season. Oregon Sea Grant (ORESU-T-95-00). Corvallis, OR.

Eitenmiller, R.R., Wallis, J.W., Orr, J.H., and Phillips, R.D. 1981. Production of histidine decarboxylase and histamine by *Proteus morganii*. J. Food Prot. 44: 815-820.

El Marrakchi, A., Bennour, M., Bouchritt, N., Hamama, A., and Tagafait, H. 1990. Sensory, chemical, and microbiological assessments of Moroccan sardines (*Sardina pilchardus*) stored in ice. J. Food Prot. 53: 600-605.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species, particulary kawawai (*Arripis trutta*). J. Aquat. Food Prod. Technol. 4: 533-574.

Food and Drug Administration. 1995. Procedures for the safe and sanitary processing and importing of fish and fishery products. Federal Register 60: 65096-65202.

Food and Drug Administration. 1998. Ch. 7. Scombrotoxin (histamine) formation. *In* Fish and Fishery Products Hazards and Controls Guide. 2nd Ed., p. 73-90. Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Food and Drug Administration. 2000. FDA's evaluation of the seafood HACCP program for 1998/1999. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Food and Drug Administration. 2001. FDA's seafood HACCP program: Mid-course correction. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Frank, H.A., J.D. Branowski, M. Chongsiriwatana, P.A. Brust, and R.J. Premaratne. 1985. Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32°C. Int. J. Food Microbiol. 2: 331-340.

Gendel, S.M. 1996. Computational analysis of the specificity of 16S rRNA-derived signature sequences for identifying food-related microbes. Food Microbiol. 13: 1-15.

Gennari, M., Tomaselli, S., and Cotrona, V. 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) sea and stored in ice. Food Microbiol. 16: 15-28.

Gram, L., Trolle, G., and Huss, H.H. 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4: 65-72.

Hwang, D.F., Chang, S.H., Shiau, C.Y., and Cheng, C.C. 1995. Biogenic amines in the flesh of sailfish (*Istiophorus platypterus*) responsible for scombroid poisoning. J. Food Sci. 60: 926-928.

Jacoby, D.R. 1987. On-board handling and preservation of frozen troll-caught albacore, *Thunnus alalunga.* Mar. Fish. Rev. 49:46-50.

Jhaveri, S.N., Leu, S., and Constantinides, S.M. 1982. Atlantic mackerel (*Scomber scombus*, L): Shelf life in ice. J. Food Sci. 47: 1808-1810.

Joosten, H.M.L.J. and Northolt, M.D. 1989. Detection, growth and amine producing capacity of lactobacilly in cheese. Appl. Environ. Microbiol. 55: 2356-2359.

Kamath, A.V., Vaaler, G.L., and Snell, E.E. 1991. Pyridoxal phosphate-dependent histidine decarboxylase: cloning, sequencing, and expression of genes from *Klebsiella planticola* and *Enterobacter aerogenes* and properties of the overexpressed enzymes. J. Biol. Chem. 266: 9432-9437.

Kim, I.S. and Bjeldanes, L.F. 1979. Amines content of toxic and wholesome canned tuna fish. J. Food Sci. 44: 922-923.

Kim, S.H., An, H., and Price, R.J. 1999. Histamine formation and bacterial spoilage of albacore harvested off the U.S. Northwest coast. J. Food Sci. 64: 340-344.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Kim, S.H., Field, K.G., Morrissey, M.T., Price, R.J., Wei, C.I., and An, H. 2001a. Source and identification of histamine-producing bacteria from temperature-abused albacore. J. Food Prot. 64: 1035-1044.

Kim, S.H., Field, K.G., Chang, D.S., Wei, C.I., and An, H. 2001b. Bacteria crucial to histamine formation in Pacific mackerel during storage. J. Food Prot. (in press).

Klausen, N.K. and H.H. Huss. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

Kvenberg, J.E. (e.d.). 1991. Ch. 9. Nonindigenous bacterial pathogens. p. 267-284. *In* Microbiology of Marine Food Products. AVI, New York, NY.

Lehane, L. and Olley, J. 2000. Histamine fish poisoning revised. Int. J. Food Microbiol. 58: 1-37.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1994. Bacteriological quality of tuna fish (*Thunnus thynnus*) destined for canning: Effect of tuna handling on presence of histidine decarboxylase bacteria and histamine level. J. Food Prot. 57: 318-323.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1996a. Sensory quality and histamine formation during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

Lopez-Sabater, 1996b. Incidence of histamine-forming bacteria and histamine content in scombroid fish species from retail markets in the Barcelona area. Int. J. Food Microbiol. 28:411-418.

Maijala, R.L. 1993. Formation of histamine and tyramine by some lactic acid bacteria in MRS-broth and modified decarboxylation agar. Lett. Appl. Microbiol. 17: 40-43.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Morii, H., Cann, D.C., and Taylor, L.Y. 1988. Histamine formation by luminous bacteria in mackerel stored at low temperatures. Nippon Suisan Gakkaishi 54: 299-305.

Morrow, J.D., Margolies, G.R., Rowland, J., and Roberts, L.J. 1991. Evidence that histamine is the causative toxin of scombroid-fish poisoning. N. Engl. J. Med. 324: 716-720.

Niven, C.F., M.B. Jeffrey, and D.A. Corlett. 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. Appl. Environ. Microbiol. 41: 321-322.

Okuzumi, M., Yamanaka, H., Kubozuka, T., Ozaki, H., and Matsubara, K. 1984. Changes in number of histamine-forming bacteria on/in common mackerel stored at various temperatures. Bull. Jpn. Soc. Sci. Fish. 50: 653-657.

Omura, Y., Price, R.J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Pacheco-Aguilar, R., Lugo-Sánchez, M.E., and Robles-Burgueño, M.R. 2000. Postmortem biochemical and functional characteristic of Monterey sardine muscle stored at 0°C. J. Food Sci. 65: 40-47.

Pérez-Villarreal, B. and Pozo, R. 1990. Chemical composition and ice spoilage of albacore (*Thunnus alalunga*). J. Food Sci. 55: 678-682.

Price, R.J., Melvin, E.F., and Bell, J.W. 1991. Postmortem changes in chilled round, bled and dressed albacore. J. Food Sci. 56: 318-321.

Price, R.J. and Melvin, E.F. 1994. Recommendations for on board handling of albacore tuna. Sea Grant Extension Program, Food Science and Technology Extension, University of California, Davis.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish shellfish. Adv. Food Nutr. Res. 39: 329-364.

Rodríguez-Jerez, J.J., Mora-Ventura, M.T., López-Sabater, E.I., and Hernández-Herrero, M. 1994. Histidine, lysine, and orinithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. J. Food Prot. 57: 784-787, 791.

Roig-Sagués, A.X., Hernández-Herrero, M., López-Sabater, E.I., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1997. Evaluation of three decarboxylating agar media to detect histamine and tyramine-producing bacteria in ripened sausages. Lett. Appl. Microbiol. 25: 309-312.

Russell, F.E. and Maretic, Z. 1986. Scombroid poisoning: Mini-review with case histories. Toxicon. 24: 967-973.

Ryder, J.M., Buisson, D.H., Scott, D.N., and Fletcher, G.C. 1984. Storage of New Zealand jack mackerel (*Trachurus novaezelandiae*) in ice: Chemical, microbiological and sensory assessment. J. Food Sci. 49: 1453-1456, 1477.

Ryser, E.T., Marth, E.H., and Taylor, S.L. 1984. Histamine production by psycrotrophic *pseudomonas* isolated from tuna fish. J. Food Prot. 47: 378-380.

Stratton, J.E. and Taylor, S.L. 1991. Ch. 13. Scombroid poisoning. p. 331-351. *In* Microbiology of Marine Food Products. van Nostrand Reinhold, N.Y.

Stratton, J.E., Hutkins, R.W., Taylor, S.L. 1991. Biogenic amines in cheese and other fermented foods. J. Food Prot. 54: 460-470.

Tanase, S., Guirard, B.M., and Snell, E.E. 1985. Purification and properties of a pyridoxal 5'-phosphate-dependent histidine decarboxylase from *Morganella morganii* AM-15. J. Biol. Chem. 260: 6738-6746.

Taylor, S.L. and Lieber, E.R. 1979. *In vivo* inhibition of rat intestinal histamine-matabolizing enzymes. Food Cosmet. Toxicol. 17: 237-240.

Taylor, S. L. 1986. Histamine food poisoning: Toxicology and clinical aspects. CRC Crit. Rev. Toxicol. 17: 91-117.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S.L. and Woychik, N.A. 1982. Simple medium for assessing quantitative production of histamine by *Enterobacteriaceae*. J. Food Prot. 45: 747-751.

Tolmasky, M.E., Actis, L.A., and Crosa, J.H. 1995. A histidine decarboxylase gene encoded by the *Vibrio anguillarum* plasmid pJM1 is essential for virulence: histamine is a precursor in the biosynthesis of anguibactin. Mol. Microbiol. 15: 87-95.

Vaaler, G.L., Brasch, M.A., and Snell, E.E. 1986. Pyridoxal 5'-phosphate-dependent histidine decarboxylase. J. Biol. Chem. 261: 11010-11014.

Van Poelje, P.D. and Snell, E.E. 1990. Pyruvoyl-dependent enzymes. Ann. Rev. Biochem. 59: 29-59.

Wolcott, M.J. 1991. DNA-based rapid methods for the detection of foodborne pathogens. J. Food Prot. 54: 387-401.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 2

Histamine Formation and Bacterial Spoilage of Albacore Harvested off the U.S. Northwest Coast

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Abstract

Iced and previously frozen albacore were monitored for histamine formation and bacterial growth during storage at 0-37°C. The optimum temperature for histamine formation in albacore tuna (*Thunnus alalunga*) was 25°C, and whole fish were more susceptible to histamine formation than dressed fish at that temperature. Storage at 25°C resulted in the highest histamine level, 60.4 mg/100 g in whole fish stored for 7 days. When albacore were frozen prior to storage, reduced amount of histamine was found at 7.14 mg/100 g after 7 day storage at 25°C, only after decomposition became obvious. No histamine was found in any of the albacore samples stored in ice for 18 days.

Introduction

Scombroid poisoning results from ingestion of foods containing high levels of histamine and is one of three most frequently reported illnesses associated with seafood consumption in the U.S. (FDA, 1994; Bean and Griffin, 1990). Among seafoods, it is mainly associated with scombroid fish species, such as, tuna, bonito and mackerel, containing high levels of free histidine in muscle (Taylor, 1986). Histidine can be converted to histamine during decomposition by histamine-producing bacteria possessing histidine decarboxylase (Rawles et al., 1996). Various types of fish implicated in scombroid poisoning have shown to contain high levels of histamine. Sailfish meat involved in a scombroid poisoning case had 168 mg histamine/100 g muscle (Hwang et al., 1995). Histamine levels up to 919 mg/100 g were found in samples of tuna sashimi incriminated in scombroid fish poisoning (Taylor and Lieber, 1979). The histamine content of marlin implicated in a poisoning incident ranged between 935 and 2760 ppm; while that of fresh

marlin did not cause symptoms of poisoning, and was undetectable (<5 ppm) (Morrow et al., 1991). The histamine content of a hot-smoked mackerel that had been implicated in a scombrotoxic incident was 2,700 ppm, whereas that of fresh hot-smoked mackerel was 25 ppm (Clifford et al., 1989). The histamine content of canned tuna implicated in human poisoning was 116 mg/100 g muscle, while that of wholesome canned tuna was 2.74 mg/100 g muscle (Kim and Bjeldanes, 1979).

Histamine formation is most often induced by high temperature abuse of fish postharvest, and the accumulated level is affected by the combination of time and temperature. Kahawai showed the highest rate of histamine formation and accumulation at 25°C, 330 mg/100 g muscle within 2 days, followed by 30°C (Fletcher et al., 1995). Mahi-mahi showed the highest histamine accumulation at 32°C, reaching 250 mg/100 g of fish in 24 hr, when incubated at 0-32°C (Baranowski et al., 1990). Oil-preserved anchovies stored at 20°C showed an increased level of histamine, 80 mg/100 g, in 2 mo storage, reaching a peak at 300 mg/100 g at 7 mo (Rodríguez-Jerez et al., 1994). At lower temperatures, 0-10°C, histamine was formed but at the reduced level mainly by psychrophilic and psychrotrophic histamine-producing bacteria (Morii et al., 1988; Baldrati et al., 1980; Baranowski et al., 1990; Frank and Yoshinaga, 1984; Ryser et al., 1984).

Albacore tuna (*Thunnus alalunga*), which belongs to *Scombridae* family, has a good eating quality and has been a highly valued fish. There has been a large effort to harvest albacore off the Northwest coast of the U.S. (Craven et al., 1995). It is known to have a relatively long shelf-life and contains negligible quantities of histamine immediately after catch. However, improper handling of albacore can cause histamine formation due to the high levels of free histidine in its muscle, >1000 mg/100 g (1%, w/w) (Fletcher et al., 1995).

The FDA (1996) established the hazards analysis and critical control point (HACCP) program and set up guidelines for histamine at 5 mg/100 g for scombroid fish species, which was tenfold less than the previous guideline (Craven et al., 1995). Our objective was to study product safety of albacore by inducing histamine production under controlled storage conditions. Effects of frozen storage and evisceration of fish were also studied.

Materials and Methods

Samples and preparation

Iced albacore samples were purchased from a commercial processor in Newport, OR and transferred in ice to the Oregon State University-Seafood Lab overnight. Although the history of fish could not be verified, in that fishing season (1996), albacore were troll-caught about 100-200 miles off the Oregon Coast. Fishermen were advised to chill the fish in slush ice immediately after catch and maintain it in ice on board. The weight of the albacore ranged between 12 and 14 kg each (avg. 13.2 kg). Upon arrival at the OSU-Seafood Laboratory, half of the fish were dressed by eviscerating guts and removing gills. Fish were rinsed with water and used as dressed fish.

Frozen albacore were obtained from a chartered boat. They had been blast-frozen on board immediately after catch and kept frozen at -30°C until used (≈4 mo). The fish were thawed at room temperature (15°C) overnight and used as whole fish.

Sample storage

The iced albacores were divided into two groups of whole and dressed fish, and both groups were stored at 0, 25, 30, and 37°C. Samples were taken from each group, storage temperature, and sampling period. The initial sampling site was the nuchal region (nape) directly behind the head above the lateral line, and the sampling site moved along the lateral line to the anterior. Muscles were removed aseptically from fish and analyzed for aerobic plate count (APC) and histamine content. Samples were taken every 12 h from 30 and 37°C storage. For 25°C, samples were taken every 24 h. At 0°C, samples were analyzed after 1 wk, and every 24 h thereafter.

Frozen albacore were thawed overnight at ambient temperature (15°C). The previously frozen fish were stored at 25 and 30°C, at which high levels of histamine were found with iced fish. Samples were taken every 24h as described and analyzed for APC and histamine content.

Bacterial enumeration

APC was determined in duplicate by the standard method (FDA, 1992). Muscle samples (10 g) were aseptically removed from fish and blended with 90 mL of saline solution (0.85%) followed by serial dilution in the same solution. Each diluted sample (1 mL) was dispensed and poured with Plate Count Agar (Difco Laboratories, Detroit, MI) supplemented with 0.5% NaCl. The APC plates were incubated at 35°C for 2 days except for those stored at 0°C or previously frozen. For iced albacore stored at 0°C, the plates were incubated at 15°C for 5 days, and for previously frozen albacore, they were incubated at 25°C for 3 days.

Histamine analysis

Histamine was analyzed in duplicate by the standard fluorometric method (AOAC, 1995). Muscle (10 g) was homogenized in 50 mL of methanol for 2 min and heated in a water bath at 60°C for 15 min. After cooling to 25°C, the volume was adjusted to 100 mL with methanol and filtered through Whatman #1 paper. The methanol filtrate was collected and loaded onto an ion exchange column (200 × 7 mm) with Dowex 1-X8 (Sigma Chemical Co., St. Louis, MO), which was converted to hydroxide form by 2 N NaOH. The column eluant was analyzed for histamine, and the fluorescence intensity was determined using a spectrophotofluorometer (Aminco Bowman, Silver Spring, MD) at excitation wavelength of 350 nm and emission wavelength of 444 nm.

Results and Discussion

Changes in bacterial counts of iced albacore

The condition of iced albacore used for storage was excellent as judged by color, odor, and firmness of muscle. The muscle was translucent white. The initial APC of the iced albacore muscle was below the colony counting range (25-250 CFU/g). Although intact muscle was sterile, APC of albacore started to increase rapidly during storage at 30 and 37°C (Fig. 2.1). After 12 h, APC was 10^3 CFU/g and increased to 10^5-10^6 CFU/g after 24 h in both whole and dressed fish. Off-odor was evident at 24 hr, and the muscle turned opaque-white as when it is cooked, due to protein denaturation. The APC reached almost 10^8 CFU/g after 36 h, and fish were completely decomposed by day 2 as determined by odor and appearance. When samples were stored at 25°C, APC increased

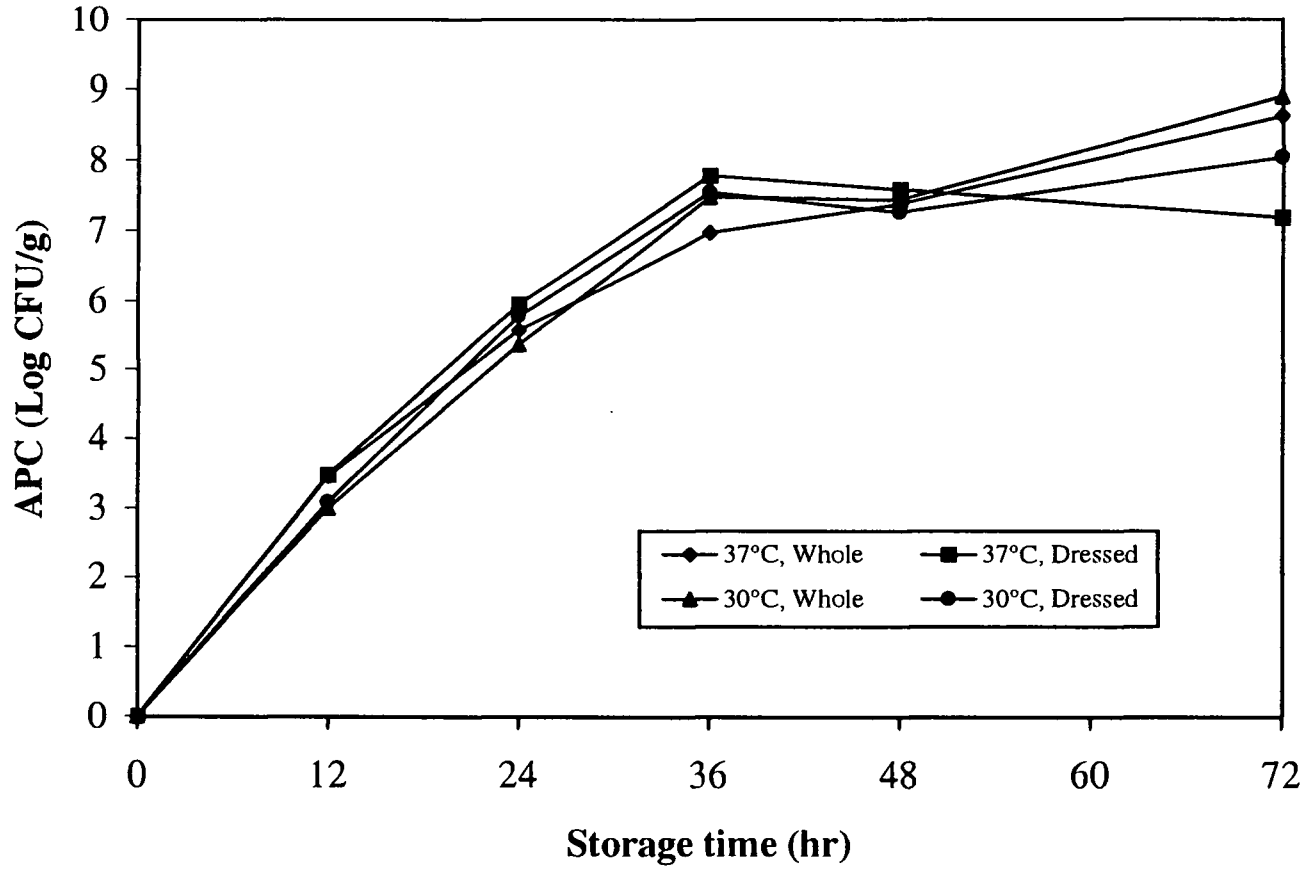

Fig. 2.1. Changes in APC of iced albacore during storage at 30 and 37°C. Fish were stored whole and dressed. APC was determined using plate count agar supplemented with 0.5% NaCl, and the plates were incubated at 35°C for 2 days.

faster in whole fish than dressed fish (Fig. 2.2). APC was 10^5 CFU/g at day 2 and increased gradually thereafter. Fish became spoiled by day 5, and APC was almost 10^7 CFU/g. Iced albacore stored at 0°C had the longest shelf-life among all the temperatures tested, 0-37°C (Fig. 2.3). APC was below 10^4 CFU/g until 7 days and increased slowly thereafter. APC of whole fish increased faster than those of dressed fish as had been observed with fish stored at 25°C. Whole fish developed thick slime on the skin by day 15, but it was not observed on dressed fish.

Pérez-Villarreal and Pozo (1990) reported that albacore had a longer shelf-life than any other scombroid fish. They reported 12 days as the maximum storage time that fish could be kept in ice with good quality. The increase in trimethylamine concentration was negligible over 25 days of storage in ice. Price et al. (1991) reported APC of albacore stored in ice increased gradually for 9 days and reached 10^6 CFU/g between 12 and 16 days. Marrakchi et al. (1990) reported that iced sardines had a shelf-life of 9 days. The initial APC of iced sardine, 3.16×10^2 CFU/g, reached the limit counts of 10^6-10^7 CFU/g at day 9 in iced storage, while the counts exceeded these limits within 24 h at ambient temperature. According to Ryder et al. (1984), jack mackerel had a shelf-life of 7 days in ice based on sensory results. During 23 days of storage in ice, APC did not exceed 10^6 CFU/g until day 11, and K value reached 20% after 7 days.

The gill and intestines of fish are the main reservoirs of normal microflora in seawater (Taylor and Speckhard, 1983). Bleeding and evisceration of fish is considered essential for some fish to maximize quality (Jacoby, 1987). In this study, APC increased showing distinctive differences between whole and dressed fish held at 0°C. Whole fish reached the maximum level, 10^8 CFU/g, in 15 days, while dressed fish reached the same

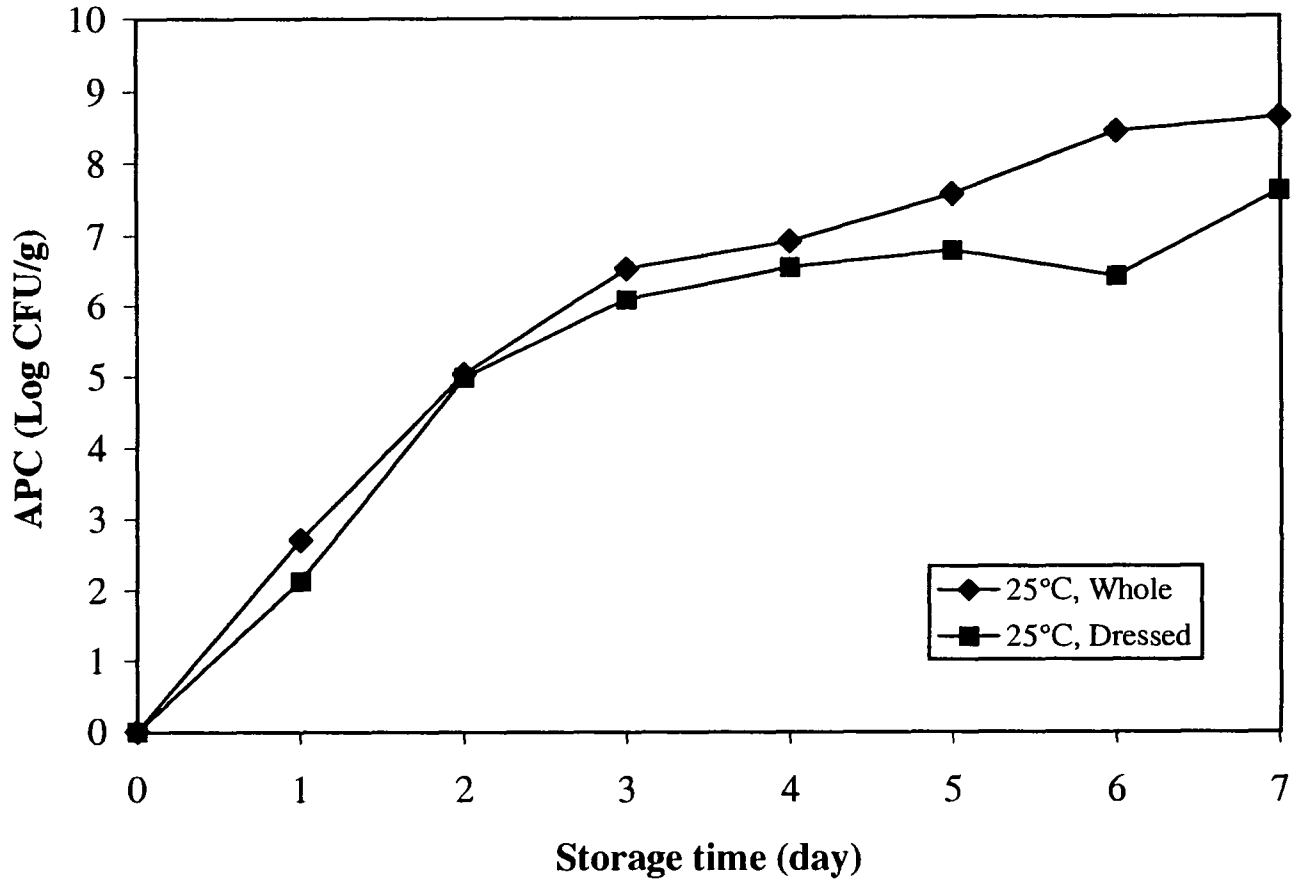

Fig. 2.2. Changes in APC of iced albacore during storage at 25°C. Fish were stored whole and dressed. APC was determined using plate count agar supplemented with 0.5% NaCl, and the plates were incubated at 35°C for 2 days.

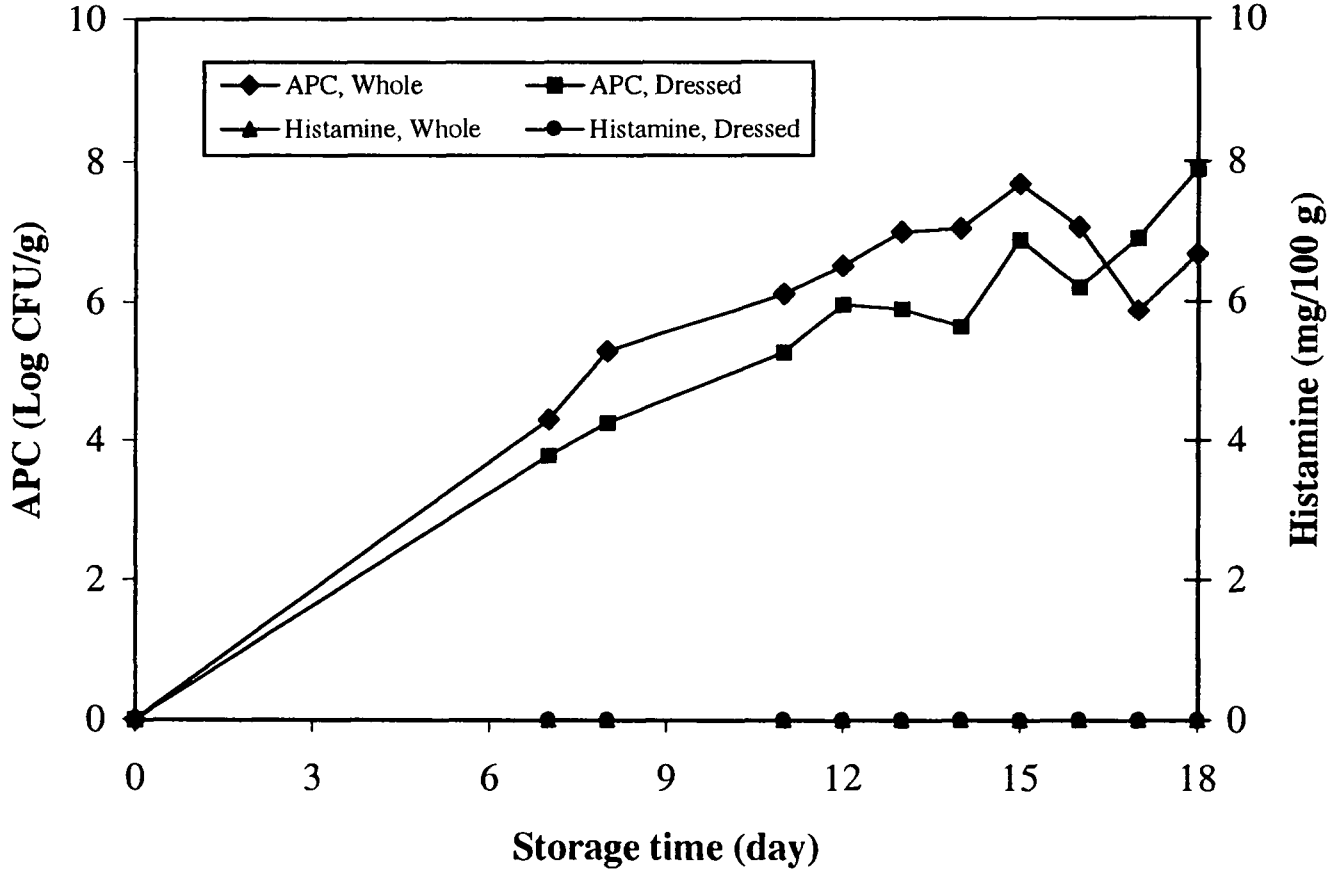

Fig. 2.3. Changes in APC and histamine content of iced albacore during storage at 0°C. Fish were stored whole and dressed. Samples were taken at 1 week and every 24 hr thereafter. APC was determined using plate count agar supplemented with 0.5% NaCl, and the plates were incubated at 15°C for 5 days. Histamine content was analyzed by the AOAC fluorometric method.

level in 18 days. However, no significant difference in APC was observed between whole and dressed fish, when incubated at 30 and 37°C. The trends in APC increase of whole and dressed albacore demonstrated that it was necessary to store fish below 4°C even after evisceration and degilling.

Histamine in iced albacore

The histamine accumulation in albacore was slow compared to the APC increase. During storage up to 3 days at 30 and 37°C, histamine levels were below 5 mg/100 g, although fish were completely decomposed by day 2 (Fig. 2.4). Fish stored whole at 30°C showed the highest level of histamine, 39.1 mg/100 g, at day 3. At 25°C, histamine level was negligible (<5 mg/100 g) until day 4, but a rapid increase was observed thereafter (Fig. 2.5). Histamine levels in fish were higher at 25°C than at 30 or 37°C. Whole fish stored at 25°C showed the highest histamine level, 60.4 mg/100 g, at day 7. We found that 25°C was optimum for histamine formation in albacore and that whole fish were more susceptible to histamine formation than dressed fish. No histamine was found in whole or dressed fish stored in ice up to 18 days (Fig. 2.3).

Haaland et al. (1990) showed the relationship between the formation of free amino acids and amines in mackerel stored at 2°C and 20°C. The contents of several amino acids decreased when fish were stored at 20°C with the resultant formation of phenylethylamine, tyramine, putrescine, histamine and cadaverine. However, no obvious changes were found in fish stored at 0°C. Wendakoon et al. (1990) reported a similar result that no amines formed in mackerel during iced storage, but several amines including histamine were found at high concentrations at 20°C. The rates of amine formation and its accumulation were

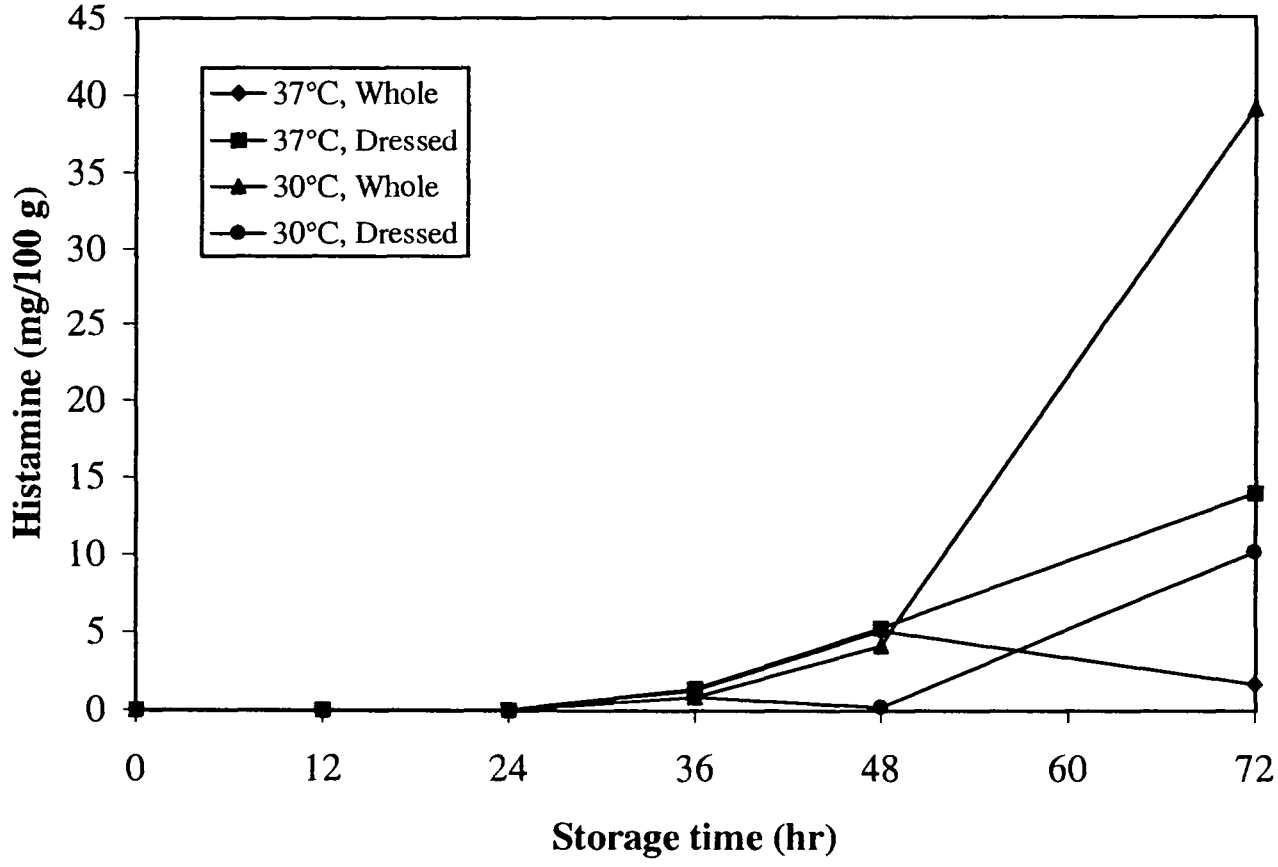

Fig. 2.4. Changes in histamine content of iced albacore during storage at 30 and 37°C. Fish were stored whole and dressed. Histamine content was analyzed by the AOAC fluorometric method.

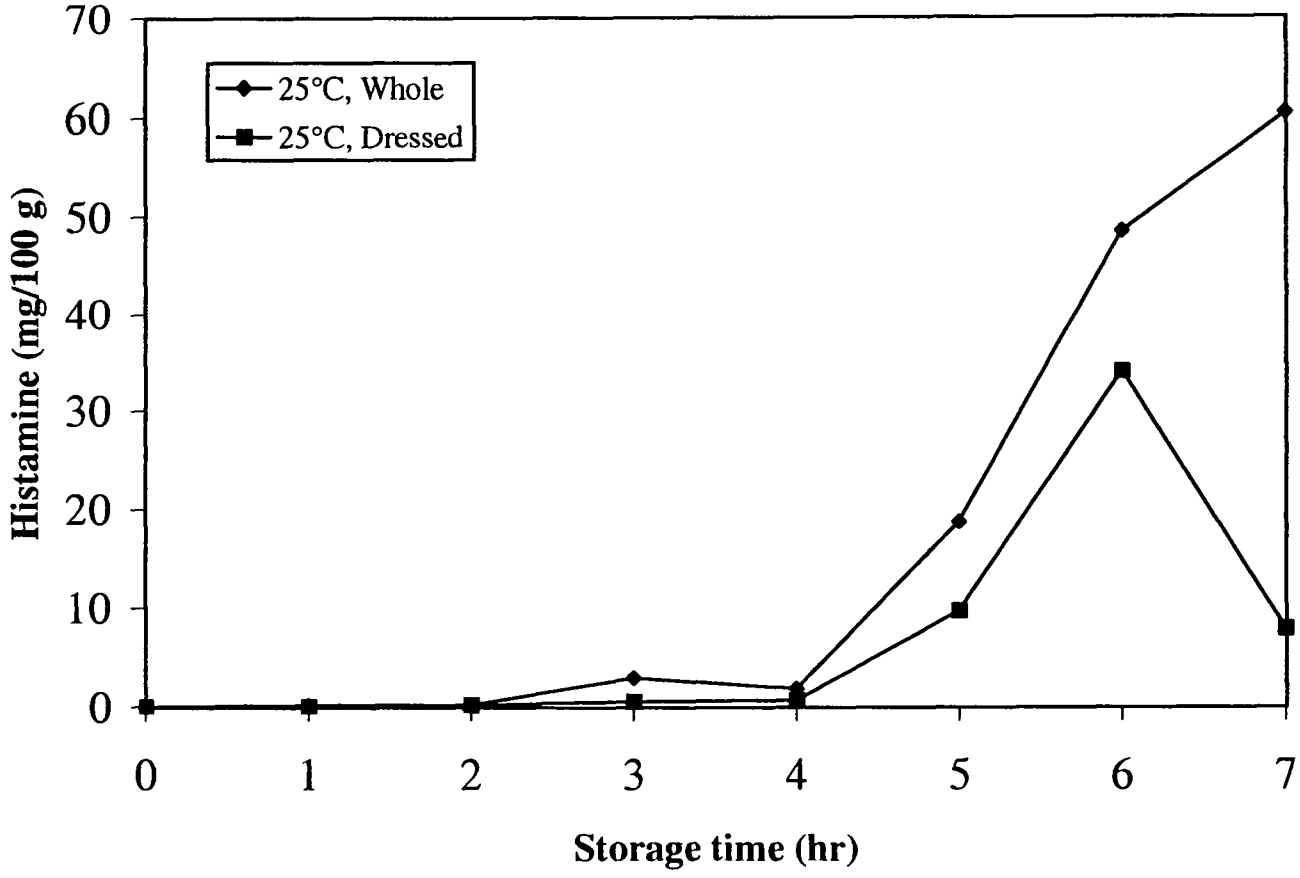

Fig. 2.5. Changes in histamine content of iced albacore during storage at 25°C. Fish were stored whole and dressed. Histamine content was analyzed by the AOAC fluorometric method.

higher in dark than in white muscle. Ababouch et al. (1991) reported that APC reached 5 $\times 10^8$ CFU/g, and the histamine level increased to 235 mg/100 g after 24 h incubation of sardine at ambient temperature. Frank et al. (1981) reported that the optimum temperature for histamine formation in skipjack tuna was 37.7°C based on microbial enzyme activity, and APC and histamine content were 2.8×10^5 CFU/g and 343 mg/100 g, respectively, on day 1 at that temperature. Price et al. (1991) reported histamine formation in round, bled, and dressed albacore stored at 0°C. Histamine was not detected for 27 days. Two bled albacore samples were found on day 33 to contain histamine at 49.2 and 82.5 mg/100 g. They suggested that histamine formation was inhibited at 0°C or below, but histamine production could continue due to preexisting histidine decarboxylase. According to López-Sabater et al. (1994), histamine levels higher than 2,000 ppm were found in canned sardine, mackerel, and tuna. In our results, the histamine content in albacore was lower than those of other scombroid fish reported, and high levels of histamine were developed after fish became decomposed and unsuitable for consumption. López-Sabater et al. (1996) reported similarly that when tuna (*Thunnus thynnus*) were properly stored at low nonfreezing temperatures, histamine formation did not present a serious health risk to consumers.

Changes in bacterial counts of frozen albacore

Initial APC of previously frozen albacore was $\approx 2.0 \times 10^2$ CFU/g. During storage at 30°C (Fig. 2.6), APC remained at 10^2 CFU/g during day 1, and then started to increase rapidly. APC reached 7.2×10^5 CFU/g within 36 hr, and fish developed apparent signs of spoilage, such as, pungent odor, slime formation, and soft texture. Although the initial APC

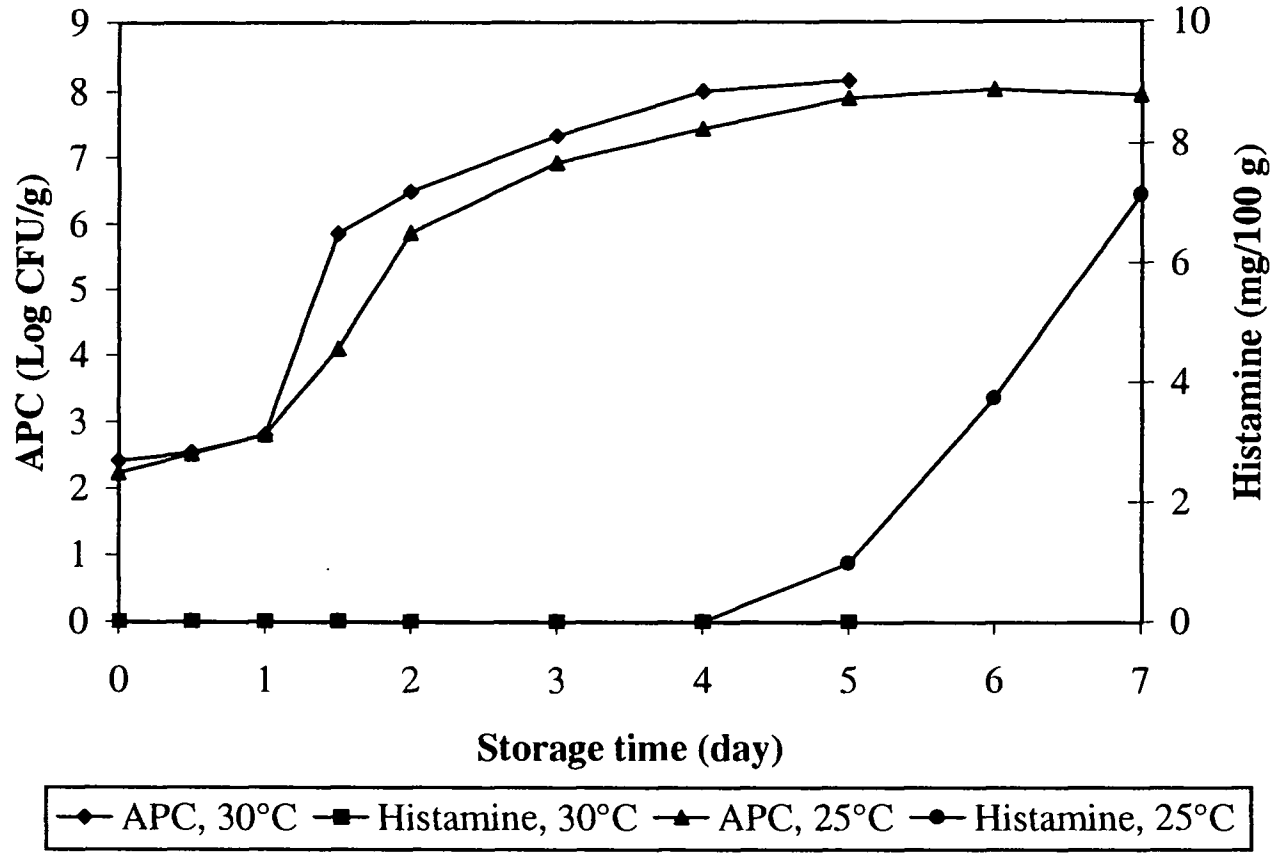

Fig. 2.6. Changes in histamine content and APC of frozen albacore during storage at 25 and 30°C. APC was determined using plate count agar supplemented with 0.5% NaCl, and the plates were incubated at 25°C for 3 days. Histamine content was analyzed by the AOAC fluorometric method.

of frozen fish were higher than those of iced fish, APC increased slowly resulting in prolonged lag periods before exponential bacterial growth occurred. Also, the pattern of APC increase in frozen albacore was quite different from that of iced albacore.

Raw frozen foods would have the same microbial flora as the raw food prior to freezing, although freezing favors survival of gram-positive bacteria (ICMSF, 1980). Dominant microflora in fish caught in cold waters are usually gram-negative psychrophilic and psychrotrophic bacteria (Bennour et al., 1991). Baranowski et al. (1990) reported that psychrotrophs were primarily spoilage bacteria at 0°C, while mesophiles predominated at 21 and 32°C. However, 30°C is not the optimum temperature for growth of psychrotrophic bacteria but the upper limit for growth. Therefore, we postulated that the rapid increase in APC of albacore after the lag phase was due to recovery of injured cells and contamination by mesophilic bacteria. APC of frozen albacore reached 7.4×10^5 CFU/g by day 2 at 25°C, and the rate of APC increase was 1 log cycle lower than that of APC in fish kept at 30°C (Fig. 2.6). No apparent differences were observed at 25°C between the iced and frozen albacore samples. Psychrotrophic bacteria in frozen fish exhibits the most rapid growth rate between 20-24°C, and mesophilic bacteria in iced fish can also grow well in that range (Jacoby, 1987).

Histamine in frozen albacore

Histamine was not detected at 30°C during 5 days storage, although APC reached the maximum level, 1.0×10^8 CFU/g, and fish developed apparent signs of spoilage (Fig. 2.6). At 25°C, histamine was not detected until day 4 (Fig. 2.6). Histamine content started

to increase slowly on day 5, when the fish was spoiled. The highest histamine content was 7.14 mg/100 g on day 7, when storage was discontinued.

Baranowski et al. (1990) reported freezing mahi-mahi, prior to storage at -20°C, inhibited subsequent histamine formation. When mahi-mahi was incubated for 24h at 32°C after frozen storage for 40 wk, a negligible level of histamine was found. In comparison, control fish, which had not been previously frozen, showed a histamine level of 266 mg/100 g. They proposed that the drastic reduction in surviving bacteria was due to microbial destruction during prolonged frozen storage. Ryser et al. (1984) isolated several psychrotrophic bacteria, *Pseudomonas fluorescens, Pseudomonas putida*, and non-fluorescent *Pseudomonas* spp. from raw sashimi tuna, and tested their abilities to form histamine at 21°C. The maximum histamine level found in their 48-hr cultures was low, 3.4 mg/100 mL. Taylor et al. (1983) reported that no histamine-producing bacteria were isolated from any of the muscle samples obtained from frozen skipjack tuna.

The production of histamine is not related to the total number of bacteria but rather to the number capable of synthesizing histidine decarboxylase (Bennour et al., 1991). The formation of histamine is mainly due to *Enterobacteriaceae* (López-Sabarter et al., 1994). Although many different bacteria can produce histamine, including *Proteus vulgaris*, *Proteus mirabillis*, *Clostridium perfringens*, *Enterobacter aerogenes*, and *Vibrio alginolyticus*, only *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei*, have been detected in foods incriminated in scrombroid poisoning (Yoshinaga and Frank, 1982; Taylor et al., 1983; Eitenmiller et al., 1982; Omura et al., 1978; Middlebrooks et al., 1988). Among them, only *M. morganii* and *K. pneumoniae* have been reported as prolific histamine former (López-Sabater et al., 1996). Several investigators showed that the optimum

temperature for the decarboxylating activity was 20 to 30°C (Middlebrooks et al., 1988; Fletcher et al., 1995). Therefore, we concluded that histamine-producing bacteria in previously frozen albacore do not recover their activity to decarboxylate histidine during incubation at 30°C but may recover it slowly at 25°C.

Acknowledgments

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References

Ababouch, L., Afilal, M.E., Benabdeljelil, H., and Busta, F.F. 1991. Quantitative changes in bacteria, amino acids and biogenic amines in sardine (*Sardina pilchardus*) stored at ambient temperature (25-28°C) and in ice. Int. J. Food Sci. Technol. 26: 297-306.

AOAC. 1995. Ch. 35. Fish and other marine products. p. 16-17. *In* Official Methods of Analysis. Vol. 2. Arlington, VA.

Baldrati, G., Fornari, M.B., Spotti, E., and Incerti, I. 1980. Effect of temperature on histamine formation in fish rich in histidine. Industr. Con. 55: 114-122.

Baranowski, J.D., Frank, H.A., Brust, P.A., Chongsiriwatana, M., and Premaratne, R.J. 1990. Decomposition and histamine content in mahimahi (*Coryphaena hippurus*). J. Food Prot. 53: 217-222.

Bean, N.H. and Griffin, P.M. 1990. Foodborne disease outbreaks in the United States, 1973-1987. J. Food Prot. 53: 804-817.

Bennour, M., Marrakchi, A. E., Bouchriti, N., Hamama, A., and Ouadaa, M. E. 1991. Chemical and microbiological assessments of mackerel (*Scomber scombrus*) stored in ice. J. Food Prot. 54: 789-792.

Clifford, M.N., Walker, R., and Wright, J. 1989. Studies with volunteers on the role of histamine in suspected scombrotoxicosis. J. Sci. Food Agric. 47: 365-375.

Craven, C., Hilderbrand, K., Kolbe, E., Sylvia, G., Daeschel, M., Gloria, B., and An, H. 1995. Understanding and controlling histamine formation in troll-caught albacore tuna: A review of preliminary findings from the 1994 season. Oregon Sea Grant (ORESU-T-95-00). Corvallis, OR.

Eitenmiller, R.R., Orr, J.H., and Wallis, W.W. 1982. Histamine formation in fish: Microbiological and biochemical conditions. Ch. 4. In *Chemictry & Biochemistry of Marine Food Products.* p. 39-50. Westport, CT, Avi Publishing Co.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species, particulary kawawai (*Arripis trutta*). J. Aquat. Food Prod. Technol. 4: 533-574.

Frank, H.A. and Yoshinaga D.H. 1984. Histamine formation in tuna. Seafood Toxins. ACS Symposium Series. No. 262. p. 443-451. American Chem. Soc., Washington, D.C.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th Ed. Arlington, VA.

Food and Drug Administration. 1994. Proposed rules. Fed. Reg. 59: 4149.

Food and Drug Administration. 1996. *In* Fish and Fisheries Products Hazards and Control Guide. p. 69-81.

Haaland, H., Arnesen, E., and Njaa, L.R. 1990. Amino acid composition of whole mackerel (*Scomber scombrus*) stored anaerobically at 20°C and at 2°C. Int. J. Food Sci. Technol. 25: 82-87.

Hwang, D.F., Chang, S.H., Shiau, C.Y., and Cheng, C.C. 1995. Biogenic amines in the flesh of sailfish (*Istiophorus platypterus*) responsible for scombroid poisoning. J. Food Sci. 60: 926-928.

ICMSF. 1980. Factors affecting life and death of microorganisms. Microbial ecology of foods. Vol. 1. Academic Press, New York.

Jacoby, D.R. 1987. On-board Handling and preservation of frozen troll-caught albacore, *Thunnus alalunga.* Mar. Fish. Rev. 49: 46-50.

Kim, I.S. and Bjeldanes, L.F. 1979. Amines content of toxic and wholesome canned tuna fish. J. Food Sci. 44: 922-923.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1994. Bacteriological quality of tuna fish (*Thunnus thynnus*) destined for canning: Effect of tuna handling on presence of histidine decarboxylase bacteria and histamine level. J. Food Prot. 57: 318-323.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1996. Sensory quality and histamine formation during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Morii, H., Cann, D.C., and Taylor, L.Y. 1988. Histamine formation by luminous bacteria in mackerel stored at low temperatures. Nippon Suisan Gakkaishi, 54: 299-305.

Morrow, J.D., Margolies, G.R., Rowland, J., and Roberts, L.J. 1991. Evidence that histamine is the causative toxin of scombroid-fish poisoning. N. Engl. J. Med. 324: 716-720.

Omura, Y., Price, R. J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Pérez-Villarreal, B. and Pozo, R. 1990. Chemical composition and ice spoilage of albacore (*Thunnus alalunga*). J. Food Sci. 55: 678-682.

Price, R.J., Melvin, E.F., and Bell, J.W. 1991. Postmortem changes in chilled round, bled and dressed albacore. J. Food Sci. 56: 318-321.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish shellfish. Adv. Food Nutr. Res. 39: 329-364.

Rodríguez-Jerez, J.J., López-Sabater, E.I., Hernández-Herrero, M.M., and Mora-Ventura, M.T. 1994. Histamine, putrescine and cadaverine formation in Spanish semipreserved anchovies as affected by time/temperature. J. Food Sci. 59: 993-997.

Ryder, J.M., Buisson, D.H., Scott, D.N., and Fletcher, G.C. 1984. Storage of New Zealand jack mackerel (*Trachurus novaezelandiae*) in ice: Chemical, microbiological and sensory assessment. J. Food Sci. 49: 1453-1456, 1477.

Ryser, E.T., Marth, E.H., and Taylor, S.L. 1984. Histamine production by psycrotrophic *seudomonas* isolated from tuna fish. J. Food Prot. 47: 378-380.

Taylor, S.L. and Lieber, E.R. 1979. *In vivo* inhibition of rat intestinal histamine-matabolizing enzymes. Food Cosmet. Toxicol. 17: 237-240.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S. L. 1986. Histamine food poisoning: Toxicology and clinical aspects. CRC Crit. Rev. Toxicol. 17: 91-117.

Wendakoon, C.N., Murata, M., and Sakaguchi, M. 1990. Comparison of non-volatile amine formation between the dark and white muscle during storage. Nippon Suisan Gakkaishi. 56: 809-818.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 3

Histamine and Biogenic Amine Production by *Morganella morganii* Isolated from Temperature-Abused Albacore

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Abstract

Histamine-producing bacteria were isolated from albacore stored at 0, 25, 30, and 37°C. They were screened using Niven's differential medium, and their histamine production was confirmed by HPLC analysis. The optimum temperature for growth of histamine-producing bacteria was 25°C. The bacterium producing the highest level of histamine was isolated from fish abused at 25°C. It was identified as *Morganella morganii* by morphological, cultural, biochemical, and antimicrobic characteristics, and the Vitek, microbial identification system. The *M. morganii* isolate was inoculated into tuna fish infusion broth medium, and the effect of temperature was determined for microbial growth and formation of histamine and other biogenic amines. The isolate produced the highest level of histamine, 5,253 ppm, at 25°C in the stationary phase. At 15°C, histamine production was reduced to 2,769 ppm. Neither microbial growth nor histamine formation was detected at 4°C. To determine whether the isolate can also produce other biogenic amines that can potentiate histamine toxicity, production of cadaverine, putrescine, serotonin, tryptamine, tyramine, phenylethylamine, spermidine, and spermine by the isolate was also monitored. Cadaverine, putrescine and phenyletylamine were detected with microbial growth in the tuna fish infusion broth medium. The optimum temperature for cadaverine, putrescine and phenyletylamine formation was found to be 25°C, as it was for histamine.

Introduction

Scombroid poisoning is one of three most frequently reported illnesses associated with seafood consumption in the U.S. (FDA, 1994). Histamine has been shown to be the cause of scombroid poisoning, and its toxicity is enhanced by the presence of other biogenic amines in foods. Histamine is formed by decarboxylation of histidine, which is found at high levels in muscles of fish belonging to the *Scombroidae* and the *Scomberesocidae* family (Rawles et al., 1996). It is most frequently associated with the consumption of tuna, skipjack, mackerel, and bonito, although non-scombroid fish, such as, mahi-mahi, bluefish, herring, and sardines often have been reported to contain high levels of histamine (Taylor et al., 1989).

Histamine formation is largely induced by high temperature abuse of fish postharvest, and the accumulated level is affected by the combination of time and temperature (Rawles et al., 1996). It generally results from proliferation of histamine-producing bacteria, which possesses histidine decarboxylase. *Enterobacteriaceae* has been reported to be the most important histamine-producing bacteria in fish (López-Sabater et al., 1994). *M. morganii, Klebsiella pneumoniae*, and *Hafnia alvei* have been isolated from fish incriminated in scombroid poisoning (Eitenmiller et al., 1981; Middlebrooks et al., 1988; Taylor, 1986; Yoshinaga and Frank, 1982). Other microorganisms, such as *Clostridium perfringens, Vibrio alginolyticus, Vibrio anguillarum, Acinetobacter lowffi, Plesiomonas shigelloides, Pseudomonas* spp., *Aeromonas* spp., *Photobacterium* spp., *Stenotrophomonas maltophilia* and N-group bacteria have also been reported as histamine producers in ish (Barancin et al., 1998;

Behling and Taylor, 1982; (López-Sabater et al., 1994; Middlebrooks et al., 1988; Okuzumi et al., 1994; Ryser et al., 1984; Yatsunami and Echigo, 1993).

Among all the histamine forming bacteria, *M. morganii* consistently has been shown to be a prolific histamine former in fish and culture broth. It is possible that the species is introduced through postharvest contamination, because it is rarely found on freshly caught fish and isolated only from spoiled fish muscles (Taylor, 1986; Yoshinaga and Frank, 1982). Rodríguez-Jerez et al. (Rodríguez-Jerez et al., 1994) isolated 200 bacteria from ripened semi-preserved Spanish anchovies, and *M. morganii* showed the highest histamine content, 2,337 ppm in the histamine evaluation medium after 24 hr of incubation at 37°C. López-Sabater et al. (1994) reported *M. morganii* was the most active histamine former among the isolates, producing 2,765 ppm of histamine, on average, in the histamine evaluation medium. According to Frank et al. (1985), eight of the nine high histamine producers isolated from decomposed mahi-mahi were *M. morganii.* They produced 1,200 ppm of histamine on average. López-Sabater et al. (1996) isolated 374 histamine-producing bacteria during tuna (*Thunnus thynnus*) spoilage. From these, 47 strains of *M. morganii* were isolated. They showed an extremely high histidine decarboxylase activity, producing >300 mg of histamine per 100 g after 18 h of incubation at 37°C.

Despite the common susceptibility reported for tuna to histamine, relatively little information has been reported for histamine formation from albacore (*Thunnus alalunga*). The objectives of our study were to isolate prolific histamine-producing bacteria in albacore and to determine the effect of temperature on the formation of histamine and other biogenic amines. Histamine-producing bacteria were screened from temperature-

abused fish using high-pressure liquid chromatography (HPLC) analysis, and the isolates were tested for microbial growth and formation of biogenic amines in tuna fish infusion broth (TFIB) medium at various temperatures.

Materials and Methods

Reagents and media

Histamine dihydrochloride, ß-phenylethylamine hydrochloride, serotonin dihydrochloride, cadaverine dihydrochloride, spermine tetrahydrochloride, spermidine tetrahydrochloride, putrescine dihydrochloride, tryptamine hydrochloride, tyramine hydrochloride, 1,7-diaminoheptamine, dansyl chloride, and ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Perchloric acid and acetonitrile were purchased from J.T. Chemical Co. (Phillipsburg, NJ). Trypticase soy broth, and Mueller-Hinton agar were purchased from BBL Microbiology System (Cockeysville, MD). All media and reagents for identification described in the "identification" section were purchased from Difco Laboratories (Detroit, MI).

Samples

Albacore, which had not been previously frozen, were obtained from a commercial processor in Newport, OR and transported in ice to the Oregon State University-Seafood Lab by overnight freight. Upon arrival at the laboratory, albacore were immediately subjected to storage at 0, 25, 30 and 37°C and were used for isolation of histamine-producing bacteria. Samples were taken every 12 h for storage at 30 and

37°C storage. For storage at 25°C, samples from the dorsal muscle were taken every 24 h. At 0°C, samples were analyzed at 1 week and every 24 h thereafter.

Isolation of histamine-producing bacteria

Isolation of histamine-producing bacteria was carried out by using a pour plate method (FDA, 1992). Muscle (10 g) was removed aseptically from the stored fish and blended with 90 ml of saline solution (0.85%), followed by serial dilution in the same solution. One milliliter of each diluted sample was dispensed into each plate and mixed with Niven's differential medium (Niven et al., 1981) for the detection of histamine-producing bacteria. After incubation, positive as well as negative colonies were picked by their morphological characteristics, as described by Niven et al. (1981) and inoculated into trypticase soy agar (TSA) slants for further confirmation.

Confirmation of histamine formation by isolates

Presumptive histamine-producing bacteria were inoculated into 10 ml of 0.1% TSB-histidine (TSBH) containing 0.5% NaCl and incubated at 37°C for 24 h, according to the method of Taylor et al. (1978). Histamine production by the isolate was confirmed by HPLC analysis according to the method of Wei et al. (1990), using the Aminex HPX-72S organic base analysis column (300 × 7.8 mm; Bio-Rad, Hercules, CA) connected with a Bio-Rad HPLC pump (Model 2700), and a UV detector (Bio-Rad Model 1706). Flow rate used was 0.6 ml/min. The mobile phase was 5% acetonitrile in 0.25 M ammonium sulfate. The injection volume was 10 μl.

To identify the histamine peak, TSBH supplemented with 5% (w/v) histamine

was injected as a control, and the spiked peak corresponding to histamine was selected. The retention time of the histamine peak was 16.67 min. Samples were prepared as described below prior to HPLC injection. Cell culture, 5 ml, was mixed with 10 ml of 6% (v/v) perchloric acid (PCA). The mixture was centrifuged at 3,000 rpm for 10 min, and the supernatant was filtered with a Whatman #1 filter paper. The filtrate was brought to 25 ml with 6% (v/v) PCA and adjusted to pH 7.0 with 30% (w/v) KOH. Potassium perchlorate precipitate was removed by filtering with Whatman #1 filter paper and further filtered with a 0.45 μm micropore filter (Millipore Corp., Bedford, MA).

Identification

The highest histamine former determined by the HPLC analysis was identified according to Middlebrooks et al. (1988), Okuzumi et al. (1994), Ryser et al. (1984), and Bergey's Manual (Holt et al., 1994). Gram stain and motility test were carried out, followed by the biochemical tests, using the following media and reagents (Difco): semisolid motility agar for motility test; 3% hydrogen peroxide for the catalase test; 1% tetramethyl-*p*-phenylenediamine reagent for the oxidase test; nutrient gelatin deep tube for the gelatin hydrolysis test; tryptone broth and Kovacs' reagent for the indole test; methyl red Voges-Proskauer (MR-VP) medium and methyl red reagent for the Methyl red test; MR-VP medium and Barritt's reagent consisted of alpha-naphthol and KOH for the Voges-Proskauer test; simmons citrate agar for the citrate test; triple sugar iron agar for H_2S production; urea broth for the urease test; phenylalanine agar and 10% phenylpyruvic acid for the phenylalanine deaminase test; deoxyribonuclease agar for the deoxyribonuclease test; decarboxylase basal medium supplemented with 0.5% orinithine,

lysine, or arginine for amino acid degradation; and phenol red broth supplemented with 1% carbohydrates for carbohydrate utility. The inoculated tubes were incubated at 37°C for 24 h and tested individually according to the test procedures. For the gelatin hydrolysis test, tubes were incubated at 22°C for 10 days.

The identified isolate was confirmed using the Vitek instrument (bioMérieux Vitek, Inc., Hazelwood, MI) according to the manufacturer's instruction. The isolate was inoculated into a trypticase soy agar slant and incubated at 37°C for 18 h. This activated culture was suspended into phosphate saline buffer, and the amount of inoculation was adjusted by the Vitek colorimeter. A suspension was inoculated into gram-negative identification (GNI) card containing medium for biochemical test in each well. The digitalized analog optical reading and identification were automatically carried out by the Vitek system.

Antimicrobic sensitivity test

Antimicrobic sensitive tests were carried out by the Kirby-Bauer method (Bauer et al., 1966) using Sensi-Disk Susceptibility Test Disks (BBL Microbiology System). The isolate was inoculated into 5 ml of Mueller-Hinton broth and incubated at 37°C for 18 h. The culture broth of isolate was swabbed with cotton on the surface of a Mueller-Hinton agar plate. High potency antimicrobic disks were placed on the plate. Antimicrobic disks tested contained chloramphenicol (10 mcg); ampicillin (10 mcg); nitrofurantoin (300 mcg); oxacillin (1 mcg); penicillin G (10 mcg); colistin (10 mcg); nalidixic acid (30 mcg); amikacin (30 mcg); tobramycin (10 mcg); cephalothin (30 mcg); cefoxitin (30 mcg); vancomycin (30 mcg); ticarcillin (75/10 mcg); and

cefoperazone/sulbactam (75/30 mcg). After 18 h of incubation at 37°C, the plates were examined and the diameters of the clear zones were measured. Significance of zones was determined using the Kirby-Bauer chart.

Medium and growth conditions for isolate

TFIB medium was prepared from fresh albacore muscle, according to the method of Arnold et al. (Arnold et al., 1980). Overnight culture of the isolate was diluted into 0.1% peptone water and inoculated into 400 ml of TFIB to the final concentration of 10^2 cells/ml. The cell culture was aseptically dispensed in the volume of 10 ml into sterile tubes, and the tubes were incubated stationary at 4, 15, 25, and 37°C. During incubation, each tube was taken every 12 h for those incubated at 25 and 37°C and every 24 h for those incubated at 4 and 15°C. Each cell culture was used for a cell count and biogenic amine analysis.

Determination of growth curve and biogenic amines analysis

Microbial growth in a culture was determined by aerobic plate counts (APC), according to the standard method (FDA, 1992). Histamine, cadaverine, putrescine, serotonin, tryptamine, tyramine, phenylethylamine, spermidine, and spermine contents were determined using the HPLC method of Eerola et al. (1993). Sample preparation and derivatization were carried out as described by Eerola et al. (1993). Cell culture, 2 ml, was mixed with 10 ml of 0.4 M PCA. The mixture was centrifuged at 3,000 rpm for 10 min and brought to 25 ml with 0.4 M PCA. The supernatant was filtered with a Whatman #1 filter paper. Medium supernatants supplemented with an internal standard,

1,7-diaminoheptane, were dansylated. Dansyl amines were analyzed on the LiChrosorb RP-18 column (4 × 125 mm; Phenomenex, Torrance, CA) mounted with a RP-18 guard column. The column was connected with a Bio-Rad HPLC pump (Model 2700) and a UV detector (Bio-Rad Model 1706). Amines were eluted by a linear gradient with the mixture of 0.1 M ammonium acetate and acetonitrile. Gradient elution was initiated with 50% acetonitrile and 50% 0.1 M ammonium acetate and terminated in 19 min with 90% acetonitrile and 10% 0.1 M ammonium acetate. Flow rate used was 1.0 ml/min. Elution of dansylated amines was detected spectrophotometrically at 254 nm.

Results and Discussion

Screening of histamine-producing bacteria

Sixty-three strains of presumptive histamine producing bacteria were isolated from albacore stored at different temperatures. The 7 and 4 strains isolated from fish stored at 25 and 30°C, respectively, were confirmed as histamine formers (data not shown). No isolates from fish stored at 0°C were confirmed as histamine-producing bacteria. Most confirmed strains were isolated from fish stored at 25 and 30°C, and they were weak histamine producers. The highest level of histamine produced by all of the isolates in TSBH was 2,154 ppm. The strain was isolated from albacore muscle completely spoiled after storage at 25°C for 7 days. This isolate was originally detected as one of the negative colonies during screening on Niven's medium. Most positive colonies detected on the medium were confirmed as false positive, based on histamine analysis using the HPLC method.

Niven's differential medium was originally developed for detection of histamine-producing bacteria in fish. Modification of this medium has been reported to adapt it for different purposes. Joosten and Northolt (1989) and Maijara (1993) modified it to enhance detection of histamine-forming lactic acid bacteria in cheese. These media have been applied to isolate histamine-producing bacteria in cheese, meat, fish and poultry products (Roig-Sagués et al., 1996). However, the histamine-producing capacity of the positive isolates was not confirmed for these media. Rodríguez-Jerez et al. (1994) reported that these media are susceptible for false-positive and false-negative reactions due to the formation of different alkaline compounds and the fermentative activity of bacteria, respectively, during screening of histamine-producing bacteria. Therefore, it has been suggested that use of a differential screening step prior to inoculating bacteria onto decarboxylating media is necessary to enhance detection rate of true histamine formers.

Bacteriological characterization

The highest histamine former was identified as *M. morganii* and designated as *M. morganii* OSL36. This strain was a gram-negative, short rod, and motility-positive in semisolid motility agar (Table 3.1). It was catalase-positive and oxidase-negative. Gelatin was not hydrolyzed by this strain. In the indole-methyl red-Voges-Proskauer-citrate test, indole and methyl red reactions were positive. However, the strain presented negative reaction in the Voges-Proskauer and citrate utilization test. H_2S was not produced. The production of urease and phenylalanine deaminase was positive. The production of deoxyribonuclease was not detected. In the amino acid degradation tests,

orinithine was decarboxylated, but lysine decarboxylase and arginine dihydrolysis were not detected. Only glucose was fermented among all of the tested carbohydrates. Gas production from glucose was detected by the inverted Durham tube in the medium.

Table 3.1. Biological characteristics of *Morganella morganii* OSL36 isolated from albacore tuna

Biological characteristics	*M. morganii* OSL36
Gram stain	-
Oxidase	-
Catalase	+
Indole production	+
Methyl Red	+
Voges-Proskauer	-
Citrate	-
H_2S production (TSI)	-
Urease	+
Phenylalanine deaminase	+
Orinithine decarboxylase	+
Lysine decarboxylase	-
Arginine dihydrolysis	-
Motility	+
Gelatin hydrolysis (22°C)	-
Acid production from :	
Glucose	+
Lactose, Arabinose, Xylose, Mannitol,	-
Rhamnose, Cellobiose, Sorbitol, Maltose	-
Gas from glucose	+
Deoxyribonuclease	-
Oxidation-Fermentation	Fermentation

Antibiotics are widely used not only for the treatment of infectious diseases, but also for the determination of bacteriological characteristics. Among the 14 antimicrobial agents tested, the *M. morganii* OSL36 strain was resistant to ampicillin, oxacillin, penicillin G, colistin, cephalothin, vancomycin and ticarcillin, but was susceptible to nalidixic acid, amikacin, tobramycin, cefoxitin, and cefoperazone/sulbactam (Table 3.2). Also, it was intermediately resistant to chloramphenicol and nitrofurantoin. According to Bergey's manual (Holt et al., 1994), *Morganella* strains are generally resistant to colistin, erythromycin, penicillin, ampicillin, and cephalothin and are susceptible to nalidixic acid, carbenicillin, and chloramphenicol. The tested strain presented results consistent with Bergey's manual in both biological and antimicrobial sensitive characteristics.

The identity of *M. morganii* OSL36 was confirmed by the Vitek system. It was shown to be *M. morganii* with 99% confidence. The strain showed the positive result in wells containing urea, polymyxin B, glucose, and orinithine, and thus showing urease production, resistance to polymyxin B, glucose utilization, and orinithin decarboxylation. Other carbohydrates and amino acids tested were not utilized, as shown by the previous identification. A miniaturized version of the conventional tests was developed for the rapid identification of microorganisms, so the commercial test kits for the identification have been commonly used in recent studies rather than the conventional tests. In this study, the Vitek system was selected for rapid identification, and the confirmation results showed consistency when compared with conventional methods for the tested strain.

Table 3.2. Antibiotic susceptibility of *Morganella morganii* 36 by various antimicrobic disks

Antimicrobic disks	Zone diameters (mm)	Susceptibility
Chloramphenicol	13	I[1)]
Ampicillin	-	R[2)]
Nitrofurantoin	15	I
Oxacillin	-	R
Penicillin G	-	R
Colistin	-	R
Nalidixic acid	28	S[3)]
Amikacin	26	S
Tobramycin	26	S
Cephalothin	-	R
Cefoxitin	18	S
Vancomycin	-	R
Ticarcillin	-	R
Cefoperazone/sulbactam	31	S

[1)]Intermediate; [2)]Resistant; [3)]Susceptible

Effect of temperature on the microbial growth and histamine formation

Histamine formation in TFIB medium followed the general pattern of microbial growth (Fig. 3.1). The highest level of histamine was produced in the stationary phase (Fig. 3.2). The temperature of 25°C was optimal for both microbial growth and histamine formation. At 25°C, the strain produced the highest histamine level, 5,253 ppm, at 48 h of incubation in the late stationary phase. The histamine level produced at 37°C was much lower than at 25°C, although the strain reached the stationary phase earlier than at 25°C at 24 h. The highest histamine level detected at 37°C was 1,949 ppm at 48 h.

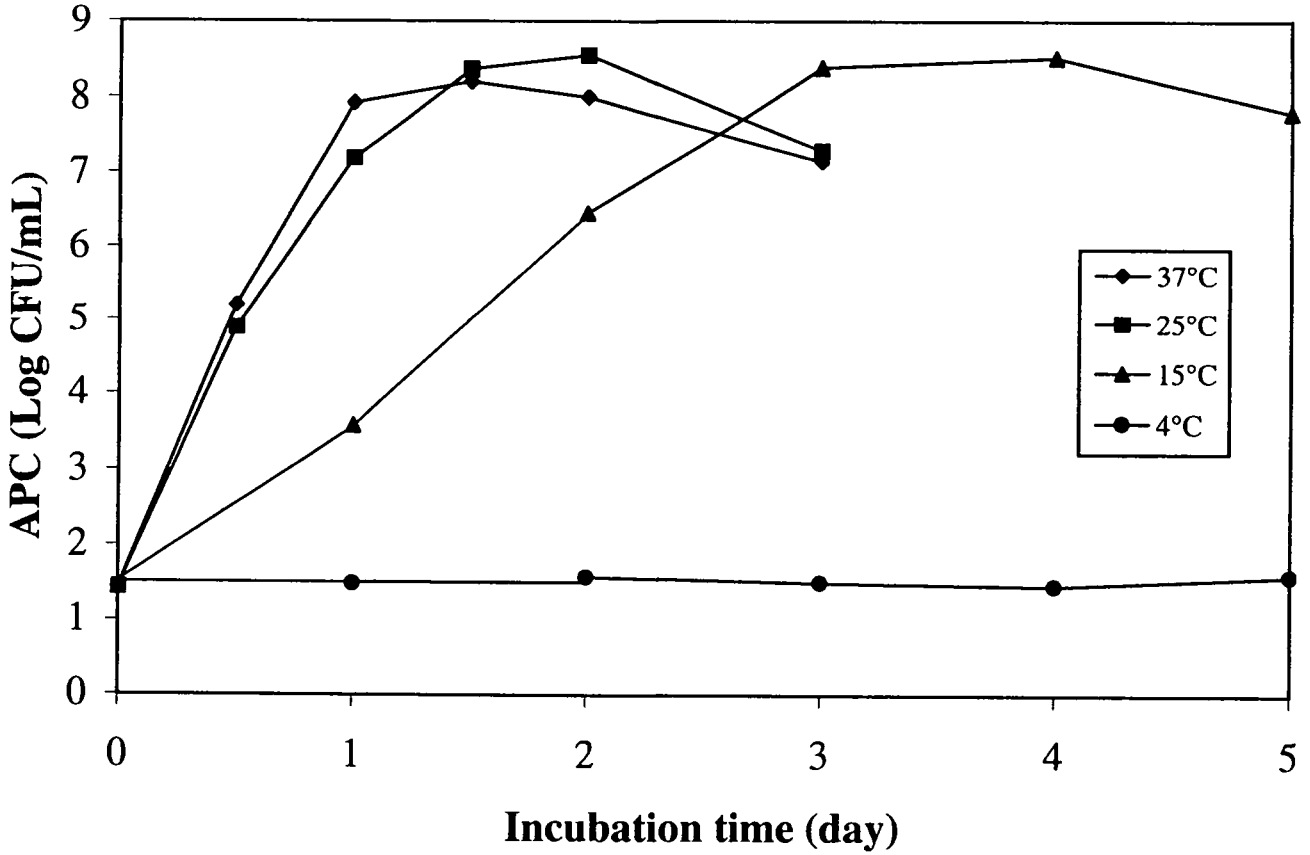

Fig. 3.1. Effect of temperature on the growth of *Morganella morganii* OSL 36. *M. morganii* OSL36 was inoculated into each 200 ml TFIB medium and incubated at 4, 15, 25 and 37°C, respectively. Cell growth was measured by APC.

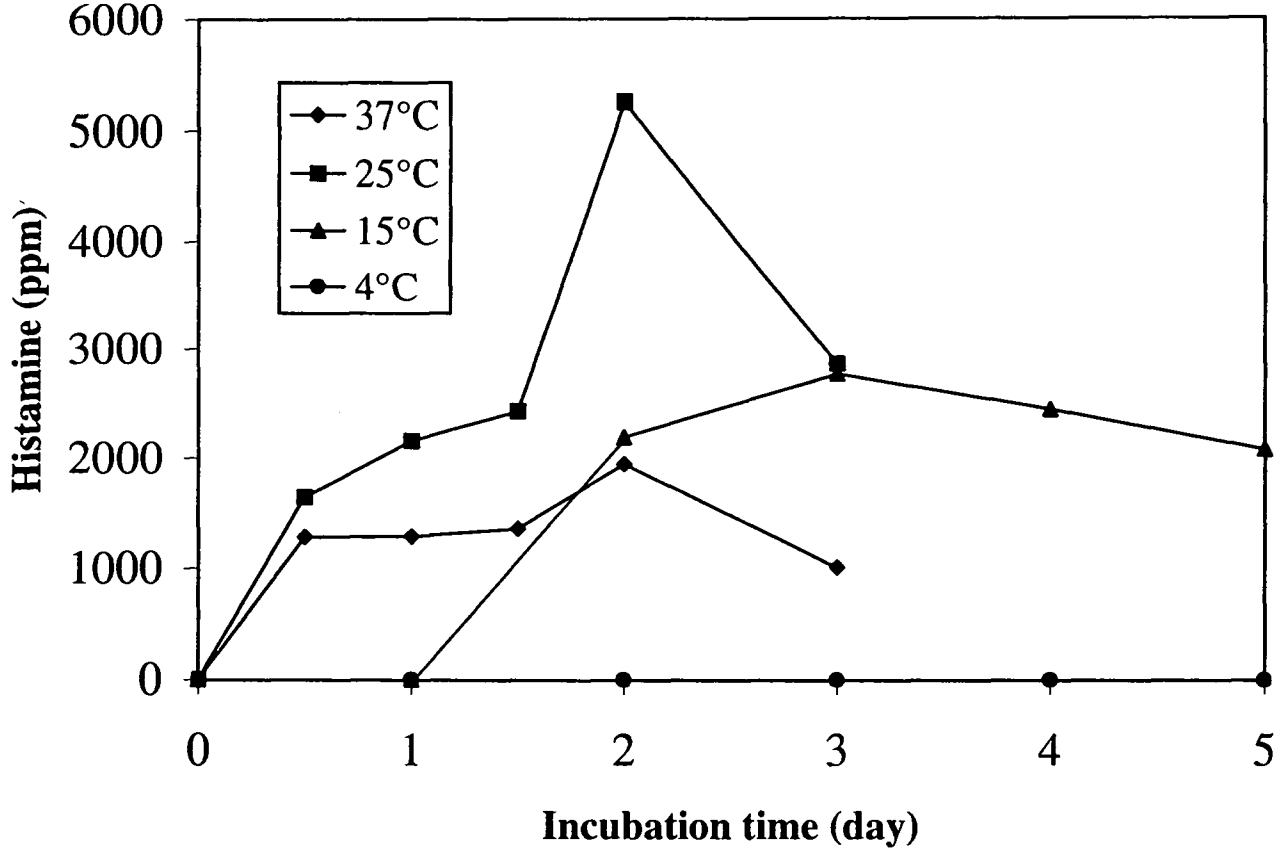

Fig. 3.2. Effect of temperature on the histamine formation of *Morganella morganii* OSL 36. *M. morganii* OSL36 was inoculated into each 200 ml TFIB medium and incubated at 4, 15, 25 and 37°C, respectively. Histamine was determined by HPLC analysis.

During incubation at 15°C, the rate of microbial growth was significantly less than at 25 and 37°C, and the APC reached 10^8 CFU/ml in 3 days. However, a significant level of histamine accumulated with microbial growth. The strain reached the stationary phase at day 3, and 2,577 ppm of histamine was detected. A temperature of 4°C showed a bacteriostatic effect on the growth of *M. morganii* OSL36. In general, this effect is induced by decreasing metabolic activity, such as enzyme activity and membrane fluidity for transportation, in mesophilic bacteria at refrigeration temperature (Jay, 1996). A negligible amount of histamine was detected after 2 weeks of incubation, although data was shown only for 5 days.

According to previous reports, the growth phase and optimal temperature for histamine production is variable due to the difference in types and levels of microbial flora in fish. Histamine production has been shown to be maximal in the middle of exponential growth phase of histamine-producing bacteria (Omura et al., 1978; Yoshinaga and Frank, 1982). In the evaluation of a *M. morganii* strain for growth and histamine formation, it was observed that the rate of histamine accumulation in the culture medium was proportional to the growth, and the histidine decarboxylase activity was maximal in the middle of the exponential growth phase (Omura et al., 1978). On the contrary, other studies have shown that histamine formation may be maximal at the later stage of culture growth. According to Kurihara et al., (1993), the decarboxylating activity of a halophilic histamine-forming bacteria, *Photobacterium histaminum*, was highest at the beginning of the stationary phase of growth and decreased thereafter.

Many investigators have reported the optimal and growth-limiting temperature for *M. morganii* isolates and other frequently reported enteric bacteria. Eitenmiller et al.

(1981) reported that the optimal temperature of *M. morganii* GRM06 for histidine decarboxylating activity was 37°C. According to Behling and Taylor (1982), at 72 h of incubation, the optimal temperature for histamine production was 37°C for *Escherichia coli* and *Citrobacter freundii*; 30°C for *M. morganii* 110SC2, *Klebsiella pneumoniae*, and *Hafnia alvei*; and 15°C for *M. morganii* JM. The lower temperature limits for the production of toxicologically significant levels of histamine in TFIB were 7°C for *K. pneumoniae*, and 15°C for the both *M. morganii* strains. According to Arnold et al. (1980), *M. morganii* isolated from spoiled skipjack tuna developed elevated levels of histamine in the skipjack infusion broth at 19 and 30°C, and no histamine was produced at 1°C in 42 days. Klausen and Huss (1987) reported that *M. morganii* grew rapidly and produced the highest histamine level in histidine decarboxylase broth at 25°C. Histamine content reached 5,400 ppm at 40 h of incubation. At 5°C, no histamine was found. However, when the culture previously incubated at 25°C for 23 h was placed at 5°C, histamine was continually accumulated, showing an increase from 800 ppm to 2,700 ppm in 120 h of incubation.

It has been reported that temperature is the most critical factor to control the growth of histamine-producing bacteria and their histamine formation. In our previous study, 25°C was the optimum temperature for the histamine formation in albacore (Kim et al., 1999), and *M. morganii* OSL36 was isolated when the sample was completely decomposed. However, no histamine was found when fish were stored in ice up to 18 days. It has been reported that storage at 0°C or below can prevent histamine formation in fish muscle (Taylor and Speckhard, 1983). Although the effect of temperature between 2 and 10°C is not clear, several studies have reported that there is little or no

formation of histamine in this temperature range (Rawles et al., 1996). *M. morganii* OSL36 showed the typical characteristics of mesophilic bacteria, in that its growth and other metabolic activities were completely inhibited at 4°C for up to 14 days of incubation. In this study, the initial APC was 10^2 CFU/ml. Although the APC was low, the results were consistent with a report by López-Sabater et al. (1996). The growth and histamine formation of *M. morganii* inoculated at 5×10^5 CFU/g was inhibited at 8°C for up to 72 h of incubation. Various species of psychrotrophic bacteria, i.e., *Pseudomonas* spp., and psychrophilic halophilic bacteria, i.e., N-group bacteria, have been reported as histamine formers (Okuzumi et al., 1994). However, their role in the overall histamine accumulation in fish may be insignificant as compared to the mesophilic histamine formers, since histamine is rarely produced in fish stored at refrigeration temperature, as described above. Frank et al. (1985) reported that psychrotrophic isolates obtained from fish incubated for 14 days at 0°C were weak histamine formers, which produced less than 1 mg/100 ml at 5 and 20°C. Although *Pseudomonas fluorescens/putida* and *Pseudomonas putrefaciens* isolated from Spanish mackerel were detected as weak histamine formers (Middlebrooks et al., 1988), the strains isolated from ripened Spanish semi-preserved anchovies did not produce histamine (Rodríguez-Jerez et al., 1994). Ryser et al. (1984) suggested that histamine production by *P. fluorescens*, *P. putida*, and non-fluorescent pseudomonads were of little health significance. The 21 of 60 psychrophilic pseudomonads isolated from frozen tuna produced low levels of histamine, 0.2-3.4 mg per 100 ml, at 21°C. *Stenotrophomonas (Pseudomonas) maltophilia* isolated from frozen albacore was also confirmed as a weak histamine former. It produced 17.2 ppm of histamine in TSB supplemented with 2% histidine (Behling and Taylor, 1982).

The 16 psychrotrophic bacteria (*Alteromonas putrefaciens*) isolated from mahi-mahi stored at 0°C produced a negligible amount (<1 mg/100 ml) of histamine (Frank et al., 1985). It has been postulated that histidine is not a primary metabolic substrate in psychrotropic bacteria. Therefore, it has been suggested that inhibition of histamine formation at refrigeration or below temperatures is mainly due to the effective inactivation of prolific mesophilic histamine formers in fish.

Formation of biogenic amines in TFIB

M. morganii OSL36 not only produced histamine but also other biogenic amines, such as, cadeverine, putrescine, and phenylethylamine in TFIB medium (Fig. 3.3). However, its production of serotonin, tryptamine, tyramine, spermidine, and spermine was negligible. The temperature of 25°C was most optimum for the biogenic amine formation, as it was for histamine. For cadaverine, the highest level, 27.8 ppm, was produced at day 1, and the level decreased thereafter (Fig. 3.4). Although the level produced was lower than that for histamine, the trend of cadaverine formation was similar to that of histamine formation at 15°C. Cadaverine content reached the maximum level, 26.7 ppm, at day 3. The highest cadaverine level was detected in the stationary phase, as was shown for the histamine formation. The highest putrescine level, 18 ppm, was produced at day 2, whereas its formation at 37 and 15°C was insignificant (<3 ppm; Fig. 3.5). Although phenylethylamine production was detected, the amount was insignificant, as compared to other biogenic amines (Fig. 3.6). These results indicated that histamine accumulation by *M. morganii* OSL36 was accompanied by other biogenic amines. All biogenic amines detected showed the highest level of accumulation at 25°C,

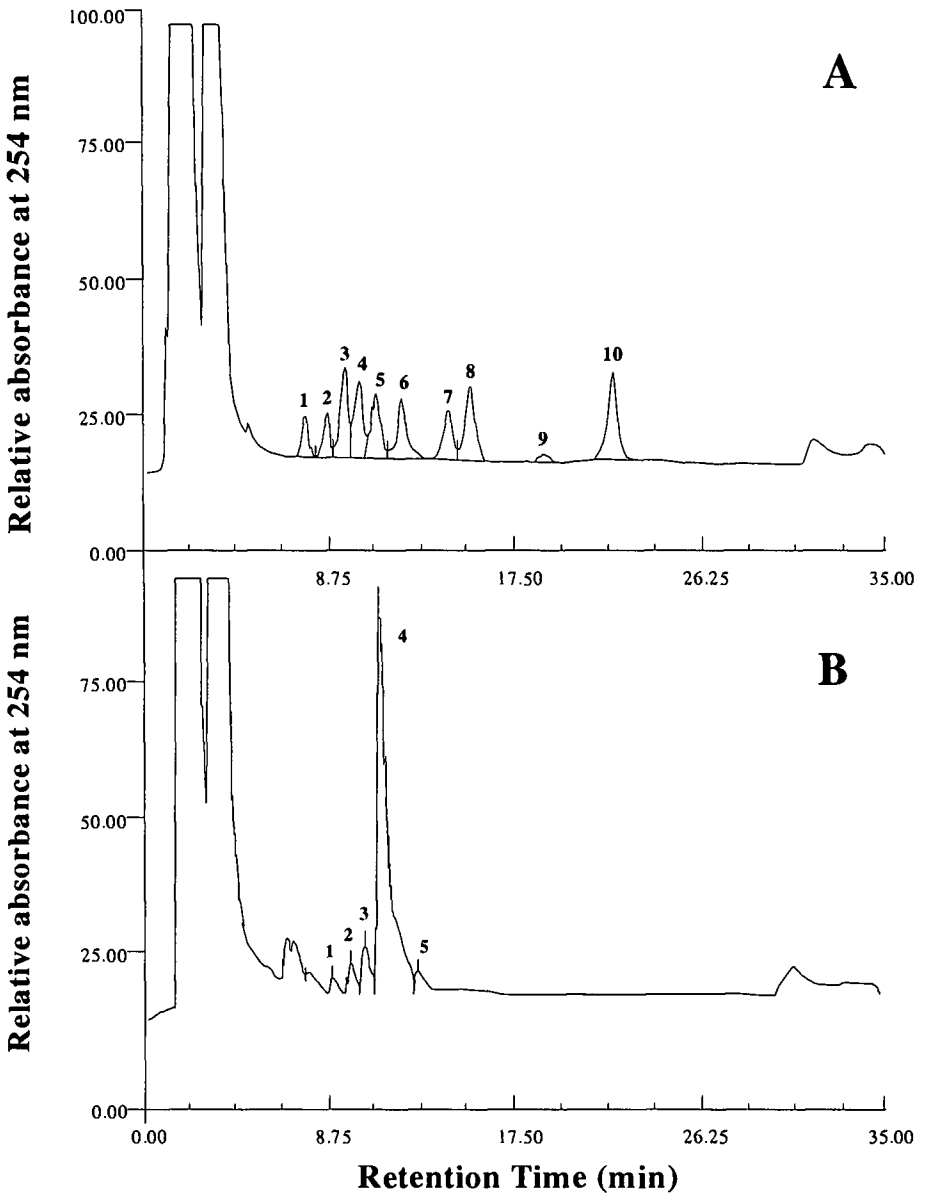

Fig. 3.3. Detection of biogenic amine in standard solution and samples by HPLC using the LiChrosorb RP-18 column. A. Chromatographic separation of dansyl derivatives of biogenic amines standards. 1. Tryptamine; 2. Phenylethylamine; 3. Putrescine; 4. Cadaverine; 5. Histamine; 6. 1,7-diaminoheptane; 7. Serotonin; 8. Tyramine; 9. Spermidine; 10. Spermine
B. Chromatographic separation of biogenic amines formed by *Morganella morganii* OSL36 in TFIB medium. 1. Phenylethylamine; 2. Putrescine; 3. Casaverine; 4. Histamine; 5. 1,7-diaminoheptane.

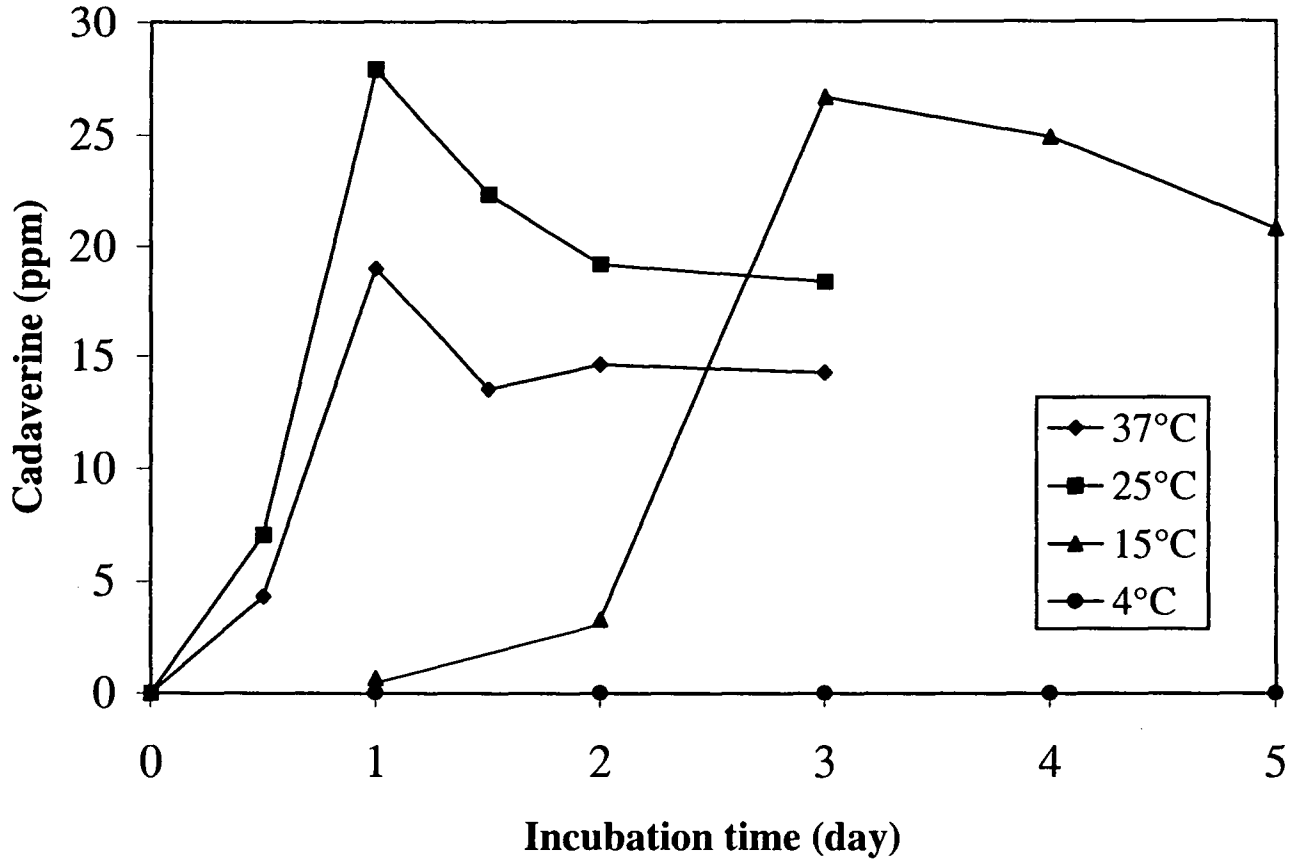

Fig. 3.4. Cadaverine formation of *Morganella morganii* OSL 36 in TFIB medium at 4, 15, 25, and 37°C. Cadaverine was determined by HPLC analysis.

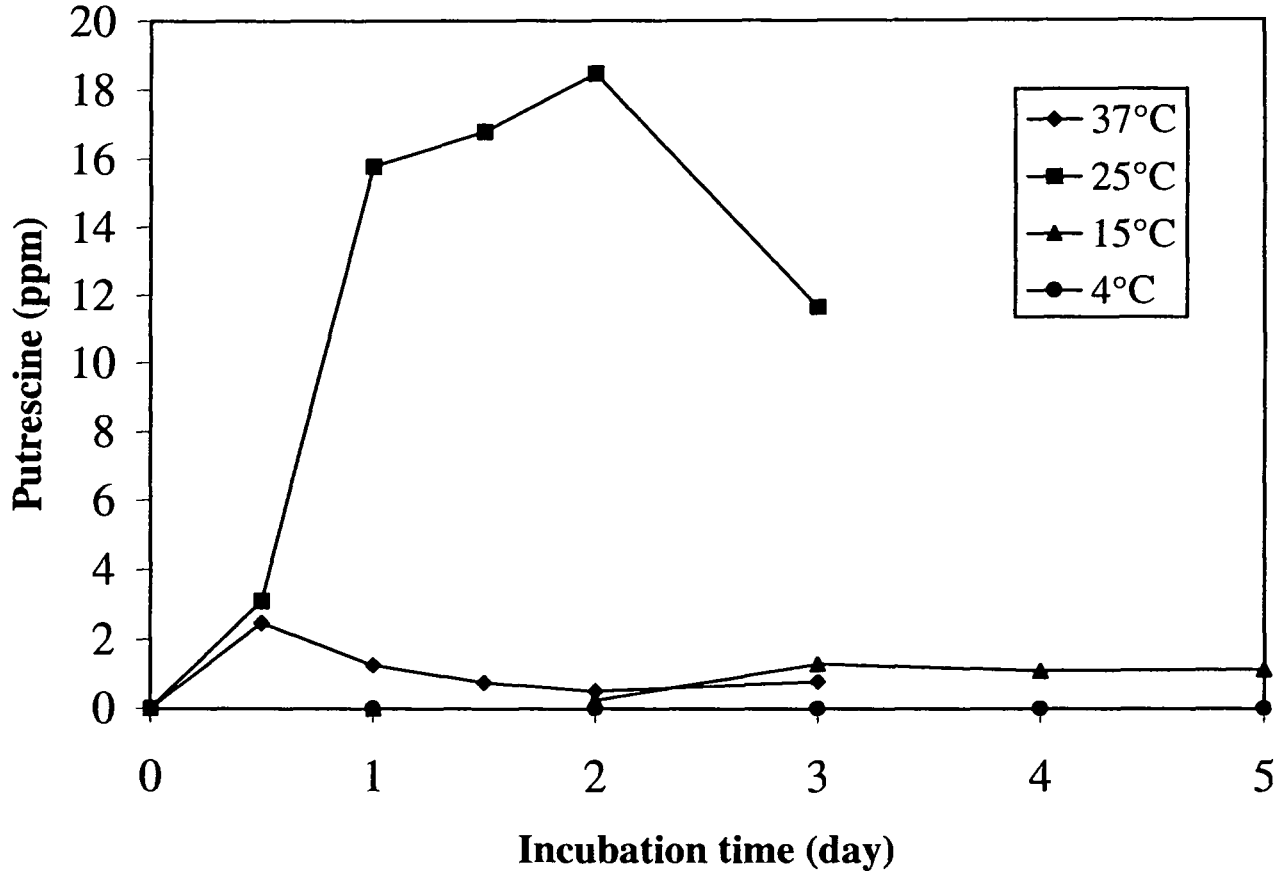

Fig. 3.5. Putrescine formation of *Morganella morganii* OSL 36 in TFIB medium at 4, 15, 25, and 37°C. Putrescine was determined by HPLC analysis.

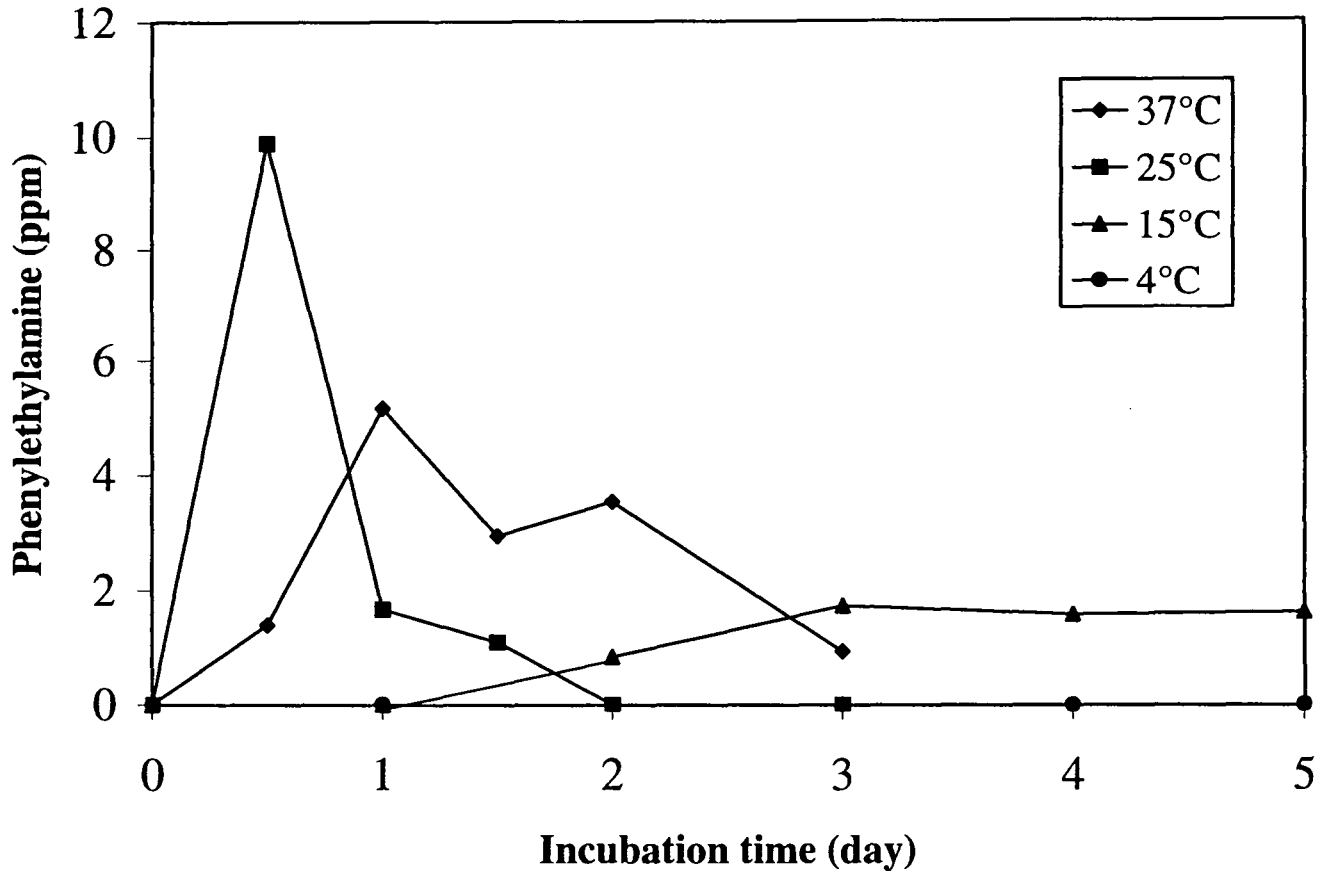

Fig. 3.6. Phenylethylamine formation of *Morganella morganii* OSL 36 in TFIB medium at 4, 15, 25, and 37°C. Phenylethylamine was determined by HPLC analysis.

like histamine, although the time required to reach the maximum levels differed.

Many microorganisms, especially within the *Enterobacteriaceae* family, are known to possess decarboxylase enzymes and, thus, are capable of producing putrescine, cadaverine, and other biogenic amines in addition to histidine decarboxylase producing histamine (Stratton et al., 1991). *Enterobacter aerogenes* produced the large amounts of histamine and cadaverine in mackerel muscle broth, 11,900 and 2,300 nmol/ml, respectively, at 24 h (Wendakoon and Sakaguchi, 1993). Babu et al. (Babu et al., 1986) reported *Streptococcus ceremoris* showed the highest growth rate and amine formation in 24 h at 30°C. The contents of histamine, tyramine, and tryptamine were 43.3, 75.8 and 3.8 μg/ml, respectively, in M-17 broth medium. According to Suzuki et al. (1988), *Aeromonas haloplanktis* produced 208 nmols/g putrescine, 31 nmols/g cadaverine, and 172 nmols/g spermidine, while *A. putrefaciens* produced 1,333; 83; and 56 nmols/g, respectively, in the nutrient broth supplemented with 50% artificial sea water at 25°C. Llano et al. (Liano et al., 1998) isolated tyramine-producing bacteria, lactococcal and leuconostoc strains, from raw milk and farmhouse cheeses. The high tyramine producer, accumulating from 370 to 807 mg/L tyramine, was also able to produce tryptamine, although it was less than 7 mg/L. *Stenotrophomonas maltophilia,* a weak histamine producer isolated from frozen albacore, produced up to 2,390 ppm of cadaverine after 24 h of incubation at 37°C in TSB supplemented with 1% lysine (Behling and Taylor, 1982).

Histamine is not the only compound responsible for scombroid poisoning. It did not trigger the toxic response typical for scombroid poisoning when the compound is given orally (Rodríguez-Jerez et al., 1994; Taylor, 1986). Scombroid poisoning is, thus, caused by histamine acting synergistically with other diamines present in the fish,

primarily putrescine and cadaverine. Its toxicity is increased in the presence of these amines by inhibiting the histamine detoxifying enzymes, i.e., diamine oxidase and histamine N-methyltransferase (Hui and Taylor, 1985; Hungerford and Arefyev, 1992). Other biogenic amines, such as tyramine and phenylethylamine, may act as potentiators by inhibiting diamine oxidase (Stratton et al., 1991).

TFIB medium was developed to evaluate bacterial ability to produce histamine in tuna (Rawles et al., 1996). In this study, TFIB was used to detect the formation of biogenic amines and to show their correlation with histamine formation. The media supported the growth and the formation of histamine and other biogenic amines by *M. morganii* OSL36. Among the free amino acids reported in albacore muscle, histidine was the highest in fresh muscle, 2,362 mg/100 g, followed by arginine, 341.1 mg/100 g; taurine, 178.7 mg/100 g; and lysine, 108.0 mg/100 g (Perez-Martin et al., 1988). The level of these free amino acids did not significantly change after cooking or sterilization. According to Fletcher et al. (1995), albacore implicated in scombroid poisoning in New Zealand contained 4,280 mg of free histidine/100 g of white muscle. Although the amino acids content in the tested medium was not determined in this study, these reports showed albacore is a good substrate for decarboxylation reaction by microorganisms. As described above, several microorganisms, including *M. morganii* OSL36 reported in this study, were determined to be multiple biogenic amine producers (Stratton et al., 1991, Wendakoon and Sakaguchi, 1993). Therefore, we speculate that toxicological effect of histamine may be enhanced by the proliferation of these bacteria capable of producing both histamine and other potentiating biogenic amines.

Acknowledgments

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References

Arnold, S.H., Price, R.J., and Brown, W.D. 1980. Histamine formation by bacteria from skipjack tuna, *Katsuwonus pelamis*. Bull. Jpn. Soc. Sci. Fish. 46: 991-995.

Babu, S., Chander, H., Batish, V.K., and Bhatis, K.L. 1986. Factors affecting amine production in *Streptococcus ceremoris*. Food Microbiol. 3: 359-362.

Barancin, C.E., Smoot, J.C., Findlay, R.H., and Actis, L.A. 1998. Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen *Vibrio anguillarum*. Plasmid. 39: 235-244.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., and Turc, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-496.

Ben-Gigirey, B., Vieites-Baptista de Sousa, J.M., Villa, T.G., and Barros-Velazquez, J. 1999. Histamine and cadaverine production by bacteria isolated from fresh and frozen albacore (*Thunnus alalunga*). J. Food Prot. 62: 933-939.

Behling, A.R. and Taylor, S.L. 1982. Bacterial histamine production as a function of temperature and time of incubation. J. Food Sci. 47: 1311-1317.

Eerola, S., Hinkkanean, R., Lindfords, E., and Hirvi, T. 1993. Liquid chromatographic determination of biogenic amines in dry sausages. J. AOAC Int. 76: 575-577.

Eitenmiller, R.R., Wallis, J.W., Orr, J.H., and Phillips, R.D. 1981. Production of histidine decarboxylase and histamine by *Proteus morganii*. J. Food Prot. 44: 815-820.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species, particularly kahawai (*Arripis trutta*). J. Aquat. Food Prod. Technol. 4: 53-74.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th Ed. Arlington, VA.

Food and Drug Administration. 1994. Proposed rules. Fed. Reg. 59: 4149.

Frank, H.A., Branowski, J.D., Chongsiriwatana, M., Brust, P.A., and Premaratne, R.J. 1985. Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32°C. Int. J. Food Microbiol. 2: 331-340.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. 1994. Bergey's manual of determinative bacteriology, 9th ed. Williams and Wilkins Co., Baltimore.

Hui, J.Y. and Taylor, S.L. 1985. Inhibition of in vivo histamine metabolism in rats by foodborne and pharmacologic inhibitors of diamine oxidase, histamine N-methyltransferase, and monoamine oxidase. Toxicol. Appl. Pharmacol. 8: 241-249.

Hungerford, Y.M. and Arefyev, A.A. 1992. Flow-injection assay of enzyme inhibition in fish using immobilized diamine oxidase. Anal. Chimica. Acta. 261: 351-359.

Jay, J.M. (ed.). 1996. Ch.15. Low temperature food preservation and characteristics of psychrotrophic microorganisms. p. 330-342. *In* Modern Food Microbiology. 5th Ed., ITP. New York, NY.

Joosten, H.M.L.J. and Northolt, M.D. 1989. Detection, growth and amine producing capacity of lactobacilly in cheese. Appl. Environ. Microbiol. 55: 2356-2359.

Kim, S.H., An, H., and Price, R.J. 1999. Histamine formation and bacterial spoilage of albacore harvested off the U.S. Northwest Coast. J. Food. Sci. 64: 340-343.

Klausen, N.K. and Huss, H.H. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

Kurihara, K., Wagatuma, Y., Fujii, T., and Okuzumi, M. 1993. Effect of reaction conditions on L-histidine decarboxylation activity of halophilic histamine-producing bacteria. Nippon Suisan Gakkaishi 59: 1745-1748.

Llano, D.G., Cuesta, P., and Rodríguez, A. 1998. Biogenic amine production by wild lactococcal and leuconostoc strains. Letters Appl. Microbiol. 26: 270-274.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., and Mora-Ventura, M.A.T. 1994. Evaluation of histidine decarboxylase activity of bacteria isolated from sardine (*Sardina pilchardus*) by an enzymatic method. Letters Appl. Microbiol. 19: 70-75.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., Roig-sagués, A.X., and Mora-Ventura, M.A.T. 1996. Sensory quality and histamine formation during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

Maijara, R.L. 1993. Formation of histamine and tyramine by some lactic acid bacteria in MRS-broth and modified decarboxylation agar. Letters Applied Microbiol. 17: 40-43.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Niven, C.F., Jeffrey, M.B., and Corlett, D.A. 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. Appl. Environ. Microbiol. 41: 321-322.

Okuzumi, M., Hiraishi, A., Kobayashi, T., and Fujii, T. 1994. *Photobacterium histaminum* sp. nov., a histamine-producing marine bacterium. Int. J. Syst. Bacteriol. 44: 631-636.

Omura, Y., Price, R.J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Perez-Martin, R.I., Franco, J.M., Aubourg, S., and Gallardo, J.M. 1988. Changes in free amino acids content in albacore (*Thunnus alalunga*) muscle during thermal processing. Z. Lebensm. Unters. Forsch. 187: 432-435.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish shellfish. Adv. Food Nutr. Res. 39: 329-364.

Rodríguez-Jerez, J.J., López-Sabater, E.I., Roig-Sagués, A.X., and Mora-Ventura, M.T. 1994. Histamine, Cadaverine and putrescine forming bacteria from ripened Spanish semi-preserved anchovies. J. Food Sci. 59: 998-1001.

Rodríguez-Jerez, J.J., Mora-Ventura, M.T., López-Sabater, E.I., and Hernández-Herrero, M. 1994. Histidine, lysine and orinithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. J. Food Prot. 57: 784-787.

Roig-Sagués, A.X., Hernández-Herrero, M., López-Sabater, E.I., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1996. Histidine decarboxylase activity of bacteria isolated from raw and ripened *Salchichón*, a Spanish cured sausage. J. Food Prot. 59: 516-520.

Ryser, E.T., Marth, E.H., and Taylor, S.L. 1984. Histamine production by psychrotrophic pseudomonads isolated from tuna fish. J. Food Prot. 47: 378-380.

Stratton, J.E., Hutkins, R.W., and Taylor, S.L. 1991. Biogenic amines in cheese and other fermented foods: a review. J. Food Prot. 54: 460-470.

Suzuki, S., Matsui, Y., and Takama, K. 1988. Profiles of polyamine composition in putrefactive Pseudomonas type III/IV. Microbios. Letters. 38: 105-109.

Taylor, S.L. 1978. Histamine production by food-borne bacterial species. J. Food Safety. 1: 173-187.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S.L. 1986. Histamine food poisoning: Toxicology and clinical aspects. CRC Crit. Rev. Toxicol. 17: 91-117.

Taylor, S.L., Stratton, J.E., and Nordlee, J.A. 1989. Histamine poisoning (Scombroid fish poisoning): An allergy-like intoxication. Clin. Toxicol. 27: 225-240.

Wei, C.I., Chen, C.M., Koburger, J.A., Otwell, W.S., and Marshall, M.R. 1990. Bacterial growth and histamine production on vacuum packaged tuna. J. Food Sci. 55: 59-63.

Wendakoon, C.N. and Sakaguchi, M. 1993. Combined effect of sodium chloride and clove on growth and biogenic amine formation of *Enterobacter aerogenes* in mackerel muscle extract. J. Food Prot. 56: 410-413.

Yatsunami, K. and Echigo, T. 1993. Changes in the number of halotolerant histamine-forming bacteria and contents of non-volatile amines in sardine meat with addition of NaCl. Nippon Suisan Gakkashi 59: 123-127.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamia*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 4

Source and Identification of Histamine-Producing Bacteria from Fresh and Temperature-Abused Albacore

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Abstract

Histamine-producing bacteria were isolated from fresh and temperature-abused albacore using two different isolation procedures. Typically the bacterial isolates on Niven's or modified Niven's medium produced negligible or low levels of histamine (<300 ppm) in histamine enumeration broth. The most frequently found species using this approach was *Hafnia alvei.* By prescreening on selective media (EMB agar for enteric bacteria; MRS agar for lactic acid bacteria; KF agar for streptococci; PI agar for pseudomonads; and S110 agar for staphylococci) prior to plating on histidine decarboxylase differential media, detection rate of true histamine formers increased. Prolific histamine producers capable of forming >1,000 ppm histamine in culture broth were isolated when PI and EMB agars were used for prescreening. Among the selective media tested, EMB agar was most effective in selecting high histamine producers as demonstrated by the highest rate of true positives based on histamine analysis. Histamine-producing isolates were mostly enteric bacteria, including *Morganella morganii*, *H. alvei*, *Klebsiella* spp., *Citrobacter freundii*, *Enterobacter* spp., and *Serratia* spp. *M. morganii* isolated on PI agar from temperature-abused albacore muscle was found to be the highest histamine former. This species was not isolated from fresh albacore, while other enteric bacteria were frequently detected on the gills. However, only a few species isolated from both fresh and temperature-abused muscles were identified as high histamine formers.

Introduction

Bacteria containing histidine decarboxylase are the main source of histamine formation in scombroid fish. This enzyme is responsible for the conversion of free histidine in fish muscle to histamine, commonly known as the scombroid toxin. Although only *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei* are isolated from fish that were incriminated in scombroid poisoning (Rawles et al., 1996; Taylor and Speckhard, 1983), a variety of bacteria capable of producing histamine have been identified in fish (Eitenmiller et al., 1981; Middlebrooks et al., 1988; Taylor, 1986; Yoshinaga and Frank, 1982). Among them, enteric bacteria have been reported to be the most important histamine-producing bacteria in fish, with *M. morganii* consistently shown as a high histamine former both in fish and culture broth (Ababouch et al., 1991; Klausen et al., 1987; López-Sabater et al., 1994). *Proteus vulgaris, P. mirabilis, Enterobacter aerogenes, E. cloacae, Serratia fonticola, S. liquefaciens,* and *Citrobacter freundii* were also isolated as histamine formers in fish (López-Sabater et al., 1996). In addition to the enteric bacteria, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lowffi*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *P. fluorescens*, *Aeromonas* spp., and *Photobacterium* spp. have been reported as histamine producers (López-Sabater et al., 1994; Middlebrooks et al., 1988; Okuzumi et al., 1994; Ryser et al., 1984; Yatsunami and Echigo, 1993).

Recovery of histamine-producing bacteria has been a great concern for rapid detection of histamine producers. Efforts to isolate histamine-producing bacteria have been hampered by the lack of reliable isolation media. Niven's medium (Niven et al., 1981) has been most widely used to isolate the bacteria. However, Niven's medium

generates extremely high rates of false-positives and false-negatives, presumably due to the presence of competing non-histamine producers (Kim et al., 2000; Taylor, 1986). The medium has been modified to develop other differential media, such as, modified Niven's, Joosten/Northolt, and Maijara, to enhance detection of histamine-forming lactic acid bacteria in cheese, meat, fish and poultry products (Joosten and Northolt, 1989; Maijala, 1993) and to screen histamine-producing bacteria from ripened sausages (Roig-Sagués et al., 1997). However, detection of histamine formers using the modified media alone is still susceptible to false reactions due to the formation of different alkaline compounds and fermentative activity of bacteria (Fletcher et al., 1995; Rodríguez-Jerez et al., 1994). Therefore, a prescreening step was included prior to screening on differential media for histidine decarboxylase, which improved the true isolation rate of histamine-forming bacteria (Roig-Sagués et al., 1997).

It is believed that histamine-producing bacteria are present in fresh fish, although they may be present in negligible numbers (Frank et al., 1985; Klausen and Huss, 1987; Omura et al., 1978). Little information has been available either for histamine formers in fresh fish or their sources in the marine environment. Therefore, the objective of this study was to detect source of histamine formers in albacore by monitoring histamine-forming bacteria in the gill, skin, and intestine of fresh and temperature-abused fish. In this study, histamine formers were isolated by the one-step method using Niven's or modified Niven's medim and the two-step method of prescreening on selective media followed by screening on differential media. Isolated histamine-producing bacteria were identified by species, and prolific histamine producers were screened for their capacities

to produce histamine above 1,000 ppm in histidine-containing culture broth according to the criteria described by Behling and Taylor (Behling and Taylor, 1982).

Materials and Methods

Reagents

Kovacs' reagent was obtained from Difco Laboratories. (Detroit, MI). Histamine dihydrochloride, leucocrystal violet, porcine kidney diamine oxidase, horseradish peroxidase, bromocresol purple, *o*-phthaldialdehyde, dowex 1-X8, and tetramethyl-*p*-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO).

Media

Trypticase soy broth and KF streptococcus (KF) agar were purchased from BBL Co. (Cockeysville, MD). Plate count agar, tryptone broth, eosin methylene blue (EMB) agar, de Man Rogosa Sharpe (MRS) agar, staphylococcus medium 110 (S110), and pseudomonas isolation (PI) agar were obtained from Difco.

Five differential media used for isolation of histamine formers were as follows: The Niven's medium was comprised of 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $CaCO_3$, 2% agar, 0.006% bromocresol purple, and 2.7% histidine 2 HCl, pH 5.3 (Niven et al., 1981). For the modified Niven's medium, we decreased the histidine concentration to 1% and supplemented with glucose at 0.5% to minimize positive reaction by non-histamine formers and adjusted pH to 5.7 to generally improve the

bacterial growth rate. The final composition used was 0.5% glucose, 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $CaCO_3$, 2% agar, 0.006% bromocresol purple, and 1% histidine HCl, pH 5.7. Joosten/Northolt medium contained 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, 0.05% Tween 80, 0.02% $MgSO_4$ 7 H_2O, 0.01% $CaCO_3$, 0.006% bromocresol purple, 0.005% $MnSO_4$ 4 H_2O, 0.004% $FeSO_4$ 7 H_2O, 2% agar, and 2% histidine (Joosten and Northolt, 1989). Maijala medium contained 0.5% tryptone, 0.8% Lab-Lemco powder (Oxoid Limited, Hampshire, U.K.), 0.4% yeast extract, 0.05% Tween 80, 0.02% $MgSO_4$ 7 H_2O, 0.01% $CaCO_3$, 0.006% bromocresol purple, 0.005% $MnSO_4 \cdot$ 4 H_2O, 0.004% $FeSO_4 \cdot$ 7 H_2O, 2% agar, and 2% histidine (Maijala, 1993). The histamine enumeration (HE) medium used to confirm histamine production by the isolates contained 0.5% tryptone, 0.5% NaCl, 0.25% K_2HPO_4, and 1% histidine HCl, pH 5.7 (Rodríguez-Jerez et al., 1994).

Samples and sample treatment

Albacore were commercially troll-caught 100 miles off the Oregon coast, kept in ice on board, and delivered to a local processing facility in Astoria, OR. Whole albacore were transported in ice to Oregon Sate University-Seafood Laboratory and used immediately for bacterial isolation or storage test without washing or further handling.

To monitor proliferation of histamine-forming bacteria and histamine formation in fish, seven whole albacore were individually placed in sterile plastic bags and stored at 25°C for 7 days. Each fish was taken every 24 h for aerobic plate count (APC), histamine analysis, and enumeration of histamine formers on Niven's differential medium. Each analysis was carried out in duplicate. For isolation and identification of histamine

formers, three fish were stored at 25°C up to 14 days, and histamine formers were isolated using both one- and two-step procedures. On days of testing (5, 8, and 14 days), 10-g samples were aseptically removed from dorsal muscle for total APC and enumeration and isolation of histamine-producing bacteria. Another set of 10-g muscle samples was removed for histamine analysis.

APC and enumeration of histamine-forming bacteria

The 10-g muscle samples were blended in 90 ml of peptone water (0.1%) and serially diluted (FDA, 1992). A 1-ml sample was taken from the diluted samples and mixed with plate count agar for enumeration of APC. Another 1-ml sample was taken for enumeration of histamine-producing bacteria and mixed with the Niven's differential medium (Niven et al., 1981). Enumeration of the APC and histamine-producing bacteria was carried out in duplicate, and plates were incubated at 25°C for 2 days.

Histamine analysis of muscle samples

Histamine in albacore muscle was analyzed in duplicate by the standard fluorometric method (AOAC, 1995). Dorsal muscle (10 g) was homogenized in 50 mL methanol for 2 min and heated in a water bath at 60°C for 15 min. After cooling to 25°C, the volume was adjusted to 100 mL with methanol and filtered through Whatman #1 paper. The methanol filtrate was collected and loaded onto an ion exchange column (200 × 7 mm) packed with Dowex 1-X8. The column eluant was derivatized with *o*-phthaldialdehyde, and the fluorescence intensity was determined using a spectrophotofluorometer (Perkin-Elmer, Norwalk, CT) at excitation wavelength of 350 nm and emission wavelength of 444 nm.

One-step isolation of histamine-producing bacteria

The gill, intestine, and skin of fresh fish were swabbed with cotton and inoculated into trypticase soy broth supplemented with 0.5% NaCl (Omura et al., 1978). After incubation at 25°C for 24 h, one loopful was streaked onto two different decarboxylase media, the Niven's and the modified Niven's medium. Dorsal muscles were sampled from fish during storage at 25°C, and APC were performed as described above. Samples were plated both on the Niven's and the modified Niven's medium. The plates were incubated at 25°C for 24 h. Positive colonies on the Niven's and the modified Niven's medium were picked based on their morphological characteristics. Only purple colonies with a purple halo on the yellow background on Niven's medium were considered positive for histamine production (Niven et al., 1981). On the modified Niven's medium, purple colonies with or without haloes were regarded as positive. On the modified Niven's medium, negative colonies appeared as yellow or white.

Two-step isolation of histamine-producing bacteria

Isolation of histamine-producing bacteria was also carried out by the two-step procedure of prescreening for a target group of bacteria on selective media and screening for histidine decarboxylase activity on differential media. The target bacteria were enteric bacteria, lactic acid bacteria, streptococci, staphylococci, and pseudomonads, which have been previously reported as histamine formers (Maijala, 1993; Middlebrooks et al., 1988; Ryser et al., 1984). The selective media used were EMB agar for enteric bacteria; MRS agar for lactic acid bacteria; KF agar for streptococci; S110 for staphylococci; and PI agar for pseudomonads. Samples were blended and diluted as

described. One milliliter of each diluent was mixed with plate count agar for enumeration of APC or selective media for isolation of histamine formers.

Colonies obtained from each plate were picked based on their morphological characteristics. They were then inoculated into tryptic soy agar slants for further testing. To screen histamine formers, the strains were further inoculated onto three differential media: Niven, Joosten/Northlot, and Maijara media. After the plates were incubated at 37°C for 24 to 48 h, purple colonies with purple halos were scored as histamine producers.

Confirmation of histamine formation by isolates

Histamine formation by the presumptive histamine producers was confirmed by analyzing the culture broth for histamine. The isolates of presumptive producers were inoculated into HE medium and incubated at 37°C for 24 h. Each bacterial culture was centrifuged at 10,000 rpm for 10 min, and the supernatant was analyzed for histamine by enzymatic analysis according to the method of Rodríguez-Jerez et al. (1994). One-half milliliter of supernatant was mixed with 1.0 ml 0.15 M phosphate buffer (0.075M KH_2PO_4 and 0.075M Na_2HPO_4, pH 6.8), 0.5 ml diamine oxidase (0.35 U/ml), 0.5 ml peroxidase (17.5 U/ml), and 0.1 ml leucocrystal violet (0.5 mg/ml), and the mixture was incubated at 37°C for 15 min. Intensity of the purple color developed was measured spectrophotometrically at 596 nm and compared to those of histamine standards (3 to 30 ppm) for quantification of histamine. Blank was prepared with HE medium instead of samples.

Identification of histamine producers

The bacterial isolates confirmed to produce histamine in HE medium were identified to species by using the Vitek instrument (bioMérieux Vitek, Inc., Hazelwood, MI) according to the protocol recommended by the manufacturer. The isolates were inoculated into tryptic soy agar slants and incubated at 37°C for 18 h. Gram staining was carried out to select a proper identification card. Oxidase tests, prerequisite for identifying gram-negative bacteria, were carried out using the filter paper method (Benson, 1992) as follows. One loopful of each culture was smeared on a piece of filter paper dampened with oxidase test reagent (1% tetramethyl-*p*-phenylenediamine dihydrochloride). A positive reaction was detected by the change of color within 10 s. After the oxidase test, the cultures were suspended in phosphate saline buffer provided with the Vitek identification kit at the concentration range determined to be suitable by the Vitek colorimeter. A suspension was placed in a Vitek identification card. The digitalized analog optical reading and the bacterial identification were automatically carried out by the Vitek system.

The additional biochemical tests necessary for definitive species identification were carried out as follows. To differentiate two species of *Klebsiella*, an indole test was carried out by the standard method (Benson, 1992). One loopful of culture was inoculated into tryptone broth. One-tenth milliliter of Kovacs' reagent was added into bacterial culture after incubation at 37°C for 24 h, and the change of color was monitored. For *Pseudomonas* species, gelatin utilization was tested by liquification of gelatin medium (Benson, 1992). One loopful of culture was inoculated into gelatin medium and incubated at 22°C for 10 days.

Results and Discussion

Proliferation of histamine-producing bacteria in decomposing albacore

Histamine levels and bacterial counts in albacore were monitored during storage at 25°C (Fig. 4.1). The highest level of histamine, 60.4 mg/100 g, was detected after fish became obviously decomposed, judged by off-odor and appearance. The APC reached 10^7 CFU/g in 5 days of storage. The presumptive histamine-producing bacteria were enumerated by using the Niven's medium. The change of color showing positive reaction on the Niven's medium was easily detectable within the 24-h incubation. On day 6 of storage, the APC reached 10^8 CFU/g, and the histamine-producing bacterial count was 10^7 CFU/g. Generally, counts of histamine-producing bacteria enumerated on the Niven's medium were one log cycle lower than the APC. Although the Niven's medium has been widely used for quantitative enumeration of histamine-producing bacteria (Ababouch et al., 1991; Fletcher et al., 1995), it has a tendency to produce false results. Previously, Kim et al. (2000) reported that most colonies detected as positive for histamine formation on the Niven's medium actually produced a negligible amount of histamine when cell culture broths were analyzed by the high-pressure liquid chromatography method. Also, *Morganella morganii*, originally detected as negative on the Niven's medium, was confirmed to produce the highest level of histamine (2,154 ppm) among the tested isolates (Kim et al., 2000).

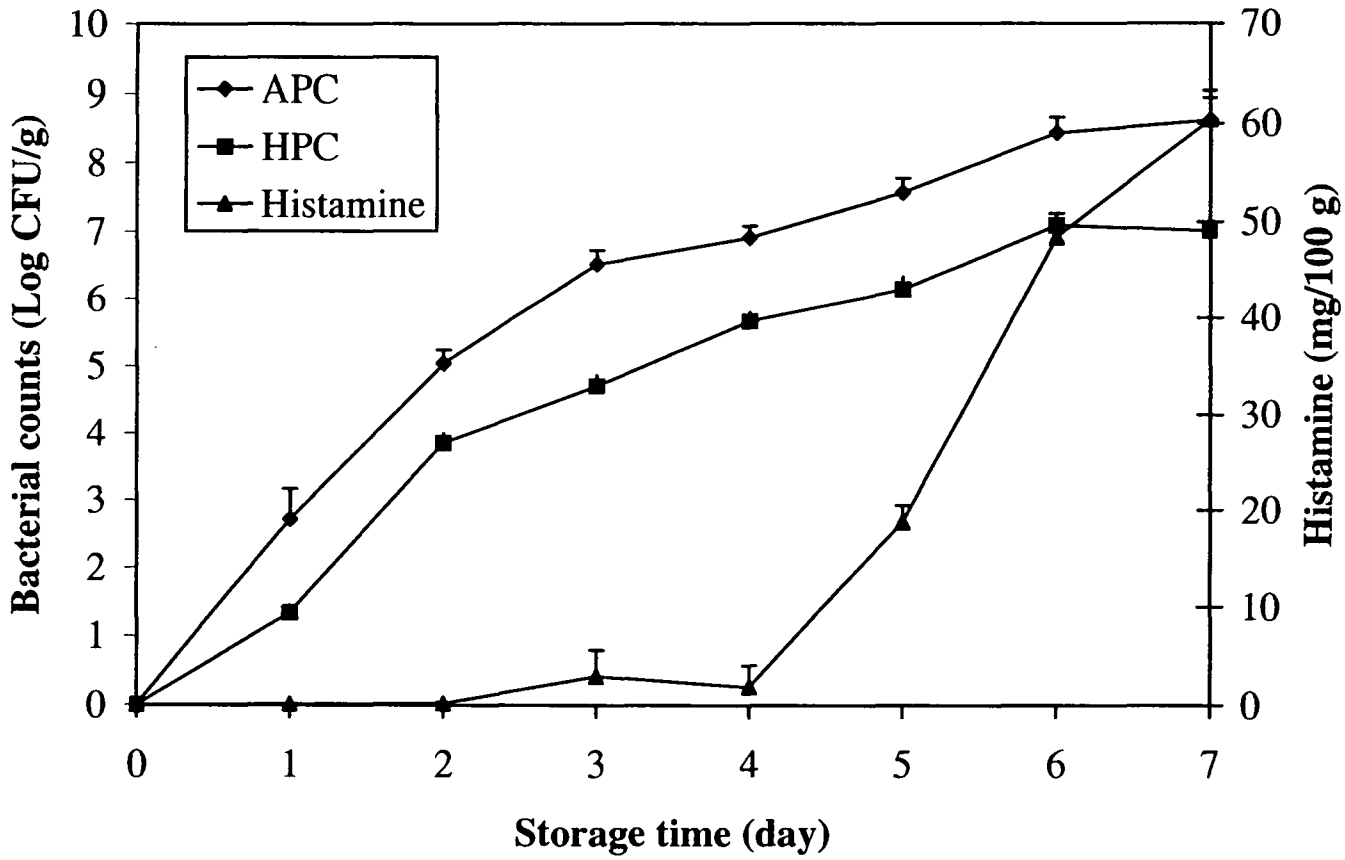

Fig. 4.1. Bacterial and histamine changes in albacore during storage at 25°C. APC and histamine-producing bacterial counts were determined using plate count agar and Niven's medium, respectively. Histamine was analyzed by the AOAC fluorometric method.

One-step screening of histamine-producing bacteria and the limitation of differential media

Most presumptive isolates from the Niven's medium were subsequently confirmed as false positives by analyzing histamine content in culture broth. The isolates only produced a negligible amount of histamine (<20 ppm) in the culture media when analyzed using the enzymatic histamine analysis method. Compared to the Niven's medium, the modified Niven's medium was more effective in isolating histamine-producing bacteria. A few true histamine producers were isolated using the medium, although the histamine concentrations were low, ranging from 30 to 300 ppm in the HE medium during the confirmation step (Table 4.1). An isolate obtained from the skin of fresh albacore, *Klebsiella oxytoca*, produced the highest level of histamine among all the isolates from fresh albacore, accumulating 343 ppm histamine in the culture broth. A psychrotroph, *Pseudomonas putida*, producing 275 ppm histamine, was also detected from the skin. *Hafnia alvei* and four *Enterobacter* isolates were identified from the gill of fresh albacore. The *H. alvei* isolate produced 108 to 153 ppm histamine, and three *Enterobacter aerogenes* isolates produced 52.9 to 97.5 ppm histamine. Three *Enterobacter* spp. that could not be differentiated to species produced similar levels of histamine to those of *E. aerogenes*.

Since histamine formers could not be isolated from fresh albacore using the Niven's medium, only the modified Niven's medium was used to isolate histamine-producing bacteria from temperature-abused albacore (Table 4.2). The isolates obtained from the modified Niven's medium produced histamine ranging from 10.2 to 76.3 ppm in culture broth, and they were confirmed as weak histamine formers. *Hafnia alvei* was

Table 4.1. One step isolation and identification of histamine-producing bacteria from fresh albacore using the modified Niven's medium

Origin	Strain[a]	Histamine (ppm)[b]
Gill	*Hafnia alvei*	153
	Hafnia alvei	123
	Hafnia alvei	119
	Hafnia alvei	112
	Hafnia alvei	108
	Enterobacter aerogenes	97.5
	Enterobacter spp.	81.8
	Enterobacter aerogenes	80.6
	Enterobacter spp.	78.9
	Enterobacter spp.	57.3
	Enterobacter aerogenes	52.9
	N. I.[c]	15.7
	N. I.	15.0
	N. I.	14.9
	N. I.	13.6
	N. I.	13.3
	N. I.	12.1
	N. I.	11.1
Skin	*Klebsiella oxytoca*	344
	Klebsiella oxytoca	282
	Pseudomonas putida	275
	Citrobacter braakii	244
	Klebsiella oxytoca	236
	Klebsiella oxytoca	70.4
	Klebsiella oxytoca	50.4
	Klebsiella pneumoniae	30.9
Gut cavity	None[d]	-

[a]Confirmed histamine-producing bacteria were identified by the Vitek system.
[b]One loopful of culture was inoculated into the histamine enumeration medium and incubated at 37°C for 24 h. Histamine production by the isolates was determined by the enzymatic histamine analysis method.
[c]Not identified
[d]Histamine-producing bacteria detected from the gut cavity produced a negligible amount of histamine in the culture media.

frequently isolated from both fresh and temperature-abused albacore on the modified Niven's medium. Other enteric bacteria identified, *Klebsiella oxytoca*, *Citrobacter braakii*, and *Serratia liquefaciens*, produced low levels of histamine in the culture medium. The weak histamine formers producing less than 15 ppm histamine were not further identified in this study.

Table 4.2. One step isolation and identification of histamine-producing bacteria from temperature-abused albacore using the modified Niven's medium

Strain[a]	Histamine (ppm)[b]	Storage days[c]
Hafnia alvei	76.3	14
Hafnia alvei	48.5	14
Hafnia alvei	32.2	14
Hafnia alvei	27.6	14
Klebsiella oxytoca	22.3	14
Hafnia alvei	19.2	14
Citrobacter braakii	17.0	14
Hafnia alvei	16.8	14
Hafnia alvei	16.7	14
Serratia liquefaciens	15.9	14
N. I.[d]	15.4	14
N. I.	13.9	8
N. I.	13.5	14
N. I.	13.4	8
N. I.	12.9	8
N. I.	11.5	8
N. I.	11.3	14
N. I.	10.2	8

[a]Confirmed histamine-producing bacteria were identified by the Vitek system.
[b]One loopful of culture was inoculated into the histamine enumeration medium and incubated at 37°C for 24 h. Histamine production by the isolates was determined by the enzymatic histamine analysis method.
[c]Albacore were stored at 25°C. Histamine formers were isolated from fish stored for 8 and 14 days.
[d]Not identified

Niven's medium was developed by modifying the conventional Moeller broth, which is used in the amino acid decarboxylation test for the identification of *Enterobacteriaceae* (Niven et al., 1981). The standard method used for the detection of amino acid decarboxylase activities is carried out in a basal medium containing glucose and the appropriate amino acid as a substrate (Shelef et al., 1998). The utilization of glucose lowers the medium pH, which in turn causes the change of the indicator color from purple to yellow. As bacteria decarboxylate the amino acids, the pH of the medium rises again. Therefore, decarboxylation is detected by the change of color to purple accompanying amine production, whereas negative reactions remain yellow. In the Niven's medium, histamine producers are differentiated from non-producers by the pH change resulting from histidine utilization. However, medium pH can also be affected by other microbial metabolic reactions. Microorganisms can use other nutrients in the medium as energy sources in the absence of carbohydrates (Cappuccino and Sherman, 1992). A semidigested protein such as peptone and tryptone can be degraded by microbial enzymes to amino acids, which, in turn, can be enzymatically converted to ketoamino acids by oxidative deamination. These are then metabolized through the Krebs cycle for energy production. These reactions liberate ammonia by forming ammonium hydroxide, providing an alkaline environment in the medium (Cappuccino and Sherman, 1992). Therefore, it has been postulated that an increase in pH can also be caused by non-histamine producers by utilization of histidine or other semidigested proteins in the absence of glucose or other carbohydrates in the medium. We speculate that the modification of the Niven's medium, by reducing histidine concentration and supplementing with glucose, helped to

reduce non-specific bacterial growth and improved detection rate of true histamine producers.

The limitation of the modified Niven's medium was that it failed to detect high histamine formers, capable of producing histamine above 1,000 ppm, in fresh or decomposed fish (Tables 4.1 and 4.2). In general, differential media do not preclude the growth of other microorganisms but can distinguish morphologically and biochemically related groups of organisms. The growth and activity of microorganisms can be modulated by competitive and inhibitory mechanisms among cells in the microflora as well. Therefore, we speculate that the high rate of false positives isolated on the differential medium alone was due to the outgrowth of non-histamine formers in the mixed culture.

Proliferation of spoilage bacteria during storage of albacore

Different groups of spoilage bacteria were monitored during storage of albacore at 25°C by using selective media to identify predominant bacterial groups. The APC reached 10^8 CFU/g in 7 days of storage (Fig. 4.2). Although the counts were high and the fish were apparently spoiled by then, the fish were further incubated up to 14 days to enhance detection rate of prolific histamine-forming bacteria. The highest bacterial count was detected on EMB agar among all the tested selective media, consistent with the common observation that gram-negative bacteria are the predominant bacterial species during fish spoilage.

Bacterial counts did not differ significantly between the APC and EMB agar after 1 week of storage. Gram-positive bacteria during storage were also enumerated by using

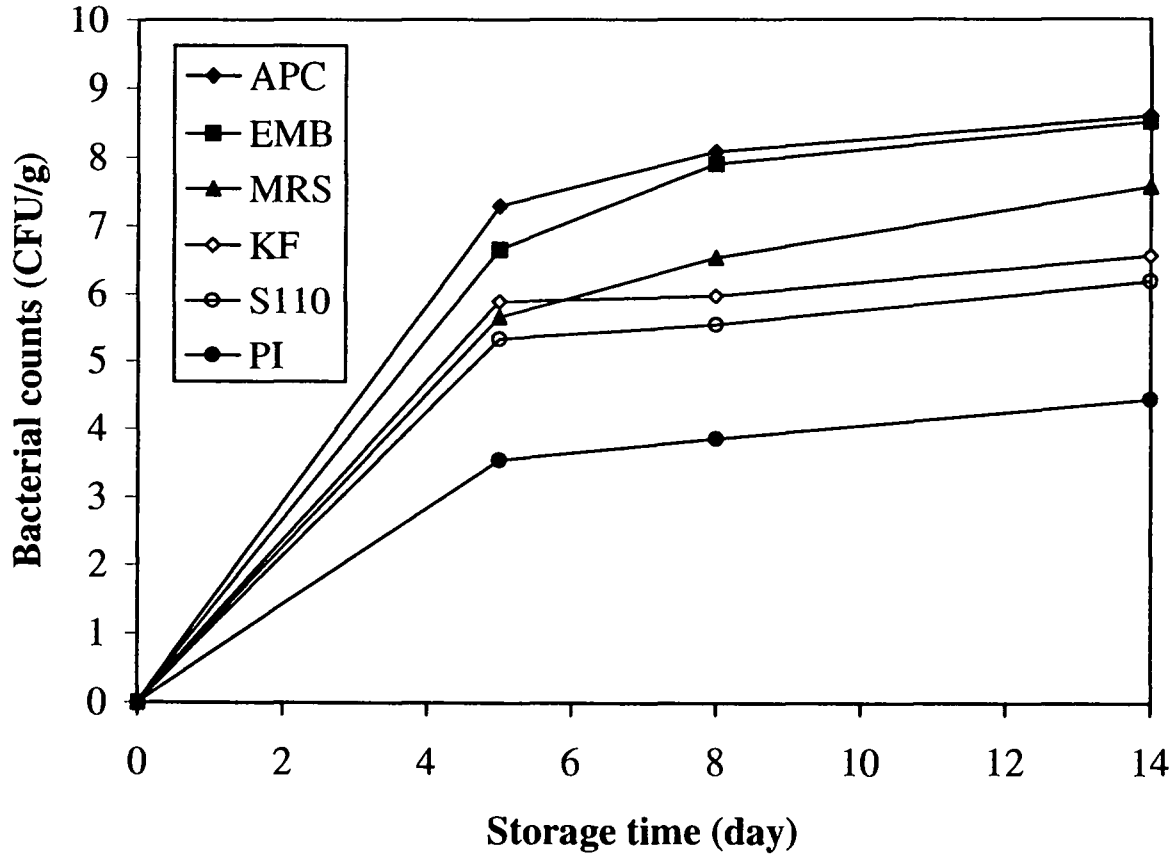

Fig. 4.2. Changes of bacterial counts in albacore stored at 25°C as determined using EMB agar, MRS agar, KF agar, S110, and PI agar. The plates were incubated at 35°C for 2 days.

KF, MRS, and S110 agar. Their counts ranged from 10^5 to 10^7 CFU/g. The bacterial count on PI agar was the lowest, and it reached 10^4 CFU/g in 14 days of storage.

Two-step screening of histamine-producing bacteria

EMB agar was most effective in selecting histamine formers among the selective media tested, followed by PI agar. When the colonies from EMB agar were transferred onto the differential media, 90% of the isolates were positive. Of the positive isolates, 74% were true histamine formers producing histamine >10 ppm, as confirmed by histamine analysis of the culture broth (Fig. 4.3). Similarly, 42% of the isolates from PI agar were positive on the differential media, and 48% of them were true histamine formers. When the isolates from KF, MRS, and S110 agar were transferred onto the differential media, very few of them were true histamine producers, although 52%, 55% and 66% of the isolates showed positive reaction on the differential media.

EMB agar has primarily been designed to prevent the growth of gram-positive bacteria in mixed cultures by incorporating an inhibitor (Difco, 1985). Many species of fastidious gram-negative organisms are inhibited as well. It also differentiates bacteria capable of utilizing lactose by the formation of characteristically colored colonies (Koneman et al., 1997). Lactose fermentative coliforms were the predominant group found on EMB agar. Black colonies both with and without a metallic sheen were most frequently detected, followed by purple colonies, indicative of weak fermenters. However, colonies typical for coliforms were not confirmed as histamine formers in this study. Although a few non-lactose fermentative colonies were detected on all the tested EMB plates, most of them were confirmed as histamine producers.

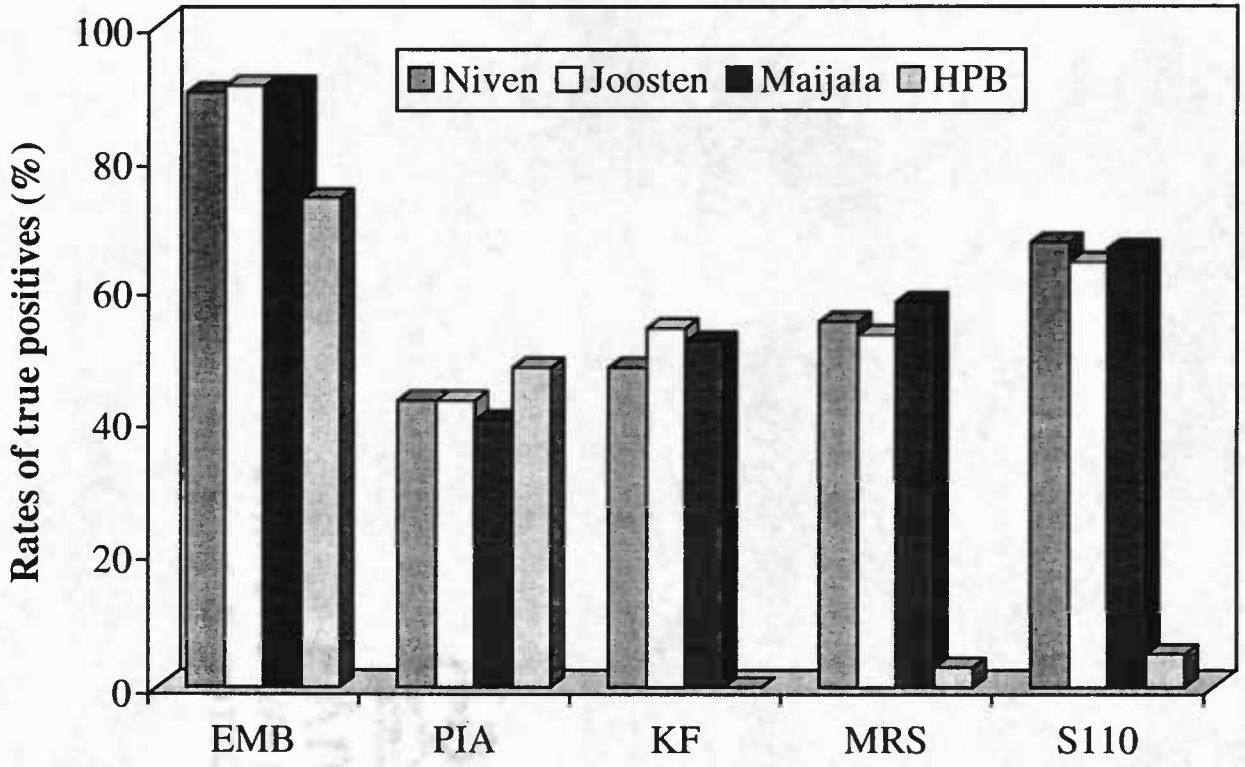

Fig. 4.3. Positive bacterial isolates from albacore in various decarboxylase media and confirmed histamine-producing bacteria (HPB). The isolates obtained from the selective media were inoculated into Niven, Joosten/Northolt, or Maijala differential media and incubated at 35°C for 2 days. The colonies showing a positive reaction on the differential media were isolated and tested for histamine production by enzymatic histamine analysis.

It is well known that the predominant spoilage organisms in fish are *Pseudomonas* spp. (Kvenberg, 1991). Gennari et al. (1999) reported *Pseudomonas* spp. were one of the most prolific microorganisms in sardines during ice storage. However, the bacterial count from PI agar was low compared to those of other media tested in this study. Thus, it appears that *Pseudomonas* spp. were not the predominant species in albacore during storage at 25°C, although the temperature used for storing the fish was within their growth temperature range. PI agar was only moderately effective in isolating histamine producers. Forty-eight percent of the isolates were confirmed as histamine formers. *M. morganii*, the most prolific histamine producer detected in this study, was isolated only from PI agar and not from EMB agar.

Lactic acid bacteria have rarely been reported as histamine formers from fish. However, histamine-producing bacteria have been isolated from other foods, such as cheeses, wine, and fermented foods, and identified as *Streptococcus faecium*, *S. mitis*, *Lactobacillus bulgarius*, *L. plantarium*, and other species of streptococci (Stratton et al., 1991). Among them, *Lactobacillus* 30a produced high levels of histamine. In this study, MRS and KF agar were used to detect these bacterial species; however, most isolates were not confirmed as histamine formers. Roig-Sagués et al. (1996) isolated histamine and other biogenic amine producers from ripened sausages and identified all the histamine producers as *Enterobacteriaceae*. They were mainly *Hafnia alvei*, *Klebsiella oxytoca*, *K. pneumoniae*, and *Serratia liquefaciens*. All the enterococci and lactic acid bacteria were confirmed as tyramine-producing bacteria, but none of them produced histamine.

S110 agar contains a high NaCl content, which is useful for the selection of staphylococci (Difco, 1985). Bacterial colonies detected on S110 agar were postulated halophilic and halotolerant bacteria. Although the counts on S110 agar were relatively high, only a few isolates were confirmed as histamine formers by histamine analysis. Some halophilic and halotolerant bacteria have been reported as histamine formers. *Staphylococcus* spp. are the most frequently reported histamine formers in fermented salted fish (Hernández-Herrero et al., 1999). Eighty-three percent of the bacteria isolated during ripening of salted anchovies (*Engraulis encrasicholus*) were identified as gram-positive, and 76% of them were identified as *Staphylococcus* spp. *Proteus cepaciae*, *Enterobacter cloacae*, and *Klebsiella oxytoca* were isolated as minor histamine formers. These results indicate that microflora can change depending on the storage conditions of fish, resulting in different types of dominant bacteria. In general, selective media for gram-positive bacteria were not effective for isolation of histamine-producing bacteria from albacore. The gram-positive bacteria that showed a positive reaction on the subsequent differential media were found to be false positives for histamine formation; thus no gram-positive bacteria were identified as histamine formers.

Overall, the limitations of the two-step screening method were as follows: i) target bacteria were difficult to detect on selective media; ii) gram-positive bacteria gave false-positive results on differential media; and iii) the method was labor-intensive and time-consuming.

Source and identification of histamine-producing bacteria

Histamine-producing bacteria were isolated from skin and gill of fresh albacore by using the two-step screening procedure. Histamine-producing bacteria isolated from gill were identified as *Enterobacter aerogenes*, *E. cloacae*, *Citrobacter braakii*, and *Hafnia alvei* (Table 4.3). *E. aerogenes* was detected as the highest histamine former, producing histamine at 4,652 ppm. *C. braakii* isolated from EMB agar also produced a high level of histamine at 2,435 ppm. *Actinobacillus ureae* isolated on S110 was a weak histamine former. It has often been isolated from aquatic environment (Holt et al., 1994).

Table 4.3. Identification of histamine-producing bacteria isolated from gill of fresh albacore by two-step screening

Strain[a]	Histamine (ppm)	Isolation Medium[c]	Positive reaction on differential media[d]
Enterobacter aerogenes	4,652	EMB[e]	N[g], J[h], M[i]
Citrobacter braakii	2,434	EMB	N, J, M
Hafnia alvei	610	EMB	N, J, M
Enterobacter aerogenes	508	EMB	J, M
Enterobacter aerogenes	315	EMB	N, J, M
Hafnia alvei	239	EMB	N, J, M
Hafnia alvei	184	EMB	N, J, M
N. I.*	62.6	EMB	N, J
Actinobacillus ureae	51.1	S110[f]	N, J, M
N. I.	13.3	EMB	N, J, M
Enterobacter cloacae	11.1	EMB	N, J, M
N. I.	10.9	EMB	N, M

[a]Confirmed histamine-producing bacteria were identified by the Vitek system.
[b]Histamine production by the isolates was determined by the enzymatic histamine analysis method.
[c]Histamine formers were isolated using selective media as a prescreening step.
[d]The isolates obtained from selective media were reinoculated on three differential media for screening of histamine formers.
[e]Eosin methylene blue agar; [f] Staphylococcus medium 110
[g]Niven's medium; [h] Joostin's medium; [i] Maijara's medium
*N.I.: Not identified

Seven strains were isolated from the skin of fresh albacore (Table 4.4). The most prolific and most frequently found histamine former was identified as *Klebsiella oxytoca*. *Klebsiella* spp. were more frequently isolated from skin by using both the modified Niven's medium and EMB agar (Table 4.1). *K. pneumoniae* was detected only once. Generally, histamine formers were more frequently isolated from the gill than the skin, and the isolates from the gill produced higher levels of histamine than those from the skin.

Table 4.4. Identification of histamine-producing bacteria isolated from skin of fresh albacore by two-step screening

Strain[a]	Histamine (ppm)[b]	Isolation Medium[c]	Positive reaction on differential media[d]
Klebsiella oxytoca	17.4	EMB[e]	N[h], J[i], M[j]
Klebsiella oxytoca	15.3	MRS[f]	N, J, M
Klebsiella oxytoca	14.0	EMB	N, J, M
Citrobacter braakii	13.1	PI[g]	J, M
N. I.*	11.5	EMB	N, J, M
N. I.	11.2	EMB	N, J, M
N. I.	11.0	EMB	N, J, M

[a]Confirmed histamine-producing bacteria were identified by the Vitek system.
[b]One loopful of culture was inoculated into the histamine enumeration medium and incubated at 37°C for 24 h. Histamine production by the isolates was determined by the enzymatic histamine analysis method.
[c]Histamine formers were isolated using selective media as a prescreening step.
[d]The isolates obtained from selective media were reinoculated on three differential media for screening of histamine formers, and positive colonies were selected.
[e]Eosin methylene blue agar; [f] de Man Rogosa Sharpe agar; [g] Pseudomonas isolation agar
[h]Niven's medium; [i] Joostin's medium; [j] Maijara's medium
*N.I.: Not identified

Histamine formers were also isolated from temperature-abused fish muscle during storage (Table 4.5). The highest histamine former among the tested strains was identified as *Morganella morganii*. All *M. morganii* isolates produced high levels of histamine

Table 4.5. Identification of histamine-producing bacteria isolated from temperature-abused albacore muscle by two-step screening

Strain[a]	Histamine (ppm)[b]	Isolation medium[c]	Positive reaction on differential media[d]	Storage days
Morganella morganii	17,650	PI[e]	N[h], J[i], M[j]	14
Morganella morganii	16,630	PI	N, J, M	8
Morganella morganii	14,170	PI	N, J, M	14
Klebsiella oxytoca	7,680	EMB[f]	J, M	14
Citrobacter braakii	4,326	PI	N, J, M	14
Klebsiella oxytoca	4,180	EMB	N, J, M	8
Serratia fonticola	2,861	EMB	N, J, M	14
Citrobacter braakii	2,638	EMB	N, J, M	8
Serratia liquefaciens	218	EMB	N, M	14
Hafnia alvei	91.7	EMB	N, J, M	8
Serratia liquefaciens	68.1	EMB	N, J, M	8
Pantoea agglomerans	52.8	EMB	N, J, M	8
Hafnia alvei	47.2	EMB	N, J, M	8
Serratia liquefaciens	37.2	EMB	N, J, M	14
N. I.*	25.5	PI	N, J, M	14
Enterobacter intermedius	21.8	PI	N, J, M	14
Klebsiella oxytoca	21.5	EMB	N, J, M	8
Serratia liquefaciens	19.5	PI	J, M	8
N. I.	19.4	EMB	N, J, M	14
Enterobacter cloacae	15.7	EMB	N, J, M	14
N. I.	14.3	PI	N, J, M	14
N. I.	14.1	PI	N, J, M	8
N. I.	14.0	EMB	N, J, M	14
N. I.	13.9	EMB	N, J, M	8
N. I.	13.3	PI	J, M	8
N. I.	13.1	EMB	N, J, M	14
N. I.	12.7	PI	N, J, M	14
N. I.	12.1	PI	N, J, M	8
N. I.	11.7	S110[g]	N, J, M	14
N. I.	11.4	EMB	N, J, M	8
N. I.	10.3	PI	N, J, M	14

[a]Confirmed histamine-producing bacteria were identified by the Vitex system.
[b]Histamine production by the isolates was determined by the enzymatic histamine analysis method.
[c]Histamine formers were isolated using selective media as a prescreening step.
[d]The isolates obtained from selective media were reinoculated on three differential media for screening of histamine formers, and positive colonies were selected.
[e]Pseudomonas isolation agar; [f] Eosin methylene blue agar; [g] Staphylococcus medium 110
[h]Niven's medium; [i] Joostin's medium; [j] Maijara's medium
*N.I.: Not identified

(>10,000 ppm). *K. oxytoca*, *Citrobacter braakii*, and *Serratia fonticola* were also identified as high histamine formers, producing histamine ranging from 2,638 to 7,680 ppm. *H. alvei* was identified as a weak histamine former (47.2 to 91.7 ppm). It was isolated from both EMB and the modified Niven's medium. Two species of *Serratia* were detected. One species was identified as *S. fonticola*, and it produced a high level of histamine. The other species not identified was a weak histamine former. A rarely reported enteric bacterium, *Pantoea agglomerans*, was also detected.

Many researchers have reported different species of histamine-producing bacteria from fish. Forty-four histamine-producing bacteria were identified from temperature-abused skipjack tuna and jack mackerel (Omura et al., 1978). Twenty-one isolates producing large amounts of histamine (1,000 ppm) were identified as *M. morganii*. *Hafnia alvei*, *Proteus* spp., and *Klebsiella* spp. were identified as weak histamine formers. Ababouch and colleagues (1991) reported that 51 out of the 55 histamine-producing isolates from temperature-abused sardine belonged to the *Enterobacteriaceae* family, and they were *M. morganii*, *Proteus vulgaris*, *P. mirabilis*, and *Providencia stuartii*. López-Sabater and coworkers (1996) reported that *M. morganii* and *K. oxytoca* were the most active histamine formers in tuna fish (*Thunnus thynnus*). Other microorganisms identified as histamine formers were *Plesiomonas shigelloides*, *Citrobacter freundii*, *Enterobacter cloacae*, *E. intermedium*, *Pseudomonas fluorescens*, *K. oxytoca*, *H. alvei*, *P. mirabilis*, *P. vulgaris*, *Serratia marcescens*, *S. plymuthica*, and *S. fonticola*. According to Yoshinaga and Frank (1982), 92% of isolates obtained from temperature-abused skipjack tuna after incubation for 24 h at 28°C were facultative or obligate anaerobes. They were identified as *Clostridium perfringens*, *E. aerogenes*, *K.*

pneumoniae, *P. mirabolis*, and *Vibrio alginolyticus*. Obligate aerobic bacteria represented only 7.5% of the microflora in fish, and most of them did not produce histamine in the culture media. López-Sabater and colleagues (1994) isolated histamine formers from tuna fish (*Thunnus thynnus*) during canning processing. *M. morganii*, *K. oxytoca*, and *K. pneumoniae* were detected as prolific histamine formers. They produced >1,000 ppm histamine in a culture broth. *E. cloacae* and *E. aerogenes* were able to form 500 to 1,000 ppm histamine. Other isolates, such as *C. freundii*, *P. mirabilis*, *P. vulgaris*, *E. agglomerans*, and *S. liquefaciens*, produced low levels of histamine (<250 ppm). They reported that *E. cloacae* was frequently isolated from tuna fish, although it was a weak histamine former. This strain produced histamine ranging from 122 to 812 ppm in culture broth. *M. morganii* has often been reported as the most prolific histamine former, and it seems to play the major role in histamine formation in fish. In this study, we also found similar results. *M. morganii* was the most prolific histamine former, and all the isolated strains consistently produced high levels of histamine. However, the other enteric bacterial species varied greatly in their ability to produce histamine. Only a few of them were identified as prolific histamine formers.

The proliferation of psychrotrophic and psychrophilic histamine producers is of great concern for the control of histamine production at refrigeration temperature. Psychrotrophic bacteria, i.e., *Pseudomonas putida* and *Hafnia alvei*, were detected as weak histamine formers in this study. Although *P. putida* was isolated only from fresh albacore, *H. alvei*, *Serratia* spp., and *Enterobacter* spp. were frequently isolated from both fresh and temperature-abused fish. According to Lindberg and colleagues (1998), these microorganisms have been found in high numbers in refrigerated foods such as

meat, fish, and milk. Although detection of natural psychrotrophic bacteria in seawater, i.e., *Vibrio alginolyticus*, *Aeromonas* spp., and *Photobacterium* spp., was excluded in this study, it is possible that they can proliferate and favor histamine formation at refrigeration temperature, although their contribution may be small.

Conclusion

The main source of histamine formers in fresh fish was the gills. However, only a few isolates from the gill were prolific histamine producers. With the progress of muscle decomposition by temperature abuse for up to 14 days, more prolific histamine producers were detected in the muscle. *M. morganii* was isolated only from decomposed muscle and was the most prolific histamine former with the consistent ability to produce histamine. Although a few were detected as prolific histamine formers, most enteric bacteria were weak histamine producers with great variability in the ability to form histamine. Bacteria belonging to gram positives were mostly non-histamine formers.

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References

Ababouch, L., Afilal, M.E., Benabdeljelil, H., and Busta, F.F. 1991. Quantitative changes in bacteria, amino acids, and biogenic amines in sardine (*Sardina pilchardus*) stored at ambient temperature (25°C) and in ice. Int. J. Food Sci. Tech. 26: 297-306.

Ababouch, L., Afilal, M.E., Rhafiri, S., and Busta, F.F. 1991. Identification of histamine-producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperature (25°C). Food Microbiol. 8: 127-136.

AOAC. 1995. Ch. 35. Fish and other marine products. p. 16-17. *In* Official Methods of Analysis. Vol. 2. Arlington, VA.

Behling, A.R. and Taylor, S.L. 1982. Bacterial histamine production as a function of temperature and time of incubation. J. Food Sci. 47: 1311-1314, 1317.

Benson, H.J. (ed.). 1992. Microbiological applications: a laboratory manual in general microbiology. WCB, Dubuque, IA.

Cappuccino, J.G. and Sherman, N. (ed.). 1992. Microbiology. Third ed. The Benjamin/Cummings Publishing Company, Inc., Redwood City, CA.

Difco. 1985. Difco manual: dehydrated culture media and reagents for microbiology, 10th ed., Difco laboratories, Detroit, MI.

Eitenmiller, R.R., Wallis, J.W., Orr, J.H., and Phillips, R.D. 1981. Production of histidine decarboxylase and histamine by *Proteus morganii*. J. Food Prot. 44: 815-820.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species, particularly kahawai (*Arripis trutta*). J. Aquat. Food Prod. Technol. 4: 53-74.

Fletcher, G.C., Summers, G., and van Veghel, P.W.G. 1998. Levels of histamine and histamine-producing bacteria in smoked fish from New Zealand markets. J. Food. Prot. 61: 1064-1070.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th ed. Arlington, VA.

Frank, H.A., Baranowski, J.D., Chongsiriwatana, M., Brust, P.A., and Premaratne, R.J. 1985. Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32°C. Int. J. Food Microbiol. 2: 331-340.

Gennari, M., Tomaselli, S., and Cotrona, V. 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in the Adriatic (Mediterranean) Sea and stored in ice. Food Microbiol. 16: 15-28.

Hernández-Herrero, M.M., Roig-Sagués, A.X., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1999. Halotolent and halophilic histamine-forming bacteria isolated during the ripening of salted anchovies (*Engraulis encrasicholus*). J. Food Prot. 62: 509-514.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. (ed.). 1994. Bergey's manual of determinative bacteriology, 9th ed. Williams and Wilkins Co., Baltimore.

Joosten, H.M.L.J. and Northolt, M.D. 1989. Detection, growth and amine producing capacity of lactobacilly in cheese. Appl. Environ. Microbiol. 55: 2356-2359.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Klausen, N.K. and Huss, H.H. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C., and Winn, W.C. Jr. (ed.). 1997. Diagnostic microbiology. 5th Ed. Lippincott. Philadelphia, PA.

Kvenberg. J.E (ed.). 1991. Microbiology of marine food products. AVI, New York, NY.

Lindberg, A.M., Ljungh, A., Ahrné, S., Löfdahl, S., and Molin, G. 1998. *Enterobacteriaceae* found in high numbers in fish, minced meat and pasteurized milk or cream and the presence of toxin encoding genes. Int. J. Food Microbiol. 39: 11-17.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., and Mora-Ventura, M.A.T. 1994. Evaluation of histidine decarboxylase activity of bacteria isolated from sardine (*Sardina pilchardus*) by an enzymatic method. Letters Applied Microbiol. 19: 70-75.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1996. Sensory quality and histamine formation during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

Maijala, R.L. 1993. Formation of histamine and tyramine by some lactic acid bacteria in MRS-broth and modified decarboxylation agar. Letters Applied Microbiol. 17: 40-43.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Niven, C.F., Jeffrey, M.B., and Corlett, D.A. 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. Appl. Environ. Microbiol. 41: 321-322.

Okuzumi, M., Hiraishi, A., Kobayashi, T., and Fujii, T. 1994. *Photobacterium histaminum* sp. nov., a histamine-producing marine bacterium. Int. J. Syst. Bacteriol. 44: 631-636.

Omura, Y., Price, R.J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish and shellfish. Adv. Food Nutr. Res. 39: 329-364.

Rodríguez-Jerez, J.J., López-Sabater, E.I., Roig-Sagués, A.X., and Mora-Ventura, M.T. 1994. Histamine, cadaverine and putrescine forming bacteria from ripened Spanish semi-preserved anchovies. J. Food Sci. 59: 998-1001.

Rodríguez-Jerez, J.J., Mora-Ventura, M.T., López-Sabater, E.I., and Hernández-Herrero, M. 1994. Histidine, lysine and orinithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. J. Food Prot. 57: 784-787.

Roig-Sagués, A.X., Hernández-Herrero, M., López-Sabater, E.I., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1996. Histidine decarboxylase activity of bacteria isolated from raw and ripened *Salchichón*, a Spanish cured sausage. J. Food Prot. 59: 516-520.

Roig-Sagués, A.X., Hernández-Herrero, M., López-Sabater, E.I., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1997. Evaluation of three decarboxylating agar media to detect histamine and tyramine-producing bacteria in ripened sausages. Letters Applied Microbiol. 25: 309-312.

Ryser, E.T., Marth, E.H., and Taylor, S.L. 1984. Histamine production by psychrotrophic pseudomonads isolated from tuna fish. J. Food Prot. 47: 378-380.

Shelef, L.A., Surtani, A., Kanagapandian, K., and Tan, W. 1998. Automated detection of amino acid decarboxylation in salmonellae and other *Enterobacteriaceae*. Food Microbiol. 15: 199-205.

Stratton, J.E., Hutkins, R.W., and Taylor, S.L. 1991. Biogenic amines in cheese and other fermented foods: a review. J. Food Prot. 54: 460-470.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S.L. 1986. Histamine food poisoning: Toxicology and clinical aspects. CRC Crit. Rev. Toxicol. 17: 91-117.

Yatsunami, K. and Echigo, T. 1993. Changes in the number of halotolerant histamine-forming bacteria and contents of non-volatile amines in sardine meat with addition of NaCl. Nippon Suisan Gakkashi 59: 123-127.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamia*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 5

Identification of Bacteria Crucial to Histamine Accumulation in Pacific Mackerel during Storage

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Abstract

Bacterial growth and histamine formation in Pacific mackerel during storage at 0, 4, 15, and 25°C were monitored. To identify bacterial species contributing to histamine formation, several groups of bacteria were isolated by using selective media under temperatures corresponding to the various storage conditions. Initially, low counts of bacteria were found in the gill, skin, and intestine of fresh fish, and only weak histamine formers were found in the gill. Histamine was found in the muscle when fish were stored above 4°C, and APC reached 10^6 CFU/g. When fish became unsuitable for human consumption by abusive storage, toxicological levels of histamine were always found. The highest level of histamine formed was 283 mg/100 g in 2 days. The optimum temperature for supporting growth of prolific histamine formers was 25°C. The most prolific and prevalent histamine former was *Morganella morganii*, followed by *Proteus vulgaris*, both of which were isolated on violet red bile glucose (VRBG) agar. At 15°C, a significant level of histamine was still produced in fish muscle, although prolific histamine formers were less frequently detected than at 25°C. The isolates on thiosulfate citrate bile salts sucrose (TCBS) agar were weak histamine formers and identified as *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. At 4°C, less than 57.4 mg/100 g of histamine was found in fish stored for 14 days. Most isolates were natural bacterial flora in the marine environment and identified as weak histamine formers. At 0°C, neither histamine former nor histamine production was detected up to 14 days of storage.

Introduction

Scombroid poisoning is a food-borne chemical intoxication. Histamine is the main causative agent in scombroid poisoning, although other biogenic amines, such as cadaverine and putrescine, have been reported to play a synergistic role with histamine (Rawles et al., 1996). Histamine is a chemical hazard monitored by the Food and Drug Administration for safety of seafood products (FDA, 1998). It can be present at high levels in fresh fish without any signs of decomposition. In general, time and temperature abuse of fish results in histamine accumulation, although the severity of symptoms varies depending on individual's sensitivity to ingested histamine (Price et al., 1991). Scombroid fish containing high levels of free histidine in the muscle are susceptible to histamine formation and are most frequently implicated in outbreaks of scombroid poisoning (Stratton and Taylor, 1991).

Histamine is rarely found in fresh fish (Kim et al., 2001; López-Sabater et al., 1996). Improper handling and storage of fish can induce histamine formation by the proliferation of bacteria possessing histidine decarboxylase, the enzyme responsible for the conversion of histidine to histamine (Rawles et al., 1996). Several species of enteric bacteria are the representative histamine formers in fish, although many other bacteria, including *Lactobacillus* spp., *Bacillus* spp., and *Clostridium* spp., contain the enzyme (Ababouch et al., 1991; Barancin et al., 1998; Middlebrooks et al., 1988; Ryser et al., 1984; Stratton and Taylor, 1991; Yoshinaga and Frank, 1982). The most prolific histamine former reported is *Morganella morganii* followed by *Klebsiella* spp. and *Enterobacter* spp. (Ababouch et al., 1991; Taylor and Speckhard, 1983). Other enteric bacteria, i.e., *Hafnia alvei*, *Citrobacter freundii*, and *Serratia* spp., are reported as weak

histamine formers (Kim et al., 1999; López-Sabater et al., 1996). Other types of histamine formers isolated from fish are psychrotrophs. *Pseudomonas* spp., *Photobacterium* spp., *Aeromonas* spp., and *Vibrio alginolyticus* have often been reported as histamine formers capable of producing histamine at refrigeration temperature (Barancin et al., 1998; Morii et al., 1988; Ryser et al., 1984). Among them, *Photobacterium phosphoreum* isolated from mackerel has been reported as an important histamine former (Morii et al., 1988).

The dominant bacterial flora in fish can change depending on handling and storage conditions. Since most prolific histamine formers are mesophiles, temperature is a critical factor affecting histamine formation in fish (Kim et al., 2000). In many studies, the optimal storage temperature reported for histamine production is 20 to 25°C (Ababouch et al., 1991; Fletcher et al., 1995; Kim et al., 1999). Low temperature storage at 0°C or below can effectively control their growth and histamine formation (Kim et al., 1999; Pacheco-Aguilar et al., 2000; Price et al., 1999). In a few studies, histamine was detected during storage at 0°C, but only after fish became unacceptable for human consumption (Bennour et al., 1991; El Marrakchi et al., 1990; Jhaveri et al., 1982; López-Sabater et al., 1996).

The effect of temperature on histamine formation between 2 and 10°C is not clear. At these temperatures, histamine formation is assumed to be formed due to the growth of psychrotrophic or psychrophilic bacteria (Bennour et al., 1991; El Marrakchi et al., 1990). However, these are mainly weak histamine formers; thus, their contribution to histamine accumulation may not be as significant as that of mesophilic histamine formers. Therefore, the objective of this study was to monitor Pacific mackerel for

product safety under various storage conditions. In this study, histamine production and bacterial growth were monitored on fish under storage at 0-25°C. Several groups of bacteria were isolated using selective media, and the confirmed histamine formers were identified to species.

Materials and Methods

Reagents

Dowex 1-X8, histamine dihydrochloride, *o*-phthaldialdehyde, and tetramethyl-*p*-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Kovacs' reagent was obtained from Difco Laboratories (Detroit, MI).

Media

Trypticase soy broth (TSB) was purchased from BBL Co. (Cockeysville, MD). Standard plate count (SPC) agar, violet red bile glucose (VRBG) agar, pseudomonas isolation (PI) agar, thiosulfate citrate bile salts sucrose (TCBS) agar, and tryptone broth were obtained from Difco.

Samples

Pacific mackerel (*Scomber japonicus*) were commercially harvested off the Oregon Coast. On the day fish were delivered to the seafood processing company (Astoria, OR), Pacific mackerel, ranging from 550 to 600 g, were obtained as samples. The fishing trips usually lasted less than 8 hours, and the mackerel samples were in

excellent quality. The fish were transported in ice to Oregon State University-Seafood Laboratory, Astoria, OR. Whole fish were immediately used for enumeration of initial bacterial counts and isolation of histamine formers. The remaining fish were separately placed in sterile plastic bags and sealed. Five and three fish were stored at 15 and 25°C, respectively, and seven fish each were stored at 0 and 4°C. Samples were taken every 24 h from fish stored at 15 and 25°C and every 48 h from those stored at 0 and 4°C for aerobic plate counts (APC), histamine analysis, and isolation of histamine formers.

Sample treatment and APC

Skin, gill, and intestine of fresh fish were taken for APC. A sterile aluminum foil template (4 × 5 cm) was placed on the surface of skin, and the area of template was swabbed with sterile cotton. The cotton swab was inoculated into 5 ml TSB (Omura et al., 1978). Aseptically, 10 g of the gill and intestine was removed and blended with 90 ml of peptone water (0.1%). For sampling muscles, the skin (3 × 8 cm^2) was excised. Immediately, 10-g portions of dorsal muscles were taken, weighed into a sterile jar, and blended with peptone water (0.1%). APC was carried out in duplicate according to the standard plate method (FDA, 1992). One milliliter aliquots from each sample were serially diluted with peptone water (0.1%) and mixed with SPC agar supplemented with 0.5% NaCl cooled down to 50°C. For fish samples stored at 15 and 25°C, plates were incubated at 25°C for 2 days. For those stored at 0 and 4°C, they were incubated at 4°C for 7 days.

Histamine analysis of fish muscles

Histamine content in fish muscles was determined in duplicate by the standard AOAC fluorometric method (AOAC, 1995). Dorsal muscles, 10 g, were homogenized in 50 ml of methanol (100%) and incubated at 60°C for 15 min. The volume of the methanol extract was adjusted to 100 ml with methanol in a volumetric flask, and the extract was filtered through Whatman no. 1 paper. The filtrate was fractionated on an ion exchange column (200 × 7 mm) packed with Dowex 1-X8, and eluted with H_2O. The column eluant was derivatized with *o*-phthaldialdehyde, and fluorescence intensity was determined using a spectrophotofluorometer (Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 350 nm and an emission wavelength of 444 nm.

Bacterial isolation

Several groups of bacteria were isolated by using a pour plate method (FDA, 1992). Sample treatments and incubation of plates for fish samples were carried out as described in the section "Sample treatment and APC." Each diluent was mixed with selective media for screening histamine formers. The selective media used for target bacteria were VRBG agar for enteric bacteria; TCBS agar for vibrios; and PI agar for pseudomonads. Colonies on each plate were picked based on their morphological characteristics, such as color, size, opacity, and form, and inoculated into trypticase soy agar (TSA) slants for histamine analysis.

Histamine formation by the isolates

Bacterial isolates were inoculated into TSB supplemented with 1% histidine and incubated at 37°C for 24 h (Taylor and Speckhard, 1983). One milliliter of bacterial culture was mixed with 9 ml of methanol. Histamine content in culture broth was determined in duplicate by the AOAC fluorometric method (AOAC, 1995) as described above.

Identification of histamine formers

Bacterial isolates confirmed to produce histamine were identified by using the Vitek instrument (bioMérieux Vitek, Inc., Hazelwood, MI) according to the protocol recommended by the manufacturer. Each isolate was inoculated in TSA slants and incubated at 37°C for 18 h. Gram staining and oxidase tests required for the selection of adequate identification cards were carried out using the standard method (Benson, 1992). Each culture was suspended in phosphate saline buffer at the concentration range determined by the Vitek colorimeter and placed in a Vitek identification card. The bacterial identification was automatically carried out by the Vitek system.

Additional biochemical tests were carried out for definitive species identification. For *Pseudomonas* species, gelatin utilization was tested by liquification of gelatin medium (Benson, 1992). One loopful of culture was inoculated into gelatin medium and incubated at 22°C for 10 days. To differentiate two species of *Proteus*, an indole test was carried out using the standard method (Benson, 1992). One loopful of culture was inoculated into tryptone broth and incubated at 37°C for 24 h. To the bacterial culture, 0.1 ml of Kovacs' reagent was added, and change of color was monitored.

Results

Initial bacterial loads and isolation of histamine formers from fresh Pacific mackerel

The Pacific mackerel samples received were in excellent condition, as judged by the sensory qualities, such as, the appearance of fish, muscle texture, and odor. All samples had bright eyes, red gills, and firm texture. Bacteria were initially found in the gill and intestine and on the skin of the fresh fish, but rarely in the muscle. The APC of the gill and skin were 1.5×10^5 CFU/g and 1.6×10^4 CFU/cm^2, respectively (Table 5.1). A relatively low APC, 2.6×10^2 CFU/g, was detected in the intestine. However, prolific histamine formers were not detected in any of the samples tested. Most isolates on the selective media were nonhistamine formers, and a few from the gill were weak histamine formers. Among 15 isolates obtained on TCBS agar, 7 strains produced 20 to 50 ppm histamine in culture broth (data not shown). The identified species were *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Aeromonas hydrophila*.

Table 5.1. Counts of total aerobic bacteria and isolation of histamine formers from fresh Pacific mackerel.

Origin of isolates	APC (CFU/g or cm^2)	Number of histamine formers[a]/ Number of isolates[b]		
		VRBG	PI	TCBS
Gill	1.5×10^5	0/31	0/14	7/15
Skin	1.6×10^4	0/18	0/18	0/16
Intestine	2.6×10^2	N. D.	N. D.	N. D.
Muscle	N. D.*	N. D.	N. D.	N. D.

[a]Histamine production by isolates was confirmed by the AOAC fluorometric method.
[b]Target groups of bacteria were isolated using selective media.
*Not detected

Changes in bacterial counts and histamine contents at various temperatures

Bacterial counts rapidly increased when fish were exposed to ambient temperature (25°C). APC reached 4.1×10^6 CFU/g in 1 day (Fig. 5.1). Fish appeared spoiled, and off-odor was detected. Cloudy slime appeared on the surface of skin. Muscle turned opaque, and its texture became soft. Belly was burst, and intestine wall was completely dissolved by autolysis. APC reached the maximum level, 9.5×10^7 CFU/g, in 2 days but decreased thereafter. The pattern of quality changes at 15°C was slightly different from 25°C. The intestinal wall of fish remained intact, and the muscle consistently showed translucent-red color. Off-odor, cloudy slime on the surface of skin, and belly burn were detected in 2 days. APC gradually increased and reached 2.8×10^7 CFU/g in 4 days. As storage temperature decreased, the rate of bacterial growth was lower, and the quality changes progressed more slowly. At 4°C, APC reached 6.8×10^5 CFU/g in 8 days of storage. By then, fish were apparently decomposed and unsuitable for human consumption. Off-odor and belly burn in fish were evident when APC reached 9.3×10^6 CFU/g in 10 days. At 0°C, APC slowly increased and did not exceed 10^6 CFU/g for 14 days. Fish developed cloudy slime on the surface of skin and slight off-odor in 10 days.

The optimal temperature for histamine formation in fish muscles was 25°C (Fig. 5.2). The histamine content exceeded 5 mg/100 g in 1 day. It increased rapidly and reached the highest level, 286 mg/100 g, in 2 days when spoilage was apparent. At 15°C storage, the histamine content increased slightly (14.0 mg/100 g) in 2 days, reaching 197 mg/100 g in 5 days. Both the APC and histamine content at 15°C were lower than 25°C. At 4°C, histamine was not produced in 6 days of storage (<1 mg/100 g), and the fish was still acceptable for human consumption. Histamine content began to increase thereafter

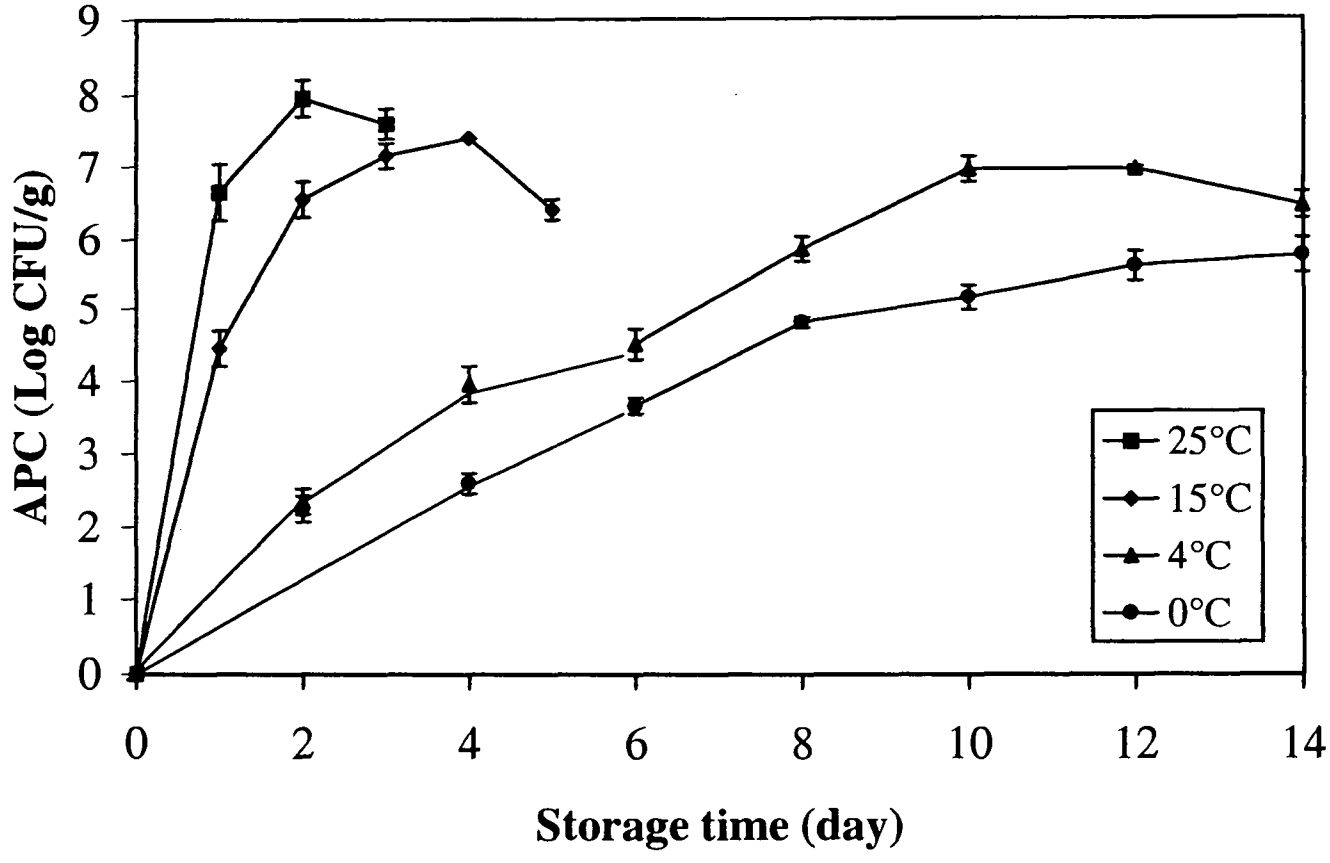

Fig. 5.1. Changes in APC of Pacific mackerel muscle during storage at 0, 4, 15, and 25°C. APC was determined in duplicate by using the standard pour plate method.

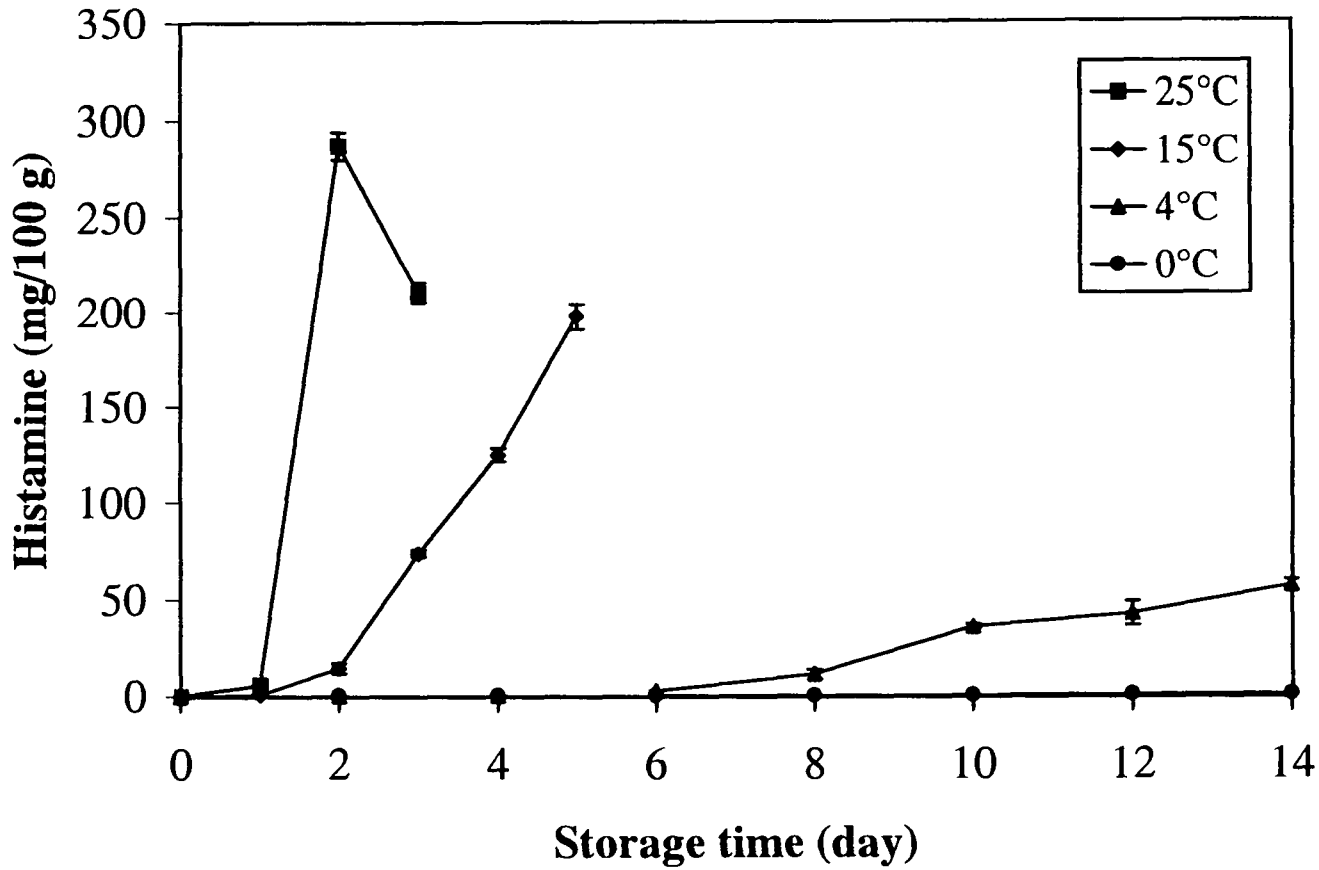

Fig. 5.2. Changes in histamine content of Pacific mackerel muscle during storage at 0, 4, 15, and 25°C. Histamine content was analyzed in duplicate by the AOAC fluorometric method.

and accumulated up to 57.4 mg/100 g in 14 days after gross spoilage. At 0°C, a negligible level of histamine (<1 mg/100 g) was formed in 14 days of storage.

Identification of prolific histamine formers during storage at 25°C

A total of 1,200 strains were isolated from the muscles of Pacific mackerel during storage by using selective media. Prolific histamine formers producing >1,000 ppm histamine at 37°C were mostly isolated on VRBG agar from fish stored at 25°C. The identified species were *Morganella morganii* and *Proteus vulgaris*. Bacterial count on VRBG agar was still low on day 1 (Fig. 5.3). Only one strain of *M. morganii* was isolated on VRBG plate (Table 5.2). *M. morganii* was frequently isolated with the progression of fish spoilage. Ten strains were isolated on day 2 and seventeen strains on day 3. All the isolates produced a significant level of histamine at 37°C, ranging from 3,209 to 3,527 ppm, in TSB supplemented with 1% histidine. Another prolific histamine former, *P. vulgaris*, produced 2,985 to 3,488 ppm histamine in culture broth at 37°C. However, it was less frequently isolated than *M. morganii*. Four isolates of *P. vulgaris* were detected on day 2, and six strains were isolated on day 3. In general, morphological characteristics of *P. vulgaris* on VRBG agar were not significantly different from those of *M. morganii*. Typically, both *M. morganii* and *P. vulgaris* colonies on VRBG agar were small (2 to 3 mm in diameter), smooth, translucent, purple with or without cloudy zone, and slightly flattened with an opaque center. Other enteric bacteria were rarely isolated. Only one strain of *Enterobacter aerogenes*, identified as a weak histamine former, was isolated on day 2.

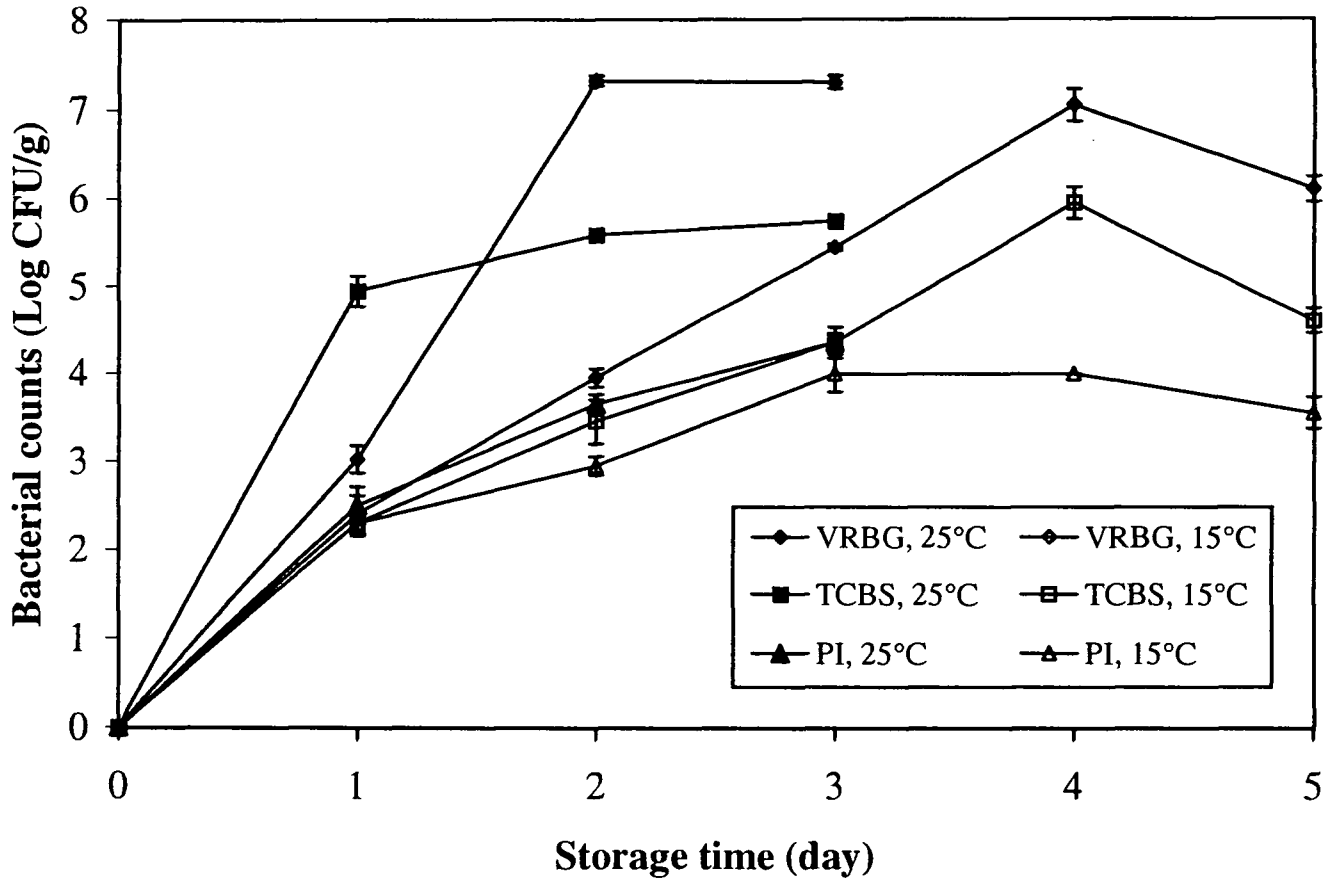

Fig. 5.3. Changes in bacterial counts of Pacific mackerel muscle during storage at 15 and 25°C as determined using VRBG, TCBS, and PI agar. Bacterial counts were determined in duplicate by the standard pour plate method.

Table 5.2. Identification of histamine formers from the muscles of Pacific mackerel during storage at 25°C

Storage day(s)	Strain isolated	Number isolated	Medium used[a]	Histamine[d] (ppm) Produced in culture
1	*Vibrio parahaemolyticus*	3	TCBS[b]	25.8 – 230 (125 ± 102)
	Morganella morganii	1	VRBG[c]	3,191
	Vibrio alginolyticus	1	TCBS	88.2
2	*Morganella morganii*	10	VRBG	3,222–3,413 (3,308 ± 62.4)
	Vibrio parahaemolyticus	5	TCBS	98.2 – 323 (178 ± 95.2)
	Proteus vulgaris	4	VRBG	3,272– 3,345 (3,299 ± 31.8)
	Vibrio alginolyticus	4	TCBS	41.5 – 78.0 (57.3 ± 16.2)
	Enterobacter aerogenes	1	VRBG	23.4
3	*Morganella morganii*	17	VRBG	3,209– 3,527 (3,306 ± 93.0)
	Proteus vulgaris	6	VRBG	2,985 – 3,488 (3,234 ± 162)
	Vibrio alginolyticus	5	TCBS	63.9 – 122 (85.8 ± 24.3)
	Vibrio parahaemolyticus	5	TCBS	11.8 – 114 (51.4 ± 44.7)

[a]Target group of bacteria was isolated by using selective medium.
[b]Thiosulfate citrate bile salt sucrose agar
[c]Violet red bile glucose agar
[d]Histamine production at 37°C by isolates was confirmed by the AOAC fluorometric method.

Weak histamine formers were frequently isolated on TCBS agar. A rapid increase in bacterial counts was shown on TCBS agar in 1 day. The highest bacterial count was also detected on TCBS agar among all the selective media tested on day 1 (Fig. 5.3). Identified histamine formers were *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, which were isolated from the gill of fresh fish in this study (Table 5.2). However, bacterial counts on TCBS agar did not increase rapidly after the first day, and they were always lower than those on VRBG agar. *V. parahaemolyticus* and *V.*

alginolyticus were frequently isolated as weak histamine formers during 3 days of storage. Among the selective media tested, the lowest bacterial count obtained was from PI agar (Fig. 5.3). Bacterial counts reached 10^4 CFU/g in 3 days of storage. Most isolates produced negligible amounts of histamine in culture broth, and thus were classified as nonhistamine formers.

Identification of dominant histamine-forming bacterial flora during storage at 0, 4, and 15°C

The highest bacterial counts were detected on VRBG agar among all the tested media during storage at 15°C (Fig. 5.3). Bacterial counts on selective media were low on day 1 for fish samples stored at 15°C, and histamine formers were not isolated from the samples (Table 5.3). *Morganella morganii* was first isolated on day 2. A total of 11 strains were isolated during 5 days of storage. Minor bacterial species identified were *Edwardsiella hoshinae*, *Pseudomonas putida*, and *Hafnia alvei*. They were confirmed as weak histamine formers. Bacterial counts on TCBS agar gradually increased over the storage time. *Vibrio parahaemolyticus* and *Vibrio alginolyticus* were isolated only from fish stored for 3 days (Table 5.3). In general, *M. morganii* was less frequently isolated, and the detection rate of vibrios decreased more substantially at 15°C than 25°C.

At 4°C, the highest bacterial counts were obtained with VRBG agar (Fig. 5.4). Although higher bacterial counts were initially detected on PI agar than on VRBG agar at the beginning of storage, their counts did not change much after 6 days of storage. Bacterial growth on TCBS was first detected in fish samples stored for 8 days, and only a slight increase in bacterial count was observed thereafter. Histamine formers were not

isolated up to 6 days of storage. They were isolated from the 8-day stored fish that contained 11.3 mg/100 g histamine (Table 5.4). All isolates were weak histamine formers, and their detection rates were low. Although they were isolated only on VRBG agar, the identified histamine formers were a part of naturally existing bacterial flora in seawater. *Actinobacillus ureae* was isolated from fish stored for 8 and 10 days, and one strain of *Pseudomonas putida* was isolated from fish stored for 10 days. *Aeromonas hydrophila* was frequently isolated at the end of the storage test. *Vibrio alginolyticus* and *Photobacterium damsela* were also isolated as histamine formers.

Table 5.3. Identification of histamine formers from the muscles of Pacific mackerel during storage at 15°C

Storage day (s)	Strain isolated	Number Isolated	Medium used[a]	Histamine[d] (ppm) Produced in culture broth
1	N.D.*	-	-	-
2	*Morganella morganii*	3	VRBG[b]	3,324–3,367 (3,348 ± 22.2)
	Edwardsiella hoshinae	1	VRBG	33.7
3	*Morganella morganii*	4	VRBG	3,201–3,340 (3,304 ± 68.9)
	Vibrio parahaemolyticus	3	TCBS[c]	42.9 – 142 (76.7 ± 56.5)
	Vibrio alginolyticus	1	TCBS	837
	Pseudomonas putida	1	VRBG	159
4	*Morganella morganii*	2	VRBG	3,203 – 3,400
	Proteus vulgaris	1	VRBG	3,225
5	*Morganella morganii*	2	VRBG	3,302 – 3,349
	Hafnia alvei	1	VRBG	148

[a]Target group of bacteria was isolated by using selective medium.
[b]Violet red bile glucose agar; [c]Thiosulfate citrate bile salt sucrose agar
[d]Histamine production at 37°C by isolates was confirmed by the AOAC fluorometric method.
*Not detected

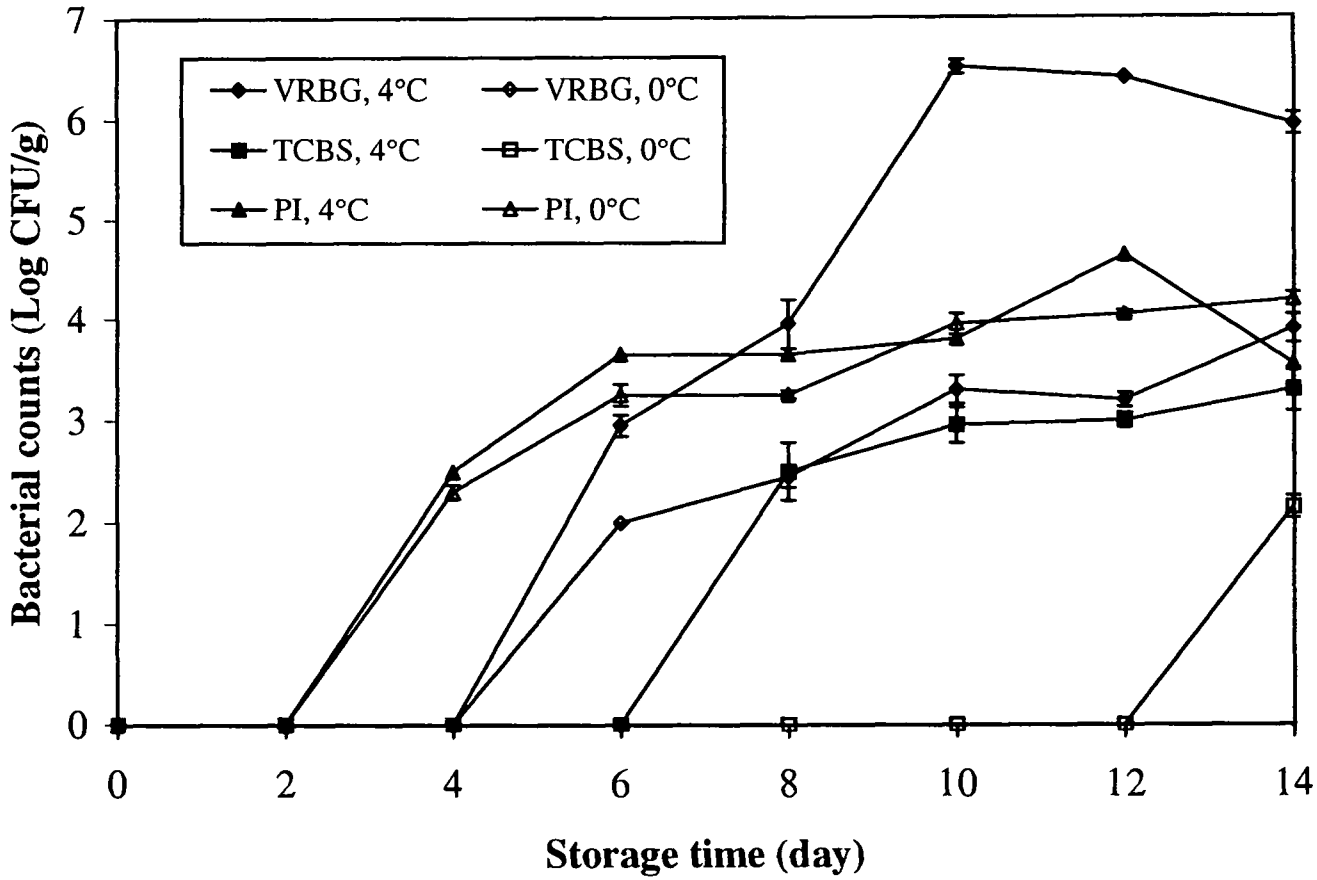

Fig. 5.4. Changes in bacterial counts of Pacific mackerel muscle during storage at 0°C and 4°C as determined by using VRBG, TCBS, and PI agar. Bacterial counts were determined in duplicate by the standard pour plate method.

At 0°C, no histamine former was isolated during storage up to 14 days. The highest bacterial count was obtained from PI agar, indicating changes in the dominant culturable bacterial flora (Fig. 5.4). Few colonies grew on VRBG agar, which was the most effective medium for the isolation of histamine formers during storage above 4°C.

Table 5.4. Identification of histamine formers from the muscles of Pacific mackerel during storage at 4°C

Storage day(s)	Strain isolated	Number isolated	Medium used[a]	Histamine[c] (ppm) Produced in culture
2	N.D.*	-	-	-
4	N.D	-	-	-
6	N.D	-	-	-
8	*Actinobacillus ureae*	2	VRBG[b]	248 – 256
10	*Pseudomonas putida*	1	VRBG	159
	Actinobacillus ureae	1	VRBG	154
12	*Aeromonas hydrophila*	1	VRBG	120
	Vibrio alginolyticus	1	VRBG	111
14	*Aeromonas hydrophila*	3	VRBG	72.9 – 182 (145 ± 62.4)
	Photobacterium damsela	1	VRBG	112
	Vibrio alginolyticus	1	VRBG	8.83

[a]Target group of bacteria was isolated by using selective medium.
[b]Violet red bile glucose agar
[c]Histamine production at 37°C by isolates was confirmed by the AOAC fluorometric method.
*Not detected

Discussion

Quality changes and product safety of Pacific mackerel during storage

Shelf life of raw seafoods depends on several factors: storage conditions (time, temperature, presence and concentration of gases, and relative humidity of the environment), intrinsic factors of fish (species, age, size, fat content, feeding, and physiological status), and number of initial microflora (Gennari et al., 1999; Gram et al., 1987). Handling fresh mackerel can be a problem due to its soft flesh, high lipid content, and delicate skin (Jhaveri et al., 1982).

In this study, we demonstrated that improper storage of mackerel resulted in the substantial deterioration of fish quality in a short period of time. The rapid quality deterioration was accompanied by bacterial proliferation at ambient temperature, 25°C, within 1 day, indicating that natural bacteria existing in the gill, skin, and intestine had spread into the muscle rich in nutrients. The quality change of mackerel progressed faster than that of larger fish, such as tuna and mahi-mahi. When albacore were stored at 25°C, APC reached only 10^7 CFU/g in 7 days (Kim et al., 1999). With mahi-mahi, the decomposition was also slow at 21°C (Baranowski et al., 1990). The APC reached 10^5 CFU/g in 2 days of storage, and histamine was not detected. The highest level of histamine, 234 mg/100 g, was found in 4 days of storage. However, the highest levels of APC and histamine content were detected in mackerel stored for only 2 days (Figs. 5.1 and 5.2).

The general recommendation of microbiological limits in fish is 5×10^5 CFU/g (ICMSF, 1986). It is well documented that histamine is a health hazard if present in fish

muscle at levels higher than 50 mg/100 g (FDA, 1998). When APC reached 10^6 CFU/g during storage above 4°C, mackerel was unacceptable for human consumption (Fig. 5.1). The levels of histamine produced exceeded 5 mg/100 g, the FDA guideline for scombroid fish (FDA, 1996). A health hazard level of histamine (>50 mg/100 g) accumulated after APC reached $>10^7$ CFU/g, although the levels were variable depending on the storage temperatures. Overall, mackerel was a highly perishable fish. Histamine above the health hazard level was produced even during storage at 4°C. However, it occurred only after fish were completely decomposed and unsuitable for human consumption, as previously reported with other fish species, i.e., tuna (López-Sabater et al., 1996) and albacore (Kim et al., 1999).

Presence of histamine formers in fresh fish

The initial bacterial loads on the gill and skin of fresh Pacific mackerel were not greatly different from previous reports of 10^2 to 10^6 CFU/cm^2 on the skin and 10^3 to 10^5 CFU/g on the gills (Kvenberg, 1991). Gennari et al. (1999) reported that 10^4 CFU/cm^2 and 10^5 CFU/g of APC were detected on the skin and gill of fresh sardine, respectively. Bacterial counts in fresh sardine were 2.5×10^6 CFU/cm^2 on the skin; 1.3×10^5 CFU/g on the gills; and 3.1×10^4 CFU/g in the intestine (Ababouch et al., 1991). However, the bacterial counts in the intestine were lower than the reported data, $>10^7$ CFU/g in feeding fish (Kvenberg, 1991).

Histamine formers may constitute a minority of the natural microflora, thus making it difficult to detect in fresh fish (Sumner and Taylor 1989). However, it is necessary to monitor the population and distribution of prolific histamine formers in fresh

fish. Many researchers suspect the presence of histamine formers in the gut and gill of fresh fish, since histamine is usually detected in fish at higher levels in tissues adjacent to the gills or intestines (López-Sabater et al., 1996, Taylor, 1986). When histamine formers were isolated from fresh albacore after the enrichment procedure, most of them were weak enteric histamine formers, and only few species, i.e., *Citrobacter braakii* and *Enterobacter aerogenes*, were prolific formers (Kim et al., 2001).

In this study, the presence of prolific histamine formers was not detected in fresh mackerel, and all the natural bacterial species isolated were confirmed as weak formers. However, prolific histamine formers were frequently isolated from temperature abused (>15°C) fish for a short period of time (Tables 5.2 and 5.3). Therefore, we suspect that the initial population of prolific histamine-producing bacteria in fresh fish was too low ($<10^2$ CFU/g) to be isolated by the culture method. Although they are present at negligible levels in fresh fish, they may proliferate, synthesize histidine decarboxylase, and produce histamine when fish are left at elevated temperatures.

Morganella morganii as the main contributor of histamine formation in fish

Morganella morganii has frequently been isolated from various types of temperature-abused fish; i.e., skipjack tuna (Omura et al., 1978), tuna (*Thunnus thynnus*) (López-Sabater et al., 1996), albacore (Kim et al., 2000), mahi-mahi (Frank et al., 1985), mackerel (Middlebrooks et al., 1988), sardine (Ababouch et al., 1991), and Spanish salted semi-preserved anchovies (Rodríguez-Jerez et al., 1994). It has been confirmed as the most prolific histamine former capable of producing >1,000 ppm histamine in culture broth. However, the isolation rate of *M. morganii* varied depending on the fish species

stored at temperatures above 25°C. *M. morganii* was identified as the most prolific histamine former in sardines, but only 7 strains were isolated from samples stored at ambient temperature (25°C) (Ababouch et al., 1991). The most frequently isolated histamine-producing bacteria were *Proteus* spp. Twenty-three strains out of 28 *Proteus* strains isolated were prolific histamine formers. Only 9% of histamine formers were *M. morganii* in mahi-mahi stored at 32°C (Frank et al., 1985). Eight strains of *M. morganii* and one strain of *P. mirabilis* were identified as prolific histamine producers (>1,000 ppm). *V. alginolyticus* comprised 90% of the mesophilic isolates, but most isolates (97 stains) did not produce histamine. Only 18 strains of *V. alginolyticus* were weak histamine formers (<100 ppm).

M. morganii played the major role in histamine accumulation in Pacific mackerel during storage at elevated temperature (>15°C). Histamine accumulation in fish muscles was proportional to the proliferation of *M. morganii* during the first 2 days. Although more *M. morganii* strains were isolated on day 3 than day 2 during storage at 25°C, the histamine content in fish muscle was lower on day 3 than on day 2. It was reported that diamine oxidase is primarily responsible for the metabolism of histamine by catalyzing oxidative deamination of histamine (Rawles, 1996; Taylor, 1986). Diamine oxidase was found in fish muscle (Hungerford and Arefyev, 1992). Therefore, it has been presumed that the decrease in histamine content in fish resulted from metabolism of histamine.

Using VRBG agar, other enteric bacteria, i.e., *Klebsiella oxytoca*, *K. pneumoniae*, *Serratia liquefaciens*, *Enterobacter cloacae* and *Citrobacter freundii*, which are commonly isolated from temperature abused fish (>25°C), were not isolated in this study. One strain each of *Enterobacter aerogenes*, *Edwardsiella hoshinae*, and *Hafnia alvei* was

isolated on VRBG agar from fish stored at 15 or 25°C. Only *Proteus vulgaris* was frequently isolated on VRBG agar and produced the comparable level of histamine to *M. morganii* in culture broth.

Effect of temperature on growth of *M. morganii* and its histamine production has been well studied. Generally, the temperature range of 25 to 37°C is optimal for histamine production in culture broth with this bacterium (Behling and Taylor, 1982; Eitenmiller et al., 1981). The minimum temperature to produce toxicological levels of histamine in culture broth is 15°C (Behling and Taylor 1981; Kim et al., 2000; Klausen and Huss, 1987). *M. morganii* showed the typical characteristics of mesophilic bacteria, in that bacterial growth as well as histamine formation were inhibited by storage below 4°C (Arnold et al., 1980; Kim et al., 2000; Klausen and Huss, 1987). These characteristics were well reflected when *M. morganii* was isolated from Pacific mackerel stored at different temperatures in this study. The isolation rate of *M. morganii* decreased as the storage temperature decreased, regardless of the length of storage. *M. morganii* was not isolated at 4°C, coinciding with the low levels of histamine found in fish muscle. However, when *M. morganii* previously incubated at 25°C for 23 h was placed at 5°C, histamine continually accumulated, increasing from 800 ppm to 2,700 ppm in culture broth in 5 days of incubation (Klausen and Huss, 1987). Baranowski et al. (Baranowski et al., 1985) also reported that histidine decarboxylase already produced by bacteria could still convert histidine to histamine even after inactivation of the cells. In this study, growth of bacteria including *M. morganii* accelerated histamine accumulation at ambient temperature (25°C) even for a short period of storage. Therefore, any delay in proper chilling would allow continuous histamine accumulation to high levels in fish muscle. It

has been recommended that fish susceptible to histamine formation be chilled below 4°C in 4 h immediately after harvest on the vessel, and that the internal temperature of fish muscle be maintained in a proper chilling system throughout storage and distribution to prevent scombroid poisoning (FDA, 1998).

Histamine production by natural bacterial flora associated with the marine environment

Many of the natural bacterial flora in the marine environment are psychrotrophs or psychrophiles. Therefore, histamine-producing psychrotrophic bacteria have been studied to control histamine formation during storage of fish at refrigeration temperature or below (Morii et al., 1988; Rawles et al., 1996). In this study, several species of natural bacteria were the main histamine producers during storage at 4°C, the limiting temperature for the growth of the enteric bacteria. However, they were weak formers and isolated only with the extended storage.

Vibrio parahaemolyticus was the only mesophilic bacteria identified. Although this species was originally isolated from fresh fish, its proliferation was notable only at 25°C due to its cold sensitivity (Oliver and Kaper, 1997). *Vibrio alginolyticus* was the only species isolated during storage at 4 to 25°C, showing the characteristics of psychrotrophic bacteria. Morii et al. (1988) reported that *Photobacterium phosphoreum* may be principally responsible for histamine formation in scombroid fish at low temperatures. However, only one strain of *Photobacterium damsela* was isolated in this study, and it produced 112 ppm histamine in culture broth. *Aeromonas* spp. are widely distributed in the aquatic environment and have been isolated from various seafoods and dairy products during refrigerated storage (Wang and Silva, 1999). Most species are

psychrotrophs and reported as emerging foodborne pathogens associated with septicemia and gastroenterities in human (Velázquez et al., 2001). Although *Aeromonas hydrophila* has not been frequently reported as histamine former, it was isolated from both fresh and spoiled fish at 4°C in this study. *Pseudomonas* spp. were not often isolated as histamine formers. Most isolates from PI agar were confirmed as non-formers. Only 2 strains were isolated on VRBG agar during storage at 4 and 15°C, and their histamine producing capacities were insignificant. Similar results were obtained with fluorescent and non-fluorescent pseudomonads when tested for histamine production (Ryser et al., 1984). They produced the low levels of histamine during incubation in the histidine decarboxylase broth at 21°C for 48 h. Overall, the naturally present bacteria could proliferate at refrigeration temperature, but their contribution to histamine accumulation was negligible.

Conclusion

Temperature greatly influenced the growth of various types of bacterial flora and, consequently, histamine accumulation in Pacific mackerel during storage. Histamine accumulation was conspicuous with prevalence of prolific histamine formers during storage at ambient temperature (25°C). *Morganella morganii* was the main contributor to histamine formation in fish muscle during storage at 15 to 25°C. Growth of prolific histamine formers was controlled by storage of fish at 4°C or below. At 4°C, natural bacterial flora was the main histamine formers rather than *M. morganii* and other enteric bacteria. Although the natural bacterial flora was weak histamine formers, a hazardous level of histamine was detected in fish muscle only after fish became unsuitable for

human consumption. At 0°C, no histamine was found, and the growth of histamine formers was effectively controlled.

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References

Ababouch, L., Afilal, M.E., Rhafiri, S., and Busta, F.F. 1991. Identification of histamine-producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperatures (25°C). Food Microbiol. 8: 127-136.

Ababouch, L., Afilal, M.E., Benabdeljelil, H., and Busta. F.F. 1991. Quantitative changes in bacteria, amino acids and biogenic amines in sardine (*Sardina pilchardus*) stored at ambient temperature (25-28°C) and in ice. Int. J. Food Sci. Tech. 26: 297-306.

AOAC. 1995. Histamine in seafood: Fluorometric method. Sec. 35.1.32, Method 977.13. *In* Official Methods of Analysis of AOAC International. 16th ed. p. 6-17. AOAC International, Gaithersburg, MD.

Arnold, S.H., Price, R.J., and Brown, W.D. 1980. Histamine formation by bacteria isolated from skipjack tuna, *Katsuwonus pelamis*. Bull. Jpn Soc. Sci. Fish. 46: 991-995.

Barancin, C.E., Smoot, J.C., Findlay, R.H., and Actis, L.A. 1998. Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen *Vibrio anguillarum*. Plasmid. 39: 235-244.

Baranowski, J.D., Brust, P.A., and Frank, H.A. 1985. Growth of *Klebsiella pneumoniae* UH-2 and properties of its histidine decarboxylase system in resting cells. J. Food Biochem. 9: 349-360.

Baranowski, J.D., Frank, M.A., Brust, P.A., Chongsiriwatana, M., and Premaratne, R.J. 1990. Decomposition and histamine content in mahimahi (*Coryphaena Hippurus*). J. Food Prot. 53: 217-222.

Behling, A.R. and Taylor, S.L. 1982. Bacterial histamine production as a function of temperature and time of incubation. J. Food Sci. 47: 1311-1314, 1317.

Bennour, M., El Marrakchi, A., Bouchriti, N., Hamama, A., and El Ouadaa, M. 1991. Chemical and microbiological assessments of Mackerel (*Scomber scombrus*) stored in ice. J. Food Prot. 54: 784, 789-792.

Benson, H.J. (ed.). 1992. Microbiological applications: a laboratory manual in general microbiology. WCB, Dubuque, IA.

Eitenmiller, R.R., Wallis, J.W., Orr, J.H., and Phillips, R.D. 1981. Production of histidine decarboxylase and histamine by *Proteus morganii*. J. Food Prot. 44: 815-820.

El Marrakchi, A., Bennour, M., Bouchritt, N., Hamama, A., and Tagafait, H. 1990. Sensory, chemical, and microbiological assessments of Moroccan sardines (*Sardina pilchardus*) stored in ice. J. Food Prot. 53: 600-605.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species particularly kahawai (*Arripis trutta*). J. Aquatic Food Prod. 4: 53-74.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th ed. Arlington, VA.

Food and Drug Administration. 1996. Decomposition and histamine raw, frozen tuna and mahi-mahi; canned tuna; and related species. CPG 7108.240, Sec. 540.525.

Food and Drug Administration. 1998. Ch.7 Scombrotoxin (histamine) formation. *In* Fish and Fishery Products Hazards and Controls Guide. 2nd ed., p. 73-90. Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Frank, H.A., Baranowski, J.D., Chongsiriwatana, M., Brust, P.A., and Premaratne, R.J. 1985. Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32°C. Int. J. Food Microbiol. 2: 331-340.

Gennari, M., Tomaselli, S., and Cotrona, V. 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) sea and stored in ice. Food Microbiol. 16: 15-28.

Gram, L., Trolle, G. and Huss, H.H. 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4: 65-72.

Hungerford, Y.M. and Arefyev, A.A. 1992. Flow-injection assay of enzyme inhibition in fish using immobilized diamine oxidase. Anal. Chimica. Acta. 261: 351-359.

ICMSF. 1986. Microorganisms in Foods 2. Sampling for microbiological analysis: principles and specific application, 2nd ed. University of Toronto Press, Buffalo, NY.

Jhaveri, S.N., Leu, S., and Constantinides, S.M. 1982. Atlantic mackerel (*Scomber scombus*, L): Shelf life in ice. J. Food Sci. 47: 1808-1810.

Kim, S.H., An, H., and Price, R.J. 1999. Histamine formation and bacterial spoilage of albacore harvested off the U.S. Northwest coast. J. Food Sci. 64: 340-344.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Kim, S.H., Field, K.G., Morrissey, M.T., Price, R.J., Wei, C.I., and An, H. 2001. Source and identification of histamine-producing bacteria from temperature-abused albacore. J. Food Prot. 64: 1035-1044.

Klausen, N.K. and Huss, H.H. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

Kvenberg. J.E (ed.). 1991. Microbiology of marine food products. AVI, New York, NY.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.T. 1996. Sensory quality and histamine forming during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Morii, H., Cann, D.C., and Taylor, L.Y. 1988. Histamine formation by luminous bacteria in mackerel stored at low temperatures. Nippon Suisan Gakkaishi 54: 299-305.

Oliver, J.D. and Kaper, J.B. 1997. Ch. 13 *Vibrio* species. *In* Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, D.C.

Omura, Y., Price, R.J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Pacheco-Aguilar, R., Lugo-Sánchez, M.E., and Robles-Burgueño, M.R. 2000. Postmortem biochemical and functional characteristic of Monterey sardine muscle stored at 0°C. J. Food Sci. 65: 40-47.

Price, R.J., Melvin, E.F., and Bell, J.W. 1991. Postmortem changes in chilled round, bled and dressed albacore. J. Food Sci. 56: 318-321.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish and shellfish. Adv. Food Nutr. Res. 39: 329-364.

Rodríguez-Jerez, J.J., Mora-Ventura, M.T., López-Sabater, E.I., and Hernández-Herrero, M. 1994. Histidine, lysine, and orinithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. J. Food Prot. 57: 784-787, 791.

Ryser, E.T., Marth, E.H., and Taylor, S.L. 1984. Histamine production by psychrotrophic pseudomonads isolated from tuna fish. J. Food Prot. 47: 378-380.

Stratton, J.E. and Taylor, S.L. 1991. Scombroid poisoning. p. 331-351. *In* Microbiology of Marine Food Products. Van Nostrand Reinhold, N.Y.

Sumner, S. and Taylor, S.L. 1989. Detection method for histamine-producing, dairy-related bacteria using diamine oxidase and leucocrystal violet. J. Food Prot. 52: 105-108.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S.L. 1986. Histamine food poisoning: toxicology and clinical aspects. CDC. 17: 91-128.

Velázquez, L.C., Escudero, M.E., and Guzmán, A.M.S. 2001. Antibacterial effects of different food-related phosphates using *Aeromonas hydrophila*. J. Food Prot. 64: 195-200.

Wang, C. and Silva, J.L. 1999. Prevalence and characteristics of *Aeromonas* species isolated from processed channel catfish. J. Food Prot. 62: 30-34.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 6

Occurrence of Histamine-Forming Bacteria in Albacore and Histamine Accumulation in Muscle at Ambient Temperature

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Abstract

Distribution of histamine-producing bacteria in fresh albacore and their proliferation in muscle during storage at 25°C were monitored. Histamine formers constituted a minor portion of the bacterial flora in albacore. Only weak histamine formers were detected in the gill and skin of fresh fish, and enrichment step was required. Histamine formers were isolated from the muscles when APC reached $>10^7$ CFU/g during storage. *Hafnia alvei* was the most prevalent specie in both the fresh and temperature-abused fish. The most prolific histamine former, *Morganella morganii*, was rarely isolated even from spoiled muscles. The prevalence of weak histamine formers in albacore resulted in low levels of histamine accumulation in muscle, 67.1 mg/100 g, up to 6 days of storage at 25°C.

Introduction

Scombroid poisoning, a food-borne chemical intoxication, is one of the most prevalent seafood illnesses in the U.S. (FDA, 1998). Histamine is the main causative agent of scombroid poisoning. It is formed mostly resulting from time and temperature abuse of fish post harvest. Histamine formed in fish is regulated by the FDA in accordance to the HACCP principles (FDA, 2001). Toxic levels of histamine can be generated within 6 to 12 hours of catch, if fish are left on deck at above 25°C, without proper chilling (Price and Melvin, 1994). Thus, handling fish on fishing vessel is a critical control point to prevent histamine formation in fish. Rapid chilling of fish to 4°C or below immediately after catch is recommended to ensure product safety (FDA, 1998). Mainly, scombroid fish, i.e., tuna (yellowfin, bluefin, and skipjack), mackerel, bonito,

and saury, are susceptible to histamine formation due to high levels of free histidine in the muscle. Tuna served in a restaurant have frequently been implicated in outbreaks of scombroid poisoning because of improper holding temperature during handling, storage, and distribution of fish (Becker et al., 2001).

Bacteria synthesizing histidine decarboxylase are the main source of histamine formation in fish (FDA, 1998). This enzyme is responsible for converting histidine to histamine. To identify the main contributors to histamine accumulation in fish, various levels and types of histamine-forming bacteria have been isolated from fish under different storage conditions. It has been reported that *Morganella morganii* (López-Sabater et al., 1996a; Rawles et al., 1996), *Klebsiella pneumoniae* (López-Sabater et al., 1996a), and *Proteus vulgaris* (Kim et al., 2001b) are prolific histamine formers, producing >1,000 ppm histamine in the culture broth. Although many researchers postulate the presence of these bacteria in the marine environment (López-Sabater et al., 1996), they have been rarely detected in fresh fish. They are mostly isolated from fish spoiled under controlled storage conditions (>20°C) (Ababouch et al., 1991; Frank et al., 1985; Kim et al., 2001b). Natural bacteria in the marine environment, i.e., *Photobacterium* spp., *Pseudomonas* spp., *Vibrio alginolyticus*, and *Aeromonas* spp., are frequently isolated from fish stored at refrigeration temperature for extended periods (Kim et al., 2001b; Middlebrooks et al., 1988; Morii et al., 1988). Generally, the identified bacteria are weak histamine formers, producing <500 ppm histamine in the culture broth (Frank et al., 1985). Growth of prolific histamine formers and histamine formation are limited under the storage conditions, and histamine is only detected after fish became unsuitable for human consumption (Kim et al., 2001b). Thus, temperatures

greatly influence the growth of prolific histamine formers during handling, distribution, and storage of fish, consequently increasing the chance to accumulate histamine in fish.

Albacore (*Thunnus alalunga*) is a valued fish characterized by its white meat and mild flavor. Although the fish has been rarely implicated in outbreaks of scombroid poisoning, there has been concerns for potential formation of histamine, since it belongs to scombroid fish species, contains high levels of free histidine in muscle, and has high body temperature (26-30°C) (Craven et al., 1995). Most of albacore are frozen on board to prevent histamine formation and supplied frozen to canneries (Price and Melvin, 1994). To develop alternative non-frozen tuna market for better eating quality, it is important to understand basic phenomenon of histamine formation in the fish. The objective of this study was to identify distribution of histamine formers in fresh albacore and to monitor prevalent histamine formers and histamine accumulation during storage at ambient temperature. In this study, histamine formers were isolated from fresh albacore with and without enrichment step. Total bacterial counts and histamine contents in fish muscles were evaluated, and histamine-producing bacteria isolated during storage of albacore at 25°C were identified to species.

Materials and Methods

Reagents and media

Dowex 1-X8, histamine dihydrochloride, *o*-phthaldialdehyde (OPT), and tetramethyl-*p*-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Kovacs' reagent was obtained from Difco Laboratories (Detroit, MI).

Trypticase soy broth (TSB) was purchased from BBL Co. (Cockeysville, MD). Standard plate count (SPC) agar, violet red bile glucose (VRBG) agar, pseudomonas isolation (PI) agar, thiosulfate citrate bile salts sucrose (TCBS) agar, and tryptone broth were obtained from Difco.

Samples and storage conditions

Fresh albacore were troll-caught off the Oregon Coast in 1999. Fish, kept in ice on-board, were purchased upon the arrival of fish at a local seafood processing company (Astoria, OR). Whole albacore weighing 10 to 13 kg were selected and transported in ice to Oregon State University-Seafood Laboratory (Astoria, OR). Two fish were immediately used for enumeration of initial bacterial counts and isolation of histamine formers. Eight fish were separately placed in sterile plastic bags and stored at 25°C. Each sample was taken every 24 hr for histamine analysis, aerobic plate counts (APC), and isolation of histamine formers.

Sample treatment for APC enumeration and bacterial isolation

Skin, gill, intestine, and dorsal muscle of fresh fish were collected for the enumeration of APC. For sampling skin, a sterile aluminum foil template (4 × 5 cm) was placed on the surface of skin, and the area of template was swabbed with sterile cotton. The swabbed cotton was inoculated into 20 ml of TSB (Omura et al., 1978). For collecting gill, intestine, and dorsal muscle samples, 10 g was removed aseptically and blended with 90 ml of peptone water (0.1%). In the enrichment step, an aliquot (10 mL)

of blended gill and intestine and swabbed skin was inoculated into 90 mL of trypticase soy broth supplemented with 0.5% NaCl and incubated at 25°C for 18 hr.

APC was carried out in duplicate. One mL aliquots from each sample were serially diluted, dispensed, and plated with SPC supplemented with 0.5% NaCl (FDA, 1992). Plates were incubated at 25°C for 2 days.

Histamine analysis of fish muscles

Histamine content in fish muscle was determined in duplicate by the standard AOAC fluorometric method (AOAC, 1995). Dorsal muscle, 10 g, was homogenized in 50 mL of methanol (100%) and incubated at 60°C for 15 min. The volume of the methanol extract was adjusted to 100 mL with methanol in a volumetric flask, and the extract was filtered through Whatman no. 1 paper. The filtrate was subjected to an ion exchange column (200 × 7 mm) packed with Dowex 1-X8 and eluted with H_2O. Histamine standards (0.5, 1.0, and 1.5 μg) and the column eluants were derivatized with OPT, and fluorescence intensity was determined using a spectrophotofluorometer (Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 350 nm and an emission wavelength of 444 nm.

Bacterial isolation

Several groups of bacteria were isolated by using a pour plate method (FDA, 1992). Sample treatments and incubation of plates for fish samples were carried out as described in the section "Sample treatment for APC enumeration and bacterial isolation." Each diluent was plated with selective media for screening histamine formers. The selective media used for target bacteria were VRBG agar for enteric bacteria; TCBS agar

for vibrios; and PI agar for pseudomonads. Colonies on each plate were picked based on their morphological characteristics and inoculated onto TSA slants for histamine analysis and identification.

Histamine formation by bacterial isolates

Bacterial isolates were inoculated into TSB supplemented with 1% histidine and incubated at 25°C for 18 h (Taylor and Speckhard, 1983). One ml of bacterial culture was taken and mixed with 9 ml methanol. Histamine content in culture broth was determined in duplicate by the AOAC fluorometric method (AOAC, 1995) as described above.

Bacterial identification

Isolates were identified by using the Vitek instrument (bioMérieux Vitek, Inc., Hazelwood, MI) according to the protocol recommended by the manufacturer. Each isolate was inoculated onto TSA slants and incubated at 25°C for 18 h. Gram staining was carried out by the standard method for the selection of adequate identification cards (Benson, 1992). Oxidase production was tested to differentiate enteric bacteria from other gram negative bacteria by using the filter paper method (Benson, 1992). For the bacterial identification, each culture was suspended in phosphate saline buffer, pH 7.0, at a suitable reading range determined by the Vitek colorimeter and subsequently placed in a Vitek identification card. The digitalized analog optical reading and bacterial identification were automatically carried out by the Vitek system.

Additional biochemical test was carried out for definitive species identification. To differentiate two species of *Proteus*, an indole test was carried out by the standard method (Benson, 1992). One loopful of culture was inoculated into tryptone broth and incubated at 37°C for 24 h. To the bacterial culture, 0.1 ml of Kovacs' reagent was added, and change of color was monitored. For *Pseudomonas* spp., gelatin utilization was tested by liquification of gelatin medium (Benson, 1992). One loopful of culture was inoculated into gelatin medium and incubated at 22°C for 10 days.

Results

Initial bacterial counts in fresh albacore

Initial bacterial counts in the gill, skin, and intestine of fresh albacore are listed in Table 6.1. Bacterial counts did not differ greatly between the two fish tested. The highest bacterial count was detected in the gill followed by the skin. APC detected were 7.7×10^4 and 6.9×10^4 CFU/g in the gill and 3.4×10^4 and 1.9×10^4 CFU/cm^2 on the skin. Relatively low APC, 1.2×10^3 and 1.5×10^3 CFU/g, were detected in the intestine. APC did not exceed 10^5 CFU/g in any of the samples tested. Bacterial counts were not detected in the muscle ($<10^2$ CFU/g).

When samples were plated with various selective media, i.e., VRBG, PI, and TCBS agars, no bacterial growth was found on TCBS agar (Table 6.1). Some bacteria grew on VRBG and PI agar, but the counts were generally low. Most isolates from the skin and gill did not produce histamine in TSB supplemented with 1% histidine. Only a

few isolates from the skin were weak histamine formers, producing histamine ranging from 10 to 30 ppm in culture broth (data not shown).

Table 6.1. Counts of total aerobic bacteria and isolation of histamine formers from fresh albacore

	Origin	APC (CFU/g)	Number of histamine formers[a]/ Number of isolates[b]		
			VRBG[c]	PI[d]	TCBS[e]
Fish 1	Skin	3.4×10^4	0/20	0/23	N.D.*
	Gill	7.7×10^4	0/10	0/8	N.D.
	Intestine	1.2×10^2	N.D.	N.D.	N.D.
	Muscle	N.D.	N.D.	N.D.	N.D.
Fish 2	Skin	1.9×10^4	4/23	0/22	N.D.
	Gill	6.9×10^4	0/9	0/13	N.D.
	Intestine	1.5×10^2	0/2	N.D.	N.D.
	Muscle	N.D.	N.D.	N.D.	N.D.

[a]Histamine production by isolates was confirmed by the AOAC fluorometric method.
[b]Target groups of bacteria were isolated using selective media.
[c]Violet red bile glucose agar
[d]Pseudomonas isolation agar
[e]Thiosulfate citrate bile salt sucrose agar
*Not detected

Isolation of histamine formers from fresh fish with enrichment

The frequently reported histamine formers were found in fresh albacore enriched in TSB supplemented with 0.5% NaCl (Tables 6.2, 6.3, and 6.4). Histamine formers were mostly isolated from the gill and skin. The pattern of growth on selective media and the identified species of the bacteria sampled from the gill were slightly different from those of the skin. More variable species of histamine formers were found in the gill than the skin.

Weak histamine formers in the gill were isolated on VRBG and TCBS agar (Table 6.2). *Hafnia alvei*, *Enterobacter cloacae*, and *Acinetobacter lwoffii*, were isolated on VRBG agar. The isolated *Hafnia alvei* strains produced 10.4 to 172 ppm histamine in the culture broth supplemented with 1% histidine. *E. cloacae* was less frequently isolated than *H. alvei* and produced histamine ranging from 20.9 to 30.0 ppm. Histamine-producing vibrios were not isolated on TCBS even with the enrichment step. The histamine formers isolated on TCBS agar were *Photobacterium damselae* and several unidentified isolates. *Photobacterium damselae* isolates produced histamine ranging from 89.5 to 340 ppm in culture broth. All isolates did not produce high levels of histamine, although *Photobacterium* spp. have been reported as prolific histamine formers (Morii et al., 1988). Unidentified weak histamine formers (11 isolates) produced histamine ranging from 47.8 to 287 ppm in culture broth (data not shown).

Table 6.2. Identification of histamine formers from the gill of fresh albacore after enrichment procedure

Origin	Strain isolated	Number isolated	Medium[a] used	Histamine[d] (ppm)
Fish 1	*Hafnia alvei*	6	VRBG[b]	10.4–153 (60.1 ± 61.5)
	Photobacterium damsela	3	TCBS[c]	89.5 – 327 (117 ± 130)
	Acinetobacter lwoffii	1	VRBG	151
	Enterobacter cloacae	1	VRBG	20.9
Fish 2	*Hafnia alvei*	13	VRBG	19.0–172 (51.4 ± 41.5)
	Photobacterium damselae	3	TCBS	325- 340 (333 ± 7.60)
	Enterobacter cloacae	3	VRBG	10.4 – 30.0 (23.3 ± 11.2)

[a]Target group of bacteria was isolated by using selective medium.
[b]Violet red bile glucose agar; [c]Thiosulfate citrate bile salt sucrose agar
[d]Histamine production by isolates was confirmed by the AOAC fluorometric method.

For skin, histamine formers were isolated only on VRBG agar (Table 6.3). All histamine formers detected were enteric bacteria. *Hafnia alvei* was most frequently isolated from the skin. Minor isolates were identified as *Enterobacter cloacae* and *Enterobacter aerogenes.* Histamine-producing capacities of the isolates from the skin were similar to those from the gill. One *Acinetobacter lowffii* was isolated on VRBG agar. Other natural bacteria in the marine environment were not isolated from the skin.

Table 6.3. Identification of histamine formers from the skin of fresh albacore after enrichment step

Sample	Strain isolated	Number isolated	Medium[a] used	Histamine[c] (ppm)
Fish 1	*Hafnia alvei*	11	VRBG[b]	6.9 – 179 (72.8 ± 55.5)
	Enterobacter clocae	5		4.9 – 105 (41.9 ± 40.1)
	Enterobacter aerogenes	1		18.6
Fish 2	*Hafnia alvei*	8	VRBG	19.0 – 178 (72.8 ± 55.5)
	Acinetobacter lwoffii	1		75.3
	Enterobacter aerogenes	1		28.5
	Enterobacter cloacae	1		11.4

[a]Target group of bacteria was isolated by using selective medium.
[b]Violet red bile glucose agar
[c]Histamine production by isolates was confirmed by the AOAC fluorometric method.

Histamine formers were rarely isolated from the intestine even with enrichment (Table 6.4). A total of 110 strains were isolated on VRBG agar, but most isolates did not produce detectable amounts of histamine in culture broth. One isolate identified was *Acinetobacter lowffii.* Three weak histamine formers could not be identified to species. The presence of *H. alvei* and other enteric histamine formers in the intestine was not

confirmed in this study. Since the intestine of fresh albacore generally remains intact even during handling of fish, non-histamine formers on VRBG agar were also identified to detect the potential presence of prolific enteric histamine formers. When oxidase test was performed to differentiate enteric bacteria from marine bacterial species, most isolates showed the oxidase-positive reaction, indicating the growth of marine bacteria on VRBG agar. Several species reported as histamine formers were identified as non-histamine formers when isolated from the intestines (Table 6.5). Among the oxidase-positive isolates, they were *Photobacterium damselae*, *Aeromonas hydrophila*, and *Vibrio* spp. Among the oxidase-negative isolates, *Proteus vulgaris*, *Enterobacter* spp., and *Providencia alcalifaciens* were frequently isolated from the first fish tested.

Table 6.4. Identification of histamine formers from the intestine of fresh albacore after enrichment step

Origin	Stain isolated	Medium[a] used	Histamine[e] (ppm)
Fish 1	*Acinetobacter lowffii*	VRBG[b]	38.8
	N.D.*	TCBS[c]	N.D.
	N.D.	PI[d]	N.D.
Fish 2	N.D.	VRBG	65.9
	N.D.	VRBG	60.2
	N.D.	TCBS	115
	N.D.	PI	N.D.

[a]Target group of bacteria was isolated by using selective medium.
[b]Violet red bile glucose agar
[c]Thiosulfate citrate bile salt sucrose agar
[d]Pseudomonas isolation agar
[e]Histamine production by isolates was confirmed by the AOAC fluorometric method.
*Not detected

Table 6.5. Identification of non-histamine formers from the intestine of fresh albacore

Origin	Strain[a] isolated	Medium used	Number isolated
Fish 1	*Proteus vulgaris*	VRBG[b]	4
	Enterobacter cloacae		7
	Acinetobacter lowffii		1
	Eikenella corrodens		1
	Providencia alcalifaciens		2
	Aeromonas hydrophila		1
	Enterobacter aerogenes		3
	Vibrio alginolyticus		1
Fish 2	*Eikenella corrodens*	VRBG	1
	Pasteurella multitocida		2
	Photobacterium damsela		1
	Aeromonas hydrophila		1
	Vibrio fluvialis		1

[a]Non-histamine formers isolated from the intestine of fresh fish were randomly identified.
[b]Violet red bile glucose agar

Overall, the identified histamine formers were not very different between the two albacore tested. Prolific histamine formers, producing histamine >1,000 ppm in culture broth, were not isolated from the fresh fish. *Hafnia alvei* on VRBG agar was mostly isolated from the skin and gill of fresh fish, but they were weak histamine formers (<500 ppm). Only *Acinetobacter lowffii* was isolated from the skin, gill, and intestine of fresh fish.

Bacterial growth, quality change, and histamine production during storage at 25°C

The quality changes in albacore progressed slowly during storage at 25°C. Fish samples were in good condition. The color of muscle was translucent-red on the first day of storage. Slight slime developed on the skin surface on the second day. At this time, APC reached 1.2×10^5 CFU/g (Fig. 6.1). Quality deterioration of albacore was accelerated on the 3rd day of storage. Off-odor and cloudy and yellow slime on the surface of skin, blood spots, and belly burn were detected, and muscle turned opaque-white. APC detected was 1.4×10^7 CFU/g. On day 4, fish were completely decomposed as judged by strong off-odor and soften muscle texture. On day 5, APC reached the highest level, 1.7×10^8 CFU/g and decreased thereafter. The quality deterioration in albacore progressed slowly. Belly burst and dissolution of intestinal wall were not observed during the storage of albacore, which is a commonly observed deterioration characteristic in smaller fish such as mackerel and sardine (Kim et al., 2001b).

Among the selective media tested, the highest bacterial counts were found on VRBG agar. The bacterial counts on VRBG agar were one log cycle lower than the total bacterial counts (data not shown). However, the bacterial counts on TCBS and PI agars were generally low ($<10^5$ CFU/g) during the storage.

The histamine level increased gradually during the storage of albacore at 25°C, the optimum temperature for histamine formation in albacore (Kim et al., 1999). Histamine production in fish muscles was still negligible when APC reached 5×10^5 CFU/g, the recommended microbiological limits in fish (ICMSF, 1986). Histamine began to accumulate when fish were apparently spoiled and became unsuitable for human consumption. On day 3, histamine content in the muscles exceeded the FDA guideline, 5

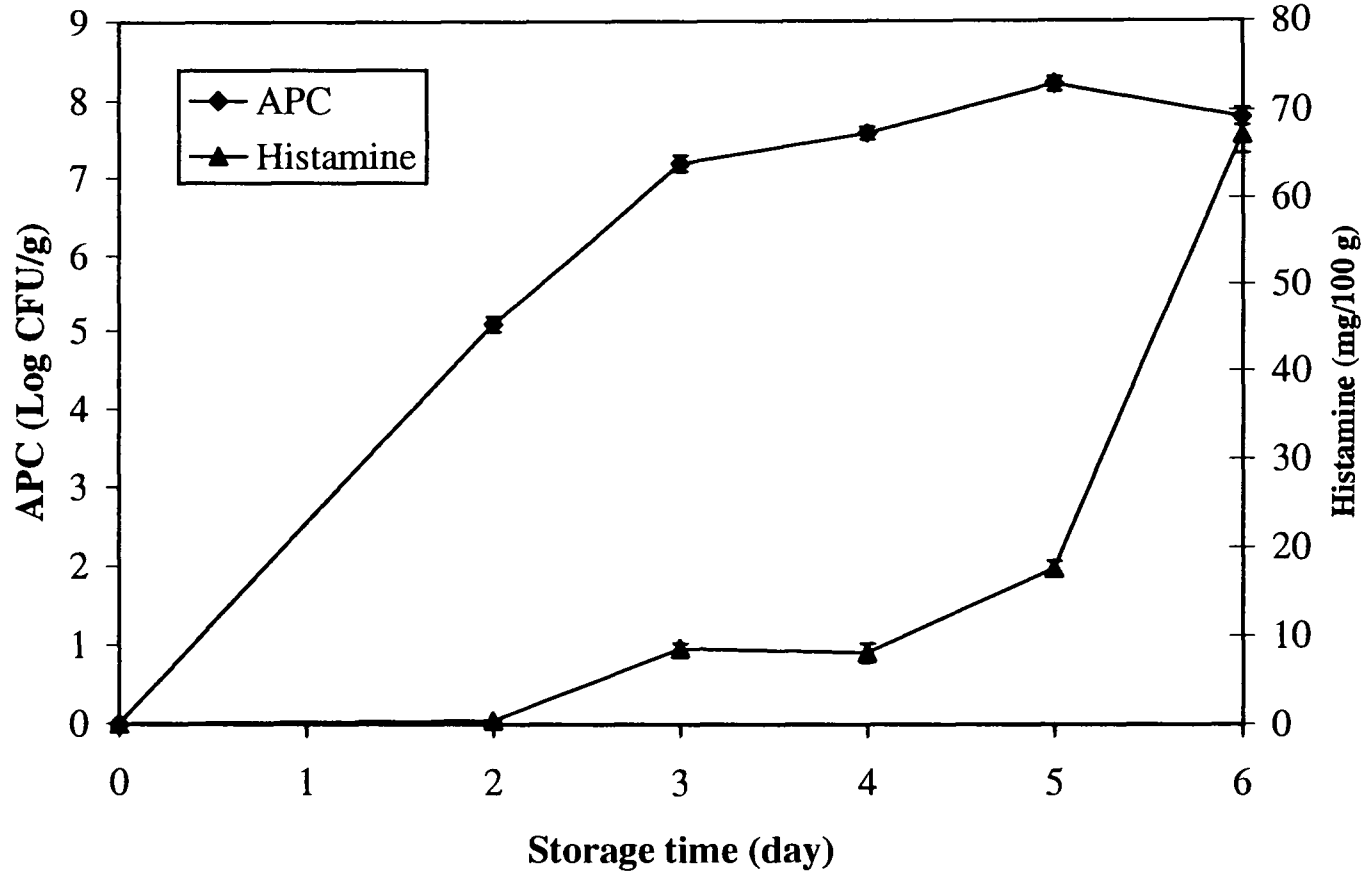

Fig. 6.1. Changes in APC and histamine content of albacore during storage at 25°C. APC was determined by using the standard pour plate method. Histamine content was analyzed by the AOAC fluorometric method.

mg/100 g (FDA, 1998). Histamine accumulation was still low on day 5. The histamine content was 17.2 mg/100 g. However, it increased rapidly thereafter and reached the highest level, 67.1 mg/100 g, on the 6th day of storage.

Histamine formers identified during storage at 25°C

As observed with fresh albacore, histamine formers constituted a minor portion of bacterial flora during fish spoilage. A total of 750 strains were isolated from albacore during the course of storage. Histamine formers were isolated only on VRBG agar. Seventy-one out of the four hundreds isolates were positive for histamine formation. Identified histamine formers in spoiled albacore were limited only to several species of enteric histamine formers. Other selective media were not efficient in screening histamine formers in albacore during storage at 25°C.

Histamine formers were frequently detected when APC reached 10^7 CFU/g in 3 days of storage (Table 6.6). *Morganella morganii* was the most prolific histamine former, producing >3,000 ppm histamine in culture broth. *M. morganii* were first isolated on day 3. However, this species was not prevalent during storage and detected as a minor species of histamine former in albacore. A total of 7 strains were isolated during the 6 days of storage. Another prolific histamine former, *Proteus vulgaris*, was isolated only from the 4-day old fish. The three isolates of *P. vulgaris* produced 2,945 to 3,474 ppm histamine in the culture broth.

Table 6.6. Identification of histamine formers from albacore during storage at 25°C

Storage day(s)	Strain isolated	Number isolated	Medium[a] used	Histamine[c] (ppm)
3	*Hafnia alvei*	8	VRBG[b]	26.4 – 497 (168 ± 171)
	Morganella morganii	1		3,875
	Edwardsiella tarda	1		393
4	*Hafnia alvei*	11	VRBG	24.4 – 351 (135 ± 142)
	Proteus vulgaris	3		2,945 – 3,474 (3,197 ± 265)
	Morganella morganii	2		3,841 - 3,940
	Actinobacillus ureae	1		203
5	*Hafnia alvei*	8	VRBG	25.8 – 429 (180 ± 134)
	Enterobacter cloacae	6		18.3 – 137 (82.9 ± 59.2)
	Enterobacter aerogenes	2		89.1 – 130
	Morganella morganii	1		3,687
	Eikenella corrodens	1		142
6	*Hafnia alvei*	13	VRBG	17.0 – 467 (144 ± 148)
	Enterobacter cloacae	7		19.3 – 366 (139 ± 141)
	Morganella morganii	3		3,582 – 3,672 (3,630 ± 45.3)
	Enterobacter aerogenes	3		29.1 – 86.1 (49.3 ± 31.9)

[a]Target group of bacteria was isolated by using selective medium.
[b]Violet red bile glucose agar
[c]Histamine production by isolates was confirmed by the AOAC fluorometric method.

Weak histamine formers were more frequently isolated than prolific histamine formers from albacore. As observed with fresh albacore, *Hafnia alvei* was the most prevalent species, and a total of 40 strains were isolated during the storage. *H. alvei* produced 17 to 497 ppm histamine. *Enterobacter cloacae* and *Enterobacter aerogenes* were not isolated at the beginning of the storage, but often isolated after 4 days of

storage. All isolates produced low levels of histamine (<150 ppm) in culture broth. The minor species was identified as *Edwardsiella tarda*, *Actinobacillus ureae*, and *Eikenella corrodens*.

Discussion

Psychrotrophic bacteria initially found in fresh albacore

The bacterial flora in fish reflects the marine environment of fish harvested, such as geographical location and season (Taylor et al., 1986). Predominant bacterial flora and initial bacterial numbers in fresh fish are influenced by the variation of salinity, temperature, organic matter, and water quality of the harvest areas (Kvenberg, 1991). The natural bacterial flora resides mainly in the outer slime layer of the skin, on the gills, and in the intestines of fish (Gram et al., 1987). The most susceptible part of fish to bacterial colonization is the gill followed by the outer skin and slime of fish (Jay, 1991).

Histamine formers have rarely been identified from fresh fish. However, it has been suspected that histamine formers would be a part of bacterial flora in fresh fish (Taylor and Speckhard, 1983; Stratton and Taylor, 1991; López-Sabater et al., 1996a; Kim et al., 2001a). In this study, we demonstrated that histamine formers were minor bacterial flora in fresh albacore, which could be isolated only with enrichment. Thus, the conventional culture method has a great limitation to identify the source of histamine formers in fresh fish due to their low bacterial numbers ($<10^2$ CFU/g). It will necessitate use of molecular-based techniques for higher sensitivity and accuracy to investigate the origin of prolific histamine formers in fish as well as the marine environment.

Albacore is a highly migratory fish species and found worldwide (Pérez-Villarreal and Pozo, 1990). In the Pacific, albacore school off in the waters of the West Coast and Hawaii. Off the Northwest Coast, albacore is generally harvested in waters with a temperature range of 15 to 16°C (Craven et al., 1995). Bacterial counts of the gill, intestine, and skin in fresh albacore were generally one log cycle lower than those in fresh Pacific mackerel and other fish species reported (Kim et al., 2001b; Kvenberg et al., 1991). Histamine formers identified were either enteric bacteria or natural bacteria found in the marine environment. They were distributed mostly in the gill and skin of fresh albacore. Most species belonged to psychrotrophic and/or psychophilic bacteria. In addition, cold sensitive *Vibrio* species such as *V. parahaemolyticus* frequently isolated from Pacific mackerel caught in July of 1999 (Kim et al., 2001b) were not isolated from any of the samples tested. This observation reflects the change of bacterial flora with seasonal variation in the marine environment. Therefore, we concluded that prolific histamine formers, mostly belonging to mesophilic bacteria, were not detected in the fish presumably because of too low water and ambient air temperatures for bacterial growth.

Prevalence of weak histamine formers in albacore during storage

Although a variety of histamine formers were initially found in fresh albacore, histamine formers detected in the muscles of albacore during storage were limited to only a few species of histamine formers. Prevalent histamine formers detected were psychrotrophic enteric bacteria. Among them, *H. alvei* and *Enterobacter* spp. were found as the main contributors to the histamine formation in albacore. Their histamine producing capacities did not differ greatly. Both species were weak histamine formers.

In general, *H. alvei* can grow in a wide range of temperatures. Optimal temperature for their growth is 37°C (Holt et al., 1994). The minimum temperature for growth ranges from 0.2 to 3.7°C with an average of 2.6°C (Ridell and Korkeala, 1997). In the refrigerated meat, *H. alvei* was the most frequently isolated enteric bacteria followed by *Serratia liquefaciens* and *Enterobacter* spp. (Lindberg et al., 1998; Gram et al., 1987).

H. alvei was often isolated from fish incriminated in scombroid poisoning along with the prolific histamine formers, *Morganella morganii* and *Klebsiella pneumoniae* (Taylor et al., 1986; Rawles et al., 1996). It was isolated from tuna fish (López-Sabater et al., 1996b), mackerel (Middlebrooks et al., 1988), albacore (Kim et al., 2001a) and Kahawai (Bremer et al., 1998). Generally, *H. alvei* has been characterized as a weak histamine former. The isolates obtained from tuna (*Thunnus thynnus*) produced 224 ppm histamine on the average in histamine enumeration broth supplemented with 1% histidine (López-Sabater et al., 1996b). Only a few isolates produced >1,000 ppm histamine in culture broth (López-Sabater et al., 1996a; Rawles et al., 1996).

Enterobacter spp. have often been isolated from various species of scombroid fish. *E. aerogenes*, *E. cloacae*, *E. agglomerans*, and *E. intermedium* have been isolated from tuna (*Thunnus thynnus*) (López-Sabater et al., 1996a) and albacore (Kim et al., 2001a). *E. aerogenes* was isolated from ripened Spanish salted anchovies with other bacteria, such as halotolerant and halophilic histamine formers (Herández-Herrero et al., 1999). Although a few isolates have been reported as prolific histamine formers, most of the isolates produced <200 ppm histamine in culture broth (López-Sabater et al., 1996a; Kim et al., 2001a).

Psychrophilic natural bacteria were not detected as often as enteric histamine formers in fish during storage. Although *Photobacterium damselae* and *Acinetobacter lowffii* were often isolated in fresh albacore, they were not detected in the muscles during storage at 25°C, their limiting growth temperature. The upper growth limit temperature for *P. damselae* is reported to be 22°C (Fouz et al., 2000; Gram et al., 1987).

Limited prolific histamine formers in albacore

M. morganii, the most prolific histamine former, shows little phenotypical strain-to-strain variations in its biochemical characteristics (Janda et al., 1996). The genus *Morganella* belongs to the tribe *Proteese* of the family *Enterobacteriaceae* along with two other genera, *Proteus* and *Providencia* (Holt et al., 1994). Most members of the *Proteus-Providencia-Morganella* group share a number of common biochemical characteristics, including a methyl red and urease positive reactions, growth in KCN broth, motility, and production of phenylalanine deaminase (Janda et al., 1996). *Proteus* spp. and *Providencia* spp. have been also reported as histamine formers. *Proteus vulgaris* was a prolific histamine former together with *M. morganii* in albacore muscle during storage (Table 6.6) as previously found in mackerel (Kim et al., 2001b). *Providencia* spp. isolated from sardines produce histamine ranging from 1,070 to 1,280 ppm (Ababouch et al., 1991).

Prolific histamine formers were not detected in fresh albacore presumably due to the low initial counts and lack of sensitivity of culture methods in this study. However, *P. vulgaris* was isolated as a non-histamine former along with *Providencia alcalifaciens* in the intestine of fresh fish, indicating the possibility that *M. morganii* may be present in

the intestine of fresh fish. *Photobacterium damselae* and *Enterobacter cloacae* were identified as non-histamine formers from the intestine of fresh fish as well. It is not clear if free histidine is essential to induce expression of the histidine decarboxylase gene.

Prevalence of prolific histamine formers was directly related to histamine accumulation in fish muscles. Histamine production increased rapidly with the proliferation of *M. morganii*, reaching up to 283 mg/100 g in 2 days in mackerel stored at 25°C (Kim et al., 2001b). *M. morganii* generally produced the highest level of histamine in fish and culture media in the stationary phase (Kim et al., 2000). Thus, large bacterial populations (>10^7 CFU/g) are required to produce significant levels of histamine (López-Sabater et al., 1996a). *M. morganii* produced 5,400 ppm histamine in culture broth, when bacterial counts reach 10^9 CFU/mL (Klausen and Huss, 1987). In this study, the isolation of *M. morganii* from albacore was negligible, thus resulting in low histamine accumulation in fish muscle. A toxicological level of histamine (>50 mg/100 g) was detected only when fish appeared completely spoiled with the prolonged storage.

Resistance of albacore to histamine formation

There has been a large effort to harvest albacore off the Northwest Coast of the U.S., and the increased production has led to continued commercial interest (Craven et al., 1995). Product safety mainly pertaining to histamine formation is of great concern to the tuna industry because of the FDA regulation. Proper handling and chilling methods have been documented as effective means to prevent histamine formation and quality deterioration on board (FDA, 1998; Price and Melvin, 1994). However, albacore harvested off the U.S. Northwest Coast has shown to be highly resistant to histamine

formation (Craven et al., 1995; Ben-Gigirey et al., 1998; Kim et al., 1999). Albacore is also one of the few fish species rarely implicated in scombroid poisoning incidences despite the considerable volume of fish consumed (Stratton and Taylor, 1991). In general, scombroid poisoning shows the wider geographic occurrence in the U.S. than any other seafood poisonings, with the incidences reported from the 45 states (Stratton and Taylor, 1991). The highest number of outbreaks and cases occurred in Hawaii. Fish imported to the U.S. from tropical areas, particularly mahi-mahi, have been implicated as a cause of scombroid poisoning resulting from the high ambient water and air temperatures in the originated area, mishandling conditions on boats, and market sanitary conditions (Ahmed, 1991).

In this study, we observed that histamine formers constituted a minor portion of bacterial flora in albacore, and prolific histamine formers were not prevalent even during storage at ambient temperature (25°C). Since the main histamine formers have not been intensively investigated in a variety of fish species, much more rigid guidelines have been established for all types of fish regardless of their harvest area and handling conditions. We suggest that avoiding contamination of prolific histamine formers including *M. morganii* from the marine environment and preventing their proliferation during handling and distribution of fish would be critical in controlling histamine accumulation and subsequently, outbreaks of scombroid poisoning associated with fish.

Conclusion

The most prolific histamine former, *Morganella morganii*, was hardly isolated even from spoiled albacore. Low population of initial histamine formers in fresh fish and the prevalence of weak histamine formers during storage resulted in low histamine accumulation in albacore. This result supports the common observation that albacore has rarely been involved in scombroid poisoning. The future regulation should target the presence and proliferation of prolific histamine formers as the main contributors of histamine formation in fish.

Acknowledgments

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References

Ababouch, L., Afilal, M.E., Rhafiri, S., and Busta, F.F. 1991. Identification of histamine-producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperatures (25°C). Food Microbiol. 8: 127-136.

Ahmed, F.E (ed.). 1991. Ch. 4. Naturally occurring fish and shellfish poisons. p.87-110. *In* Seafood Safety. National Academy Press, Washington, D.C.

AOAC. 1995. Histamine in seafood: Fluorometric method. Sec. 35.1.32, Method 977.13. *In* Official Methods of Analysis of AOAC International. 16th ed. p. 6-17. AOAC International, Gaithersburg, MD.

Becker, K., Southwick, K., Reardon, J., Berg, R., MacCormack, J.N. 2001. Histamine poisoning associated with eating tuna brgers. JAMA. 285: 1327-1330.

Ben-Gigirey, B., Craven, C., and An, H. 1998. Histamine formation in albacore muscle analyzed by AOAC and enzymatic methods. J. Food Sci. 63: 210-214.

Benson, H.J (ed.). 1992. Microbiological applications: a laboratory manual in general microbiology. WCB, Dubuque, IA.

Bremer, P.J., Osborne C.M., Kemp R.A., van Veghel, P. and Fletcher, G.C. 1998. Thermal death times of *Hafnia alvei* cells in a model suspension and in artificially contaminated hot-smoked kahawai (*Arripis trutta*). J. Food Prot. 61: 1047-1051.

Craven, C., Hilderbrand, K., Kolbe, E., Sylvia, G., Daeschel, M., Gloria, B., and An, H. 1995. Understanding and controlling histamine formation in troll-caught albacore tuna: A review of preliminary findings from the 1994 season. Oregon Sea Grant (ORESU-T-95-00). Corvallis, OR.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species particularly kahawai (*Arripis trutta*). J. Aquatic Food Prod. 4: 53-74.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th ed. Arlington, VA.

Food and Drug Administration. 1998. Ch.7. Scombrotoxin (histamine) formation. *In* Fish and Fishery Products Hazards and Controls Guide. 2nd ed., p.73-90. Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Food and Drug Administration. 2001. FDA's Seafood HACCP Program: Mid-Course Correction. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Fouz, B., Toranzo, A.E., Milán, M., and Amaro, C. 2000. Evidence that water transmits the disease caused by the fish pathogen *photobacterium damselae* subsp. *damselae*. J. Appl. Microbiol. 88: 531-535.

Frank, H.A., Baranowski, J.D., Chongsiriwatana, M., Brust, P.A., and Premaratne, R.J. 1985. Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32°C. Int. J. Food Microbiol. 2: 331-340.

Gram, L., Trolle, G., and Huss, H.H. 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4: 65-72.

Hernández-Herrero, M.M., Roig-Sagués, A.X., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1999. Halotolerant and halophilic histamine-forming bacteria isolated during the ripening of salted anchovies (*Engraulis encrasicholus*). J. Food Prot. 62: 509-514.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. 1994. Bergey's manual of determinative bacteriology, 9th ed. Williams and Wilkins Co., Baltimore.

ICMSF. 1986. Microorganisms in Foods 2. Sampling for microbiological analysis: principles and specific application, 2nd ed. University of Toronto Press, Buffalo, NY.

Janda, J.M., Abbott, S.L., Khashe, S., and Robin, T. 1996. Biochemical investigation of biogroups and species of *Morganella morganii*. J. Clin. Microbiol. 34: 108-113.

Jay, J.M (ed.). 1996. Ch.6. Seafoods. p. 118-130. *In* Modern Food Microbiology. Fifth Ed., ITP. New York, NY.

Kim, S.H., An, H., and Price, R.J. 1999. Histamine formation and bacterial spoilage of albacore harvested off the U.S. Northwest coast. J. Food Sci. 64: 340-344.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Kim, S.H., Field, K.G., Morrissey, M.T., Price, R.J., Wei, C.I., and An, H. 2001a. Source and identification of histamine-producing bacteria from fresh and temperature-abused albacore. J. Food Prot. 64: 1035-1044.

Kim, S.H., Field, K.G., Chang, D.S., Wei, C.I., and An, H. 2001b. Identification of bacteria crucial to histamine accumulation in Pacific mackerel during storage. J. Food Prot. (in press).

Klausen, N.K. and Huss, H.H. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

Kvenberg, J.E (ed.). 1991. Microbiology of marine food products. AVI, New York, NY.

Lindberg, A.M., Ljungh, Å., Ahrné, S., Löfdahl, S., and Molin, G. 1998. *Enterobacteriaceae* found in high numbers in fish minced meat and pastured milk or cream and the presence of toxin encoding genes. Int. J. Food Microbiol. 39: 11-17.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.T. 1996a. Sensory quality and histamine forming during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., and Mora-Ventura, M.T. 1996b. Incidence of histamine-forming bacteria and histamine content in scombroid fish species from retail markets in the Barcelona area. Int. J. Food Microbiol. 28: 411-418.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Morii, H., Cann, D.C., and Taylor, L.Y. 1988. Histamine formation by luminous bacteria in mackerel stored at low temperatures. Nippon Suisan Gakkaishi 54: 299-305.

Omura, Y., Price, R.J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Pérez-Villarreal, B. and Pozo, R. 1990. Chemical composition and ice spoilage of albacore (*Thunnus alalunga*). J. Food Prot. 55: 678-682.

Price, R.J. and Melvin, E.F. 1994. Recommendations for on board handling of albacore tuna. Sea Grant Extension Program, Food Science and Technology Extension, University of California, Davis.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish and shellfish. Adv. Food Nutr. Res. 39: 329-364.

Ridell, J. and Korkeala, H. 1997. Minimum growth temperatures of *Hafnia alvei* and other *Enterobacteriaceae* isolated from refrigerated meat determined with a temperature gradient incubator. Int. J. Food Microbiol. 35: 287-292.

Stratton, J.E. and Taylor, S.L. 1991. Scombroid poisoning. p.331-351. *In* Microbiology of Marine Food Products. van Nostrand Reinhold, N.Y.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S.L. 1986. Histamine food poisoning: toxicology and clinical aspects. CDC. 17: 91-128.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 7

Histamine Production by *Morganella morganii* in Mackerel, Albacore, Mahi-mahi, and Salmon at Various Storage Temperatures

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Abstract

Morganella morganii was studied for its growth and histamine formation in mackerel, albacore, mahi-mahi, and salmon stored at various temperatures from –30°C to 37°C. The optimal temperature for histamine formation was 25°C. Mackerel, albacore, and mahi-mahi were shown as good substrates for histidine decarboxylation by *M. morganii* at elevated temperatures (>15°C). *M. morganii* inoculated in all fish species including salmon formed histamine above the FDA guideline. Their growth was controlled by cold storage of the fish at 4°C or below, but histamine formation was controlled only by frozen storage. Although histamine was not detected in any frozen samples, it accumulated rapidly in the previously frozen fish stored at 25°C.

Introduction

Histamine is a causative agent of scombroid poisoning, a chemical health hazard (FDA, 1998). Scombroid poisoning is usually a mild illness with a variety of symptoms although death may ensue (Stratton and Taylor, 1991). The most frequently encountered symptoms are rash, nausea, diarrhea, flushing, sweating, and headache (FDA, 1998). Severity of the symptoms can vary considerably with the amount of histamine ingested and the individual's sensitivity to histamine (Russell and Maretic, 1986).

Fish is most frequently involved in the outbreaks of scombroid poisoning (CDC, 2000). Particularly, scombroid fish are the type of fish commonly involved in scombroid poisoning due to the high content of free histidine in the muscle (CDC, 2000a). These fish species include tuna (yellowfin, big-eye, bluefin, skipjack, and albacore), mackerel, bonito, and saury. Yellowfin, skipjack, and mackerel are frequently involved in

scombroid poisoning, because of their wide distribution and consumption throughout the world (Stratton and Taylor, 1991). Several species of non-scombroid fish such as mahi-mahi, bluefish, herring, and sardine have often been implicated in incidents of scombroid poisoning (Price and Melvin, 1994). In the U.S., mahi-mahi, bluefish, and tuna are the most frequently implicated fish species in scombroid poisoning (Ahmed, 1991).

A toxic level of histamine may be found before the fish appears spoiled or is organoleptically unacceptable (López-Sabater et al., 1994). Histamine is formed post-mortem in fish by proliferation of bacteria synthesizing histidine decarboxylase to convert histidine to histamine (Rawles et al., 1996). These bacteria have been isolated not only from fish and other seafood products but also from other types of foods such as cheese, fermented sausage, and wine (Taylor, 1986). Types of histidine decarboxylase found in these bacteria are either PLP-dependent enzymes containing pyridoxal-5'-phosphate as the essential coenzyme or Pvr-dependent enzymes requiring bound pyruvoyl residue as a coenzyme (van Poelje and Snell, 1990). The PLP-dependent enzymes have been identified from *Morganella morganii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Vibrio anguillarum* (Barancin et al., 1998). The Pvr-dependent enzymes have been found in *Lactobacillus* 30a, *Clostridium perfringens*, *Lactobacillus buchneri*, and *Micrococcus* sp. (van Poelje and Snell, 1990). Histamine is a competitive inhibitor of the Pvr-dependent enzymes but not of the PLP-dependent enzymes (Vaaler et al., 1986). Histamine formers synthesizing PLP-dependent enzymes are frequently isolated from fish (Ababouch et al., 1991; Stratton and Taylor, 1991; Klausen and Huss, 1987), thus making it possible to accumulate histamine in fish muscle without inhibiting histidine decarboxylase activity.

Although many different species of bacteria can form histamine, their histamine-producing capacities vary greatly. Only three species, *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei*, have been isolated from fish incriminated in scombroid poisoning (Rawles et al., 1996). Among them, *M. morganii* is the most prolific histamine former capable of producing >1,000 ppm histamine in culture broth (Kim et al., 2001). All the isolates have consistently been shown to form high levels of histamine in culture broth and isolated frequently from spoiled fish stored above 15°C (Rawles et al., 1996; López-Sabater et al., 1996). Thus, it has been proposed that *M. morganii* plays an important role in histamine production due to its prevalence and histamine-producing capacity (Kim et al., 2001). The objective of this study was to characterize histamine formation and proliferation of *M. morganii* in various fish species under controlled storage conditions. In this study, *M. morganii* isolated from mackerel was inoculated into fish muscles such as mackerel, albacore, mahi-mahi, and salmon. The changes of bacterial growth and histamine content were monitored during storage at -20, -30, 4, 15, 25, and 37°C

Materials and Methods

Reagents and media

Dowex 1-X8, histamine dihydrochloride, and *o*-phthaldialdehyde (OPT) were purchased from Sigma Chemical Co. (St. Louis, MO). Standard plate count (SPC) agar was obtained from Difco Co. (Detroit, MI). Trypticase soy broth (TSB) was purchased from BBL Co. (Cockeysville, MD).

Morganella morganii strain

M. morganii was previously isolated by using violet red bile glucose agar from Pacific mackerel during storage at 25°C (Kim et al., 2001). To confirm histamine production by the isolates, bacterial isolates were inoculated into TSB supplemented with 1% histidine and incubated at 37°C for 24 hr. Histamine content in culture broth was determined in duplicate by the AOAC fluorometric method (AOAC, 1995). Histamine formers were identified by the Vitek identification system (bioMérieux Vitek, Inc., Hazelwood, MI) following the manufacturer's instruction. Among all *M. morganii* isolates, the highest histamine former producing 3,527 ppm histamine in culture broth was selected for this study.

Bacterial culture was prepared for bacterial inoculation into fish muscles. One loopful of culture was inoculated in TSB and incubated at 37°C for 18 hr. Overnight culture was serially diluted with peptone water (0.1%) to adjust bacterial inoculation level.

Samples preparation and storage conditions

Fresh fish fillets were obtained from a local seafood market in Astoria, Oregon. The skin of fish was aseptically removed under a vertical laminar flow hood. The muscles were washed with ethanol-acetone (1:1, v/v) and rinsed with sterile water (López-Sabater et al., 1996). Each fish muscle was placed in a sterile food processor and ground to a mince. Serially diluted bacterial culture was inoculated into ground muscle at 10^6 CFU/g and homogenized. The inoculated fish muscles were aseptically dispensed in the weight of 30 g into sterile petri-dish and stored on each storage condition. Growth

of *M. morganii* and histamine formation were monitored during storage at 4, 15, 25, and 37°C. For the analyses, each muscle was taken every 12 hr from the samples incubated at 15, 25, and 37°C and every 24 hr from those incubated at 4°C. The viability of *M. morganii* during frozen storage was determined at -20 and -30°C. Their subsequent recovery after frozen storage was determined by replacing previously frozen samples at 25°C. Samples were taken every week from the samples stored at -20 and -30°C and every 12 hr from those incubated at 25°C.

Aerobic plate count (APC)

APC was determined in duplicate by the standard pour plate method (FDA, 1992). Aseptically, 10-g portions of muscles in plates were taken and blended with 90 ml of peptone water (0.1%). One mL of aliquots from each sample were serially diluted, dispensed, and mixed with SPC agar supplemented with 0.5% NaCl. The plates were incubated at 25°C for 2 days except for the frozen samples. For the frozen samples, plates were incubated at 15°C for 2-3 days.

Histamine analysis

Histamine content in fish muscle was determined in duplicate by the AOAC fluorometric method (AOAC, 1995). Samples, 10 g, were homogenized in 50 mL of methanol (100%) and incubated at 60°C for 15 min. The volume of the methanol extract was adjusted to 100 mL in a volumetric flask with methanol, and the extract was filtered through Whatman no. 1 paper. The filtrate was subjected to an ion exchange column (200 × 7 mm) packed with Dowex 1-X8 and eluted with H_2O. Histamine standards (0.5, 1.0, and

1.5 μg) and the column eluant were derivatized with OPT, and fluorescence intensity was determined using a spectrophotofluorometer (Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 350 nm and an emission wavelength of 444 nm.

Results

Histamine production by *Morganella morganii* in fish muscles during storage at 4, 15, 25, and 37°C

All fish muscles used in this study, i.e., mackerel, albacore, mahi-mahi, and salmon, supported growth of *M. morganii* at temperatures above 15°C. The trend of bacterial growth was not greatly different among the fish species tested (data not shown). *M. morganii*, initially inoculated at 10^6 CFU/g, grew rapidly in mackerel in 6 h of storage at 37°C (Fig. 7.1). *M. morganii* reached the stationary phase in the muscles in 12 hr of storage at both 25 and 37°C. Bacterial counts were slightly higher in the muscles stored at 25°C than 37°C under the storage conditions. Bacterial counts detected were 4.9×10^9 CFU/g and 1.4×10^9 CFU/g at 25 and 37°C, respectively. At 15°C, *M. morganii* grew gradually and reached the stationary phase in 48 hr of storage. Bacterial counts were 2.1×10^9 CFU/g in 48 hr of storage but decreased thereafter. The growth of *M. morganii* was inhibited in all fish species tested at 4°C up to 14 days of storage, as shown by no changes in counts from the initial inoculation level of 10^6 CFU/g.

As bacterial counts reached 10^8 CFU/g, histamine was found in mackerel, albacore, and mahi-mahi. Among the species, the most susceptible fish to histamine formation was mackerel followed by albacore and mahi-mahi (Fig. 7.2a). The highest

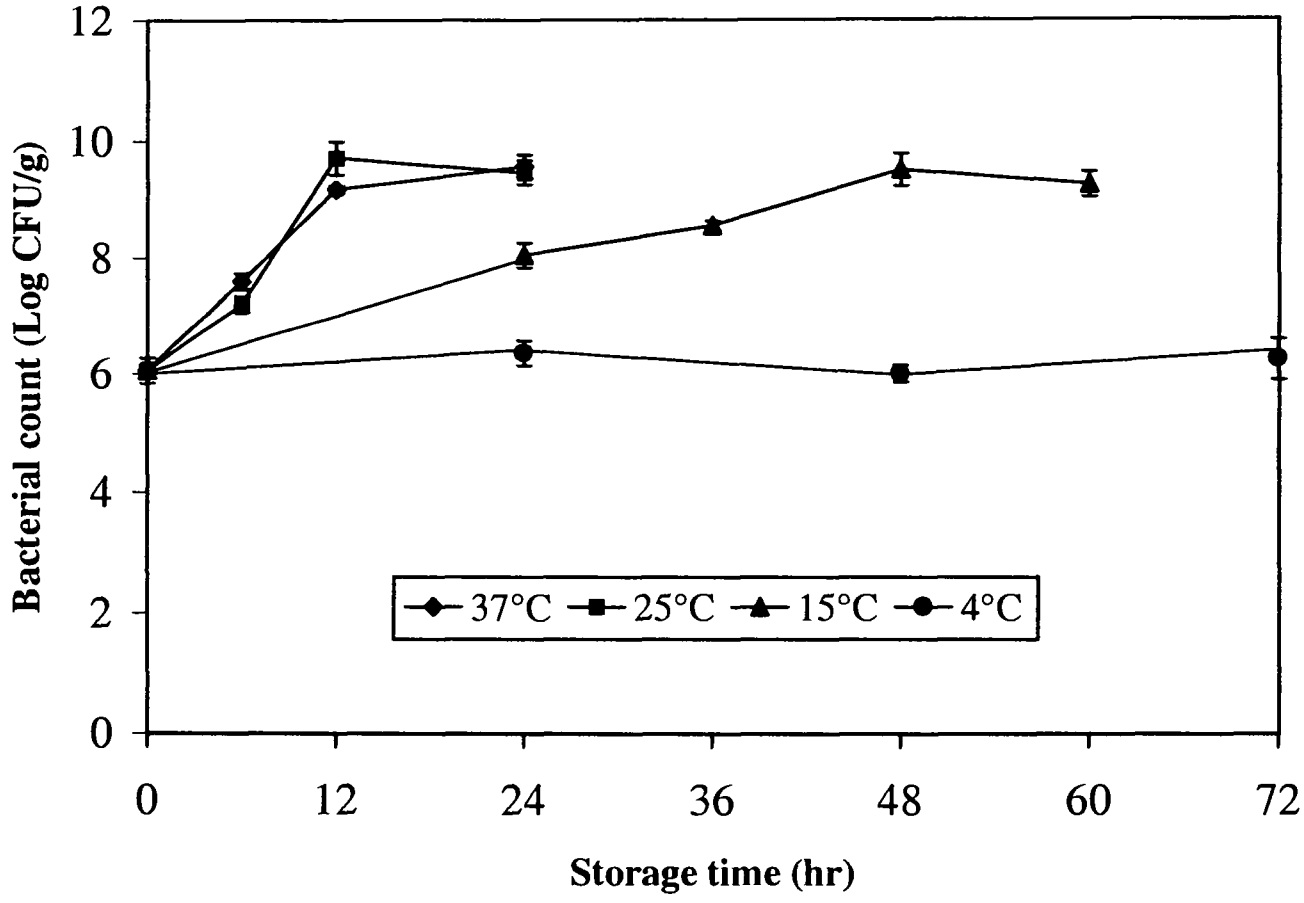

Fig. 7.1. Changes in APC of mackerel mince inoculated with *Morganella morganii* at 10^6 CFU/g during storage at 4, 15, 25, and 37°C. APC was determined in duplicate by using the standard pour plate method.

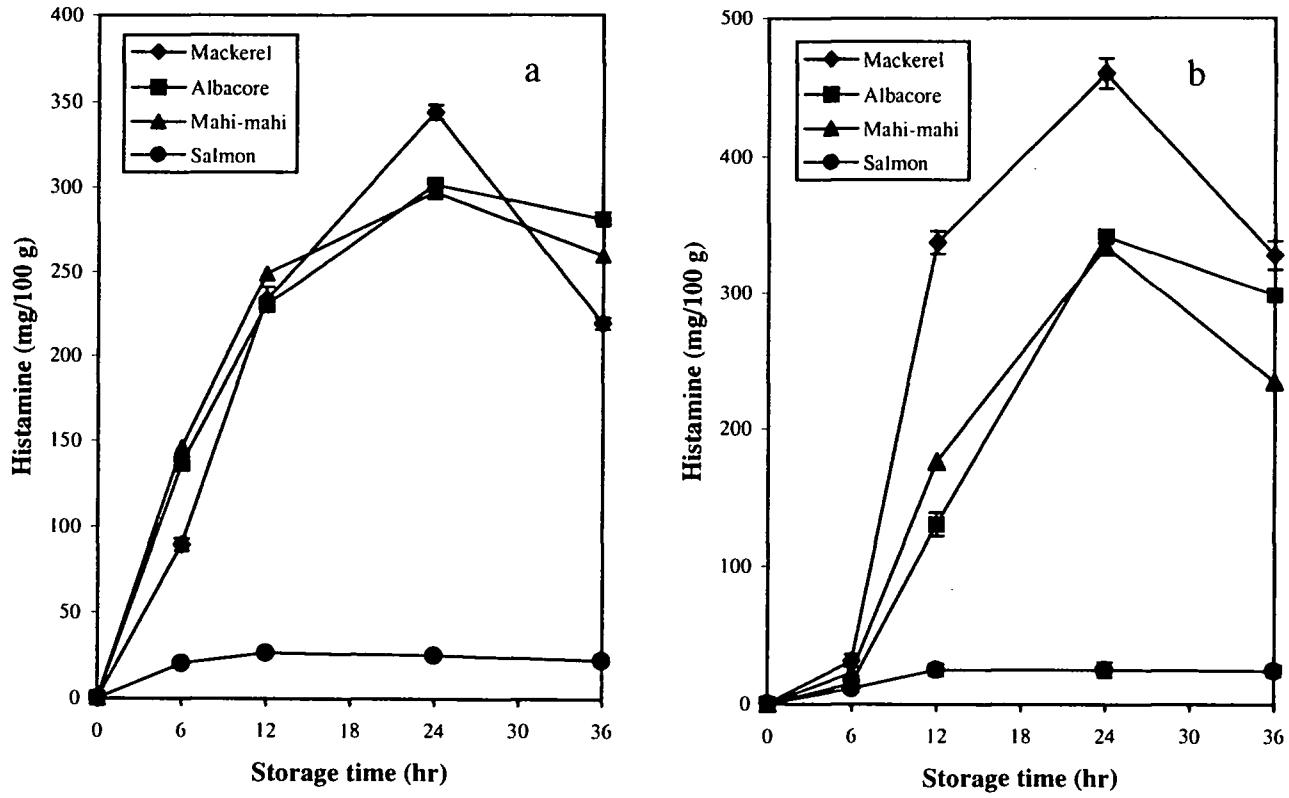

Fig. 7.2. Changes in histamine content of mackerel, albacore, mahi-mahi, and salmon mince inoculated with *Morganella morganii* at 10^6 CFU/g during storage at 37°C (a) and 25°C (b). Histamine content was analyzed in duplicate by the AOAC fluorometric method.

level of histamine was detected in the fish muscles, after *M. morganii* reached the stationary phase during storage at temperatures above 15°C. At 37°C, histamine production was accelerated along with bacterial growth, showing histamine levels ranging 90-146 mg/100 g in 6 hr of storage. Histamine content in mackerel, albacore, and mahi-mahi exceeded the health hazard level, 50 mg/100 g (FDA, 1998). In mackerel, 145 mg histamine/100 g was detected in 6 hr of incubation. The highest level of histamine detected in each fish muscles was 342 mg/100 g in mackerel; 301 mg/100 g in albacore; and 297 mg/100 g in mahi-mahi in 24 hr of storage. Optimal temperature for histamine production by *M. morganii* was found at 25°C (Fig. 7.2b). At the beginning of storage, histamine production was negligible in fish muscle but increased rapidly after 6 hr of storage. The highest level of histamine detected in 24 hr of storage was 461 mg/100 g in mackerel; 343 mg/100 g in albacore; and 334mg/100 g in mahi-mahi.

At 15°C, histamine content increased gradually with extended storage. Histamine was detected in 24 hr of storage, and its contents in mackerel and albacore exceeded 50 mg/100 g (Fig. 7.3a). The highest level of histamine was found at 309 mg/100 g in mackerel; 260 mg/100 g in albacore; and 151 mg/100 g in mahi-mahi in 48 hr of storage. In general, histamine levels detected in mahi-mahi were slightly lower than those in mackerel and albacore. At 4°C, the level of histamine detected in fish muscles during storage was insignificant. Histamine content detected was negligible in all fish species tested during 6 days of storage (<1 mg/100 g) (Fig. 7.3b). The histamine levels increased slightly thereafter, although histamine contents detected were still below 5 mg/100 g of fish muscle in 12 days of storage. The highest level of histamine detected in

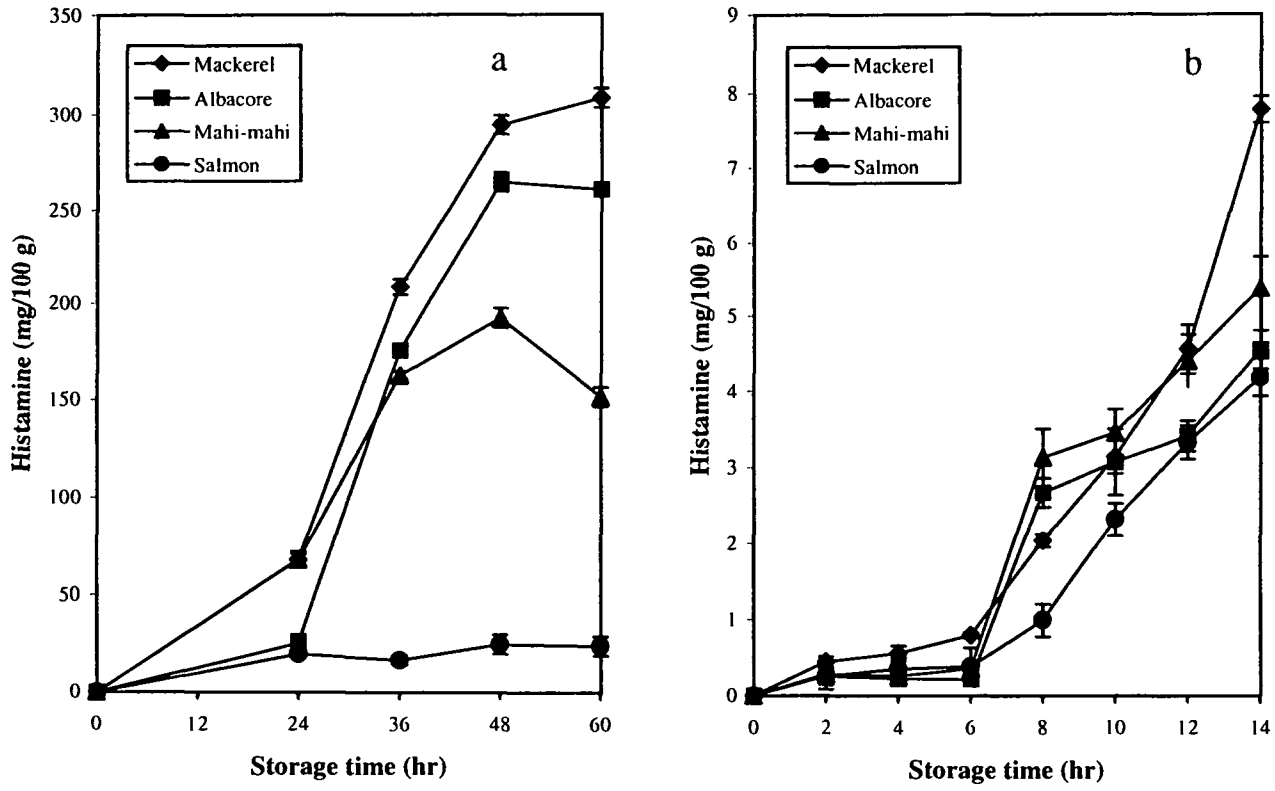

Fig. 7.3. Changes in histamine content of mackerel, albacore, mahi-mahi, and salmon mince inoculated with *Morganella morganii* at 10^6 CFU/g during storage at 15°C (a) and 4°C (b). Histamine content was analyzed in duplicate by the AOAC fluorometric method.

each fish species was 7.75 mg/100 g in mackerel; 4.56 mg/100 g in albacore; and 5.37 mg/100 g in mahi-mahi in 14 days.

M. morganii produced the least amount of histamine in salmon than any other fish species tested. The highest level of histamine produced was 24.5 mg/100 g, 25.5 mg/100 g, and 26.3 mg/100 g during storage at 15, 25, and 37°C, respectively. In general, histamine content reached 25 mg/100 g in the exponential growth of *M. morganii*. However, histamine levels did not change greatly, even after bacterial growth reached stationary phase with extended storage. During storage at 4°C, histamine content increased slightly, as shown in other fish muscle tested. The highest level of histamine accumulated was 4.19 mg/100 g in salmon in 14 days of storage.

Viability of *Morganella morganii* and control of histamine production during frozen storage

The reduction of bacterial counts was not significant during 3 month frozen storage. Bacterial counts in each fish muscle did not differ greatly during storage at -20 and -30°C. Bacterial counts gradually decreased during storage at -30°C (Fig. 7.4). Counts of *M. morganii* detected were 1.4×10^5 CFU/g in mackerel, 1.6×10^5 CFU/g in albacore; 2.4×10^5 CFU/g; in mahi-mahi; and 1.2×10^5 CFU/g in salmon in 2 months of storage. After 3 months of storage, bacterial counts were 2.1×10^4 CFU/g in mackerel, 2.7×10^4 CFU/g in albacore, 4.1×10^4 CFU/g in mahi-mahi, and 1.6×10^4 CFU/g in salmon at -30°C. Bacterial counts in various fish muscles were slightly lower at -20°C than at -30°C (data not shown). Bacterial counts in fish muscles were 1.0×10^5 CFU/g in mackerel, 9.8×10^4 CFU/g in albacore, 1.1×10^5 CFU/g in mahi-mahi, and 9.9×10^4

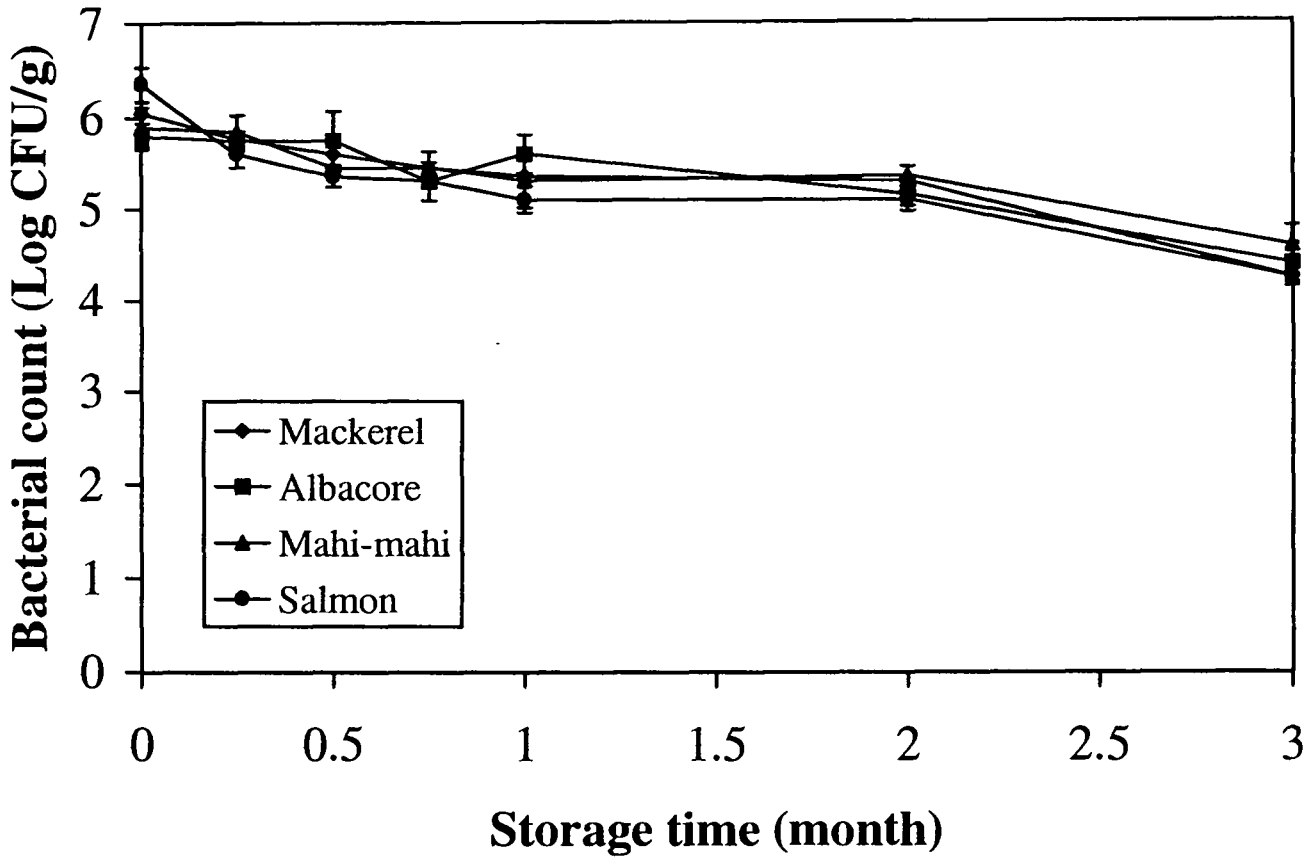

Fig. 7.4. Changes in APC during storage at -30°C of mackerel, albacore, mahi-mahi, and salmon mince inoculated with *Morganella morganii* at 10^6 CFU/g. APC was determined by using the standard pour plate method.

CFU/g in salmon at -20°C in 2 months storage. After 3 months of storage, bacterial counts were 1.6×10^4 CFU/g in mackerel, 1.2×10^4 CFU/g in albacore, 1.8×10^4 CFU/g in mahi-mahi, and 1.5×10^4 CFU/g in salmon at -30°C.

Histamine was effectively controlled in muscles tested during frozen storage. No histamine was detected in any fish species tested during 3 months of storage both at -20 and -30°C (data not shown).

Recovery of *Morganella morganii* in previously frozen samples during storage at 25°C

M. morganii began to grew rapidly, when fish muscles were thawed after 3 months of frozen storage and held at 25°C, an optimum temperature for histamine formation and bacterial recovery (Fig. 7.5a). The trend of bacterial growth was not obviously different among the fish species tested as previously observed with the muscles stored at –20 to 37°C. *M. morganii* reached the stationary phase in the fish muscles in 36 h of storage. The highest bacterial count detected in each muscle was 7.4×10^9 CFU/g in mackerel; 1.7×10^9 CFU/g in albacore; 1.7×10^9 CFU/g in mahi-mahi; and 2×10^9 CFU/g in salmon.

Although histamine was not detected in any of the frozen samples, histamine began to accumulate following the bacterial growth. When the bacterial counts reached 10^7 CFU/g in 12 hr of storage, histamine formation in all the fish muscles was still insignificant (<5 mg/100 g) (Fig. 7.5b). However, histamine began to accumulate greatly thereafter. As was observed with the fish never been frozen, mackerel was the most susceptible fish species to histamine production. The highest amount of histamine

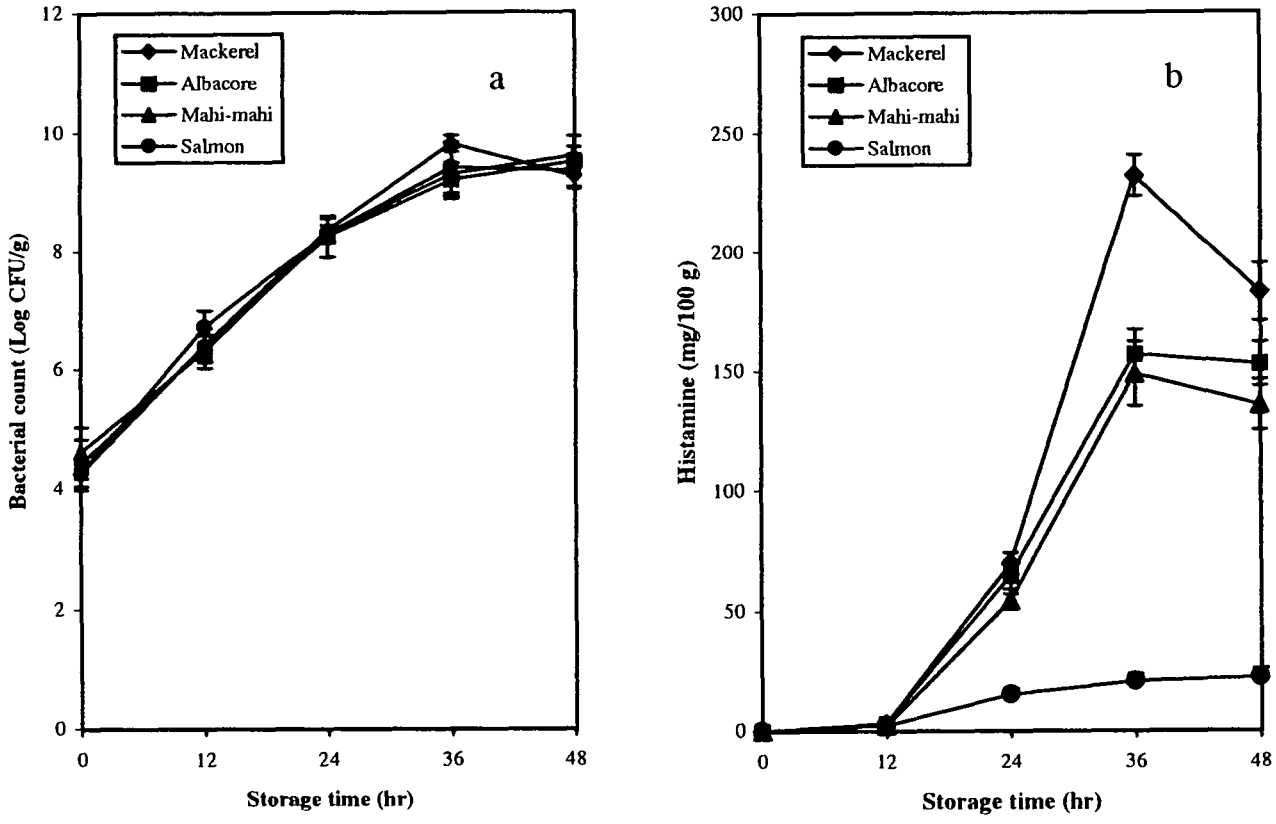

Fig. 7.5. Changes in APC (a) and histamine content (b) of mackerel, albacore, mahi-mahi, and salmon mince inoculated with *Morganella morganii* at 10^6 CFU/g, subjected to frozen storage, and stored at 25°C. APC was determined by using the standard pour plate method. Histamine content was analyzed in duplicate by the AOAC fluorometric method.

detected in each fish species was 232 mg/100 g in mackerel; 159 mg/100 g in albacore; and 149 mg/100 g in mahi-mai in 24 h of storage. Salmon was the least susceptible species, and it showed the similar pattern of histamine production to fish that has never been frozen. Level of histamine detected was 15.3 mg/100 in salmon in 24 h of storage. The highest level, 21.2 mg of histamine /100 g, was found in the muscle in 48 h.

Discussion

Control of histamine formation for wholesome seafood products

In December 1995, FDA issued seafood regulations based on the principles of Hazard Analysis and Critical Control Point (HACCP) (FDA, 1998). This regulation became effective in December 1997. The aim of this regulation is to produce wholesome products and to prevent hazards that could cause foodborne illnesses (FDA, 2000). A HACCP system is designed to identify hazards, establish critical control points and critical limits, monitor procedures, and keep record for procedures. Among many hazards identified in seafood products, histamine is one of the major causes of seafood-related illnesses in the U.S (FDA, 1998; CDC, 2000a). Histamine does not have odor or color to indicate its presence in food (Stratton and Taylor, 1991). It is extremely stable; thus it cannot be easily removed or destroyed by cooking, retorting, or freezing. Therefore, monitoring histamine is a critical task in seafood industry. To control histamine formation, the HACCP guideline for histamine is established at 5 mg/100 g of fish muscle (FDA, 1995). Fish containing histamine above this level should be discarded and cannot be used for human consumption.

Histamine formation mostly results from time and temperature abuse of fish during handling, processing, and distribution (FDA, 1998). Troll-caught fresh tuna usually shows a negligible amount of histamine, since they are brought up on board immediately after catch (Price and Melvin, 1994). In most incidents of scombroid poisoning, the period between the fish harvest and chilling to 0°C was either prolonged (>12 hr) or the temperature of fish muscles was never reached below 20°C (Russell and Maretic, 1986). In one case, yellowfin tuna caught by long line in a commercial fishing boat was retained on lines up to 20 hr in the water temperature of 25.8°C. Although the tuna was shipped in iced vats from the fishing boat to a processor, the fish was implicated in outbreak of scombroid poisoning in Pennsylvania in 1998 (CDC, 2000b).

Most histamine-producing bacteria are mesophiles, and thus temperature is the most important factor to support their growth and histamine formation among many environmental parameters (Kim et al., 2000). Prolific histamine formers play an important role in accumulating histamine toxicity in fish. *Morganella morganii* has been isolated as the most prolific histamine formers from fish and other seafood products (López-Sabater et al., 1996; Kim et al., 2001). Its growth directly results in high levels of histamine accumulation in mackerel during storage at 25°C (Kim et al., 2001).

In this study, when mackerel, albacore, mahi-mahi, and salmon were artificially contaminated with *M. morganii*, the trends of bacterial growth were similar in all the fish species tested during storage. *M. morganii* grew rapidly in the muscles during storage at both 25 and 37°C. However, histamine levels detected varied greatly depending on fish species. Mackerel, albacore, and mahi-mahi were shown as good substrates for decarboxylation reaction of *M. morganii* at elevated temperatures (>15°C). The ambient

temperature (25°C) is the most favorable condition to histamine production by *M. morganii*. Higher levels of histamine accumulated in fish muscles during storage at 25°C than at 37°C, although bacterial population was not greatly different. At 15°C, a significant level of histamine accumulated with the increase in bacterial population. Although bacterial growth was inhibited, histamine accumulated slightly in all the fish muscles tested with the prolonged storage at 4°C. It is reported that the histidine decarboxylation activity of *M. morganii*, required for histamine formation, is fairly stable for 96 hr at 4 to 24°C, but reduced to 5% after 24 hr of incubation at 37°C (Baranowski et al., 1985).

Generally, it takes seven days to transfer fish through the distribution process to consumer, and trips lasting over five days before reaching the consumer render inferior fish quality (Price and Melvin, 1994). On-board handling technique is the most critical step to prevent histamine formation in fish, since any delay in proper storage of fish leads to histamine formation in fish muscles (Price et al., 1991; Kim et al., 2001). Thus, it is recommended to chill fish rapidly below 4°C on the vessel (FDA, 1998). In addition, special handling is required to deliver fresh, unfrozen fish destined for specialty products such as sushi. The internal temperature of fish muscles should be maintained in a proper chilling system and measured by periodically throughout the fishing trips (Price and Melvin, 1994).

Bacterial histamine formation in various fish species

Bacterial histamine formation is affected by free histidine contents in fish muscles, serving as a substrate for histidine decarboxylase. Autolysis or bacterial

proteolysis can also promote histamine formation in fish muscles, because it accelerates the release of histidine from tissue proteins (Stratton and Taylor, 1991). The minimum histidine concentration for histidine decarboxylase activity is 100 to 200 mg/100 g (Chen et al., 1989). Free histidine contents in fish muscles reported vary considerably due to the differences in feeding, season, sex, and stage of maturity (Antonine et al., 1999). Dark-fleshed mackerel and horse mackerel contain histidine ranging from 210 to 726 mg/100 g (Arnold and Brown, 1978). With skipjack tuna, free histidine contents are detected at 1,389mg/100 g in the white tissue and 268 mg/100 g in the red tissue (Abe, 1983). In another study, distribution of free histidine in fresh skipjack tuna is uniform at concentrations of 564-611 mg/100 g (Yoshinaga and Frank, 1982). Free histidine contents in dark-muscle fish are 182-541 mg/100 g in mahi-mahi; and 220-708 mg/100 g in tuna (Antoine et al., 1999). Mahi-mahi contains 490-940 mg of free histidine/100 g in the muscles (Baranowski et al., 1985). Three species of fish, kahawai (*Arripis trutta*), kingfish (*Seriola grandis*), and albacore (*Thunnus alalunga*), implicated in scombroid poisoning in New Zealand, contain free histidine in the muscle at the concentrations >1,000 mg/100 g (Fletcher et al., 1995). On contrast, sea perch, flounder, and red snapper, which were never implicated in outbreaks of scombroid poisoning, contain free histidine less than 5 mg/100 g (Arnold and Brown, 1978).

Histamine levels higher than 2,000 ppm have usually been reported in sardines, mackerel, and tuna, consistent to their high levels of free histidine in the muscles (López-Sabater, 1996). In this study, mackerel was the most susceptible fish species to histamine formation by *M. morganii* at all the storage temperatures tested. Mackerel is not commonly consumed in the U.S. However, it has been widely consumed throughout the

world and processed into canned, marinated, smoked, or sausage-type products (Venugopal and Shahidi, 1994). Thus, mackerel have frequently been involved in scombroid poisoning in Japan and U.K. (Stratton and Taylor, 1991). Albacore has rarely been involved in the incidents of scombroid poisoning. Relatively little histamine is accumulated in albacore compared to other scombroid fish species (Kim et al., 1999). However, in this study, albacore obviously supported histamine formation for *M. morganii*, showing that resistance of albacore to histamine formation is not due to intrinsic factors of the muscles. Mahi-mahi imported to the U.S. from the tropical areas has frequently been implicated in scombroid poisoning (CDC, 2000a). It mainly results from the high ambient water and air temperatures in the originated area, mishandling conditions on boats, and the sanitary conditions of market (Ahmed, 1991). Mahi-mahi also acted as a good substrate for histidine decarboxylation observed in this study. Thus, good fish handling techniques should be applied to prevent proliferation and cross contamination of *M. morganii* during handling and distribution. Salmon was the least susceptible fish species to histamine formation among the tested fish species; however, *M. morganii* still produced histamine, >25 mg/100 g, well above the FDA guideline. Since salmon have also been involved in the incidents of scombroid poisoning (Stratton and Taylor, 1991), monitoring growth of *M. morganii* and histamine production may not be restricted to only the fish species rich in histidine if to ensure product safety.

Histamine formation in fish during frozen storage, thawing, and processing

In the fishery industry, bacterial histamine production is primarily controlled by chilling or low temperature storage. Freezing has commonly been used to prevent

histamine formation in the canning industry. Fish destined to be canned are frequently preserved by frozen storage prior to delivery to the canneries (Price et al., 1991). Freezing fish may be partially responsible for reduction of bacterial loads, especially in the number of gram-negative microorganisms (FDA, 1998). In this study, counts of *M. morganii* did not decrease rapidly due to the presence of cryoprotectants in fish muscles (Jay, 1996). Histamine formation was effectively controlled up to 3 months of storage by inhibiting induction of histidine decarboxylase. However, histamine can be accumulated by mishandling of fish before and/or after frozen storage. Once the enzyme has been formed before freezing of fish, it can continue to produce histamine in fish regardless of the injury of bacterial cell during frozen storage (Baranowski et al., 1985; FDA, 1998). In this study, histamine formation was followed by bacterial proliferation in the muscles, when previously frozen fish were placed at 25°C, optimum temperature for histamine formation and bacterial recovery. Histamine levels in the previously frozen samples were always less than those which had not been previously frozen. However, *M. morganii* still produced toxicological levels of histamine (>50 mg/100g of fish) under these conditions.

In general, frozen fish are thawed before processing and subjected to additional handling, which may result in histamine accumulation as well as quality loss in processed products (FDA, 1998). Due to its heat resistance, histamine can be remained in sterilized cans or other products (López-Sabater et al., 1994). In some instances, the presence of toxic amounts of histamine in cans results from the use of poor hygienic quality fish as raw material or a defective handling of high quality fish during processing (Price and Melvin, 1994). When frozen tuna fish are defrosted and processed, an increase in bacterial population are observed until they are subjected to the heating process during

canning (López-Sabater et al., 1994). The highest bacterial count is found in samples during the tuna cutting and skin peeling steps, which allows a rapid recovery and growth of injured bacteria or a cross contamination from the environment. Histamine contents detected in canned fish are 1.35 ppm in herring, 30.9 ppm in tuna, 16.3 ppm in sardine, and 28.3 ppm in mackerel (López-Sabater et al., 1994). High level of histamine, was also found in semi-preserved anchovies (219 ppm) (Viciana-Nogues et al., 1989); and cold-smoked salmon stored at 5°C (>100 ppm) (Jørgensen et al., 2000).

Conclusion

Morganella morganii formed significant amounts of histamine (>200 mg/100 g) in artificially contaminated fresh and frozen mackerel, albacore, and mahi-mahi, when the fish were improperly stored at ambient temperatures (25°C). *M. morganii* produced >20 mg histamine/100 g, well above the FDA guidelines even in salmon. Monitoring the presence and proliferation of *M. morganii* would greatly assist in the implement of HACCP in the fishery industry to control histamine formation in fish products.

Acknowledgments

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References

Ababouch, L., Afilal, M.E., Rhafiri, S., and Busta, F.F. 1991. Identification of histamine-producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperatures (25°C). Food Microbiol. 8: 127-136.

Abe, H. 1983. Distribution of free L-histidine and its related compounds in marine fishes. Bull. Jpn. Soc. Sci. Fish. 49: 1683-1687.

Ahmed, F.E (ed.). 1991. Ch. 4. Naturally occurring fish and shellfish poisons. p.87-110. *In* Seafood Safety. National Academy Press, Washington, D.C.

Antoine, F.R., Wei, C.I., Littell, R.C., and Marshall, M.R. 1999. HPLC method for analysis of free amino acids in fish using *o*-Phthaldialdehyde precolumn derivatization. J. Agric. Food Chem. 47: 5100-5107.

AOAC. 1995. Histamine in seafood: Fluorometric method. Sec. 35.1.32, Method 977.13. *In* Official Methods of Analysis of AOAC International. 16th ed. p. 6-17. AOAC International, Gaithersburg, MD.

Arnold, S.H. and Brown, W. 1978. Histamine (?) toxicity from fish products. Adv. Food Res. 24: 113-154.

Barancin, C.E., Smoot, J.C., Findlay, R.H., and Actis, L.A. 1998. Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen *Vibrio anguillarum.* Plasmid. 39: 235-244.

Baranowski, J.D., Brust, P.A., and Frank, H.A. 1985. Growth of *Klebsiella pneumoniae* UH-2 and properties of its histidine decarboxylase system in resting cells. J. Food Biochem. 9: 349-360.

Centers for Disease Control and Prevention. 2000a. Surveillance for foodborne-disease outbreaks-United States, 1993-1997. *In* CDC surveillance summaries, MMWR, 49: 1-72.

Centers for Disease Control and Prevention. 2000b. Scombroid fish poisoning-Pennsylvania, 1988. MMWR, 49: 398-400.

Chen, C.M., Wei, C.I., Koburger, J.A., and Marshall, M.R. 1989. Comparison of four agar media for detection of histamine-producing bacteria in tuna. J. Food Prot. 52: 808-813.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species, particularly kawawai (*Arripis trutta*). J. Aquat. Food Prod. Technol. 4: 533-574.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th ed. Arlington, VA.

Food and Drug Administration. 1995. Procedures for the safe and sanitary processing and importing of fish and fishery products. Federal register. 60: 65096-65202.

Food and Drug Administration. 1998. Scombrotoxin (histamine) formation. Ch. 7 In Fish and Fishery Products Hazards and Controls Guide. 2nd ed., p. 73-90. Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Food and Drug Administration. 2000. FDA's evaluation of the seafood HACCP program for 1998/1999. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Jay, J.M. (ed.). 1996. Ch.15. Low temperature food preservation and characteristics of psychrotrophic microorganisms. p. 330-342. *In* Modern Food Microbiology. 5th Ed., ITP. New York, NY.

Jørgensen, L.V., Dalgaard, P., and Huss, H.H. 2000. Multiple compound quality index for cold-smoked salmon (*Salmo salar*) developed by multivariate regression of biogenic amines and pH. J. Agric. Food Chem. 48: 2448-2453.

Kim, S.H., An, H., and Price, R.J. 1999. Histamine formation and bacterial spoilage of albacore harvested off the U.S. Northwest coast. J. Food Sci. 64: 340-344.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Kim, S.H., Field, K.G., Chang, D.S., Wei, C.I., and An, H. 2001. Proliferation of histamine-producing bacteria and histamine production in Pacific mackerel during storage. J. Food Prot. (in press).

Klausen, N.K. and Huss, H.H. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1994. Bacteriological quality of tuna fish (*Thunnus thynnus*) destined for canning: Effect of tuna handling on presence of histidine decarboxylase bacteria and histamine level. J. Food Prot. 57: 318-323.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1996. Sensory quality and histamine formation during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

Price, R.J., Melvin, E.F., and Bell, J.W. 1991. Postmortem changes in chilled round, bled and dressed albacore. J. Food Sci. 56: 318-321.

Price, R.J. and Melvin, E.F. 1994. Recommendations for on board handling of albacore tuna. Sea Grant Extension Program, Food Science and Technology Extension, University of California, Davis.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish shellfish. Adv. Food Nutr. Res. 39: 329-364.

Russell, F.E. and Maretic, Z. 1986. Scombroid poisoning: Mini-review with case histories. Toxicon, 24: 967-973.

Stratton, J.E. and Taylor, S.L. 1991. Scombroid poisoning. p.331-351. *In* Microbiology of Marine Food Products. van Nostrand Reinhold, N.Y.

Taylor, S. L. 1986. Histamine food poisoning: Toxicology and clinical aspects. CRC Crit. Rev. Toxicol. 17: 91-117.

Vaaler, G.L., Brasch, M.A., and Snell, E.E. 1986. Pyridoxal 5'-phosphate-dependent histidine decarboxylase. J. Biol. Chem. 261: 11010-11014.

Van Poelje, P.D., and Snell, E.E. 1990. Pyruvoyl-dependent enzymes. Annu. Rev. Biochem. 59: 29-59.

Veciana-Nogués, M.T., Vidal-Carou, M.C., Mariné-Font, A. 1989. Histamine and tyramine in preserved and semi-preserved fish products. J. Food Sci. 54: 1653-1655.

Venugopal, V. and Shahidi, F. 1994. Thermostable water dispersions of Myofibrillar proteins from Atlantic mackerel (*Scomber scombus*). J. Food Sci. 59: 265-268, 276.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). Appl. Environ. Microbiol. 44: 447-452.

Chapter 8

Development of 16S rDNA Targeted Primers for Detection of *Morganella morganii*, a Prolific Histamine Former, with PCR

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Abstract

A PCR assay was developed for rapid and sensitive detection of the most prolific histamine former, *Morganella morganii*. 16S rDNA target PCR primers were designed, and primer specificity and sensitivity of the PCR assay were evaluated. The 16S rDNA sequence (1,503 bp) of *M. morganii* showed 95% identity to those of enteric bacteria, i.e., *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Hafnia alvei*, *Proteus* spp., and *Providencia* spp. Among the three primer sets designed based on the variable regions in the 16S rDNA sequence, unique primers found for *M. morganii* were: the forward primer, 5'-CTC GCA CCA TCA GAT GAA CCC ATA T-3'; and the reverse primer, 5'-CAA AGC ATC TCT GCT AAG TTC TCT GGA TG-3'. The primers showed positive reactions with all *M. morganii* strains tested. However, no PCR amplification was detected when the primers were tested with other enteric bacteria or marine bacteria. When the sensitivity of the assay was evaluated, 9 CFU/ml of *M. morganii* in albacore homogenate were detected with a 6 h-enrichment of sample in trypticase soy broth at 37°C.

Introduction

In December, 1995, the U.S. Food and Drug Administration issued seafood regulations based on the principles of Hazard Analysis and Critical Control Point (HACCP) (FDA, 1998). The regulation became effective in December, 1997. To increase the margin of safety, a mandatory HACCP system is designed to identify hazards, establish critical control points and critical limits, monitor procedures, and keep records for procedures. Histamine was identified by FDA as a major chemical hazard in seafood products, causing one of the three most frequent seafood-related illnesses in the U.S. (FDA, 1998; CDC, 2000). The FDA is intensifying its inspection efforts to focus particularly on controlling histamine and pathogens after two rounds of inspection for all hazards occcurred in seafood (1998-1999) (FDA, 2000). Thus, monitoring histamine at each handling and processing step is a critical task for the seafood industry.

Histamine is formed in fish by proliferation of bacteria that synthesize histidine decarboxylase to convert free histidine to histamine (Rawles et al., 1996). Fish containing high levels of free histidine in the muscle are particularly susceptible to histamine formation due to improper handling and storage. Various levels and types of histamine-forming bacteria, *Morganella morganii*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Hafnia alvei*, *Citrobacter freundii*, and *Serratia* spp., have been isolated from fish under controlled storage conditions. Most frequently isolated histamine-producing bacteria are mesophilic enteric bacteria; thus, temperature is the most important environmental parameter to influence their growth and histamine formation. Natural bacteria in the marine environment, including *Photobacterium* spp., *Pseudomonas* spp., *Vibrio alginolyticus*, and *Aeromonas* spp., are isolated from fish stored at refrigeration temperature for extended

periods (Kim et al., 2001b; Middlebrooks et al., 1988; Morii et al., 1988). Among them, *Morganella morganii* was identified as the most important prolific histamine former in fish (Kim et al., 2001b). Histidine decarboxylase in *M. morganii* contains pyridoxal-5′-phosphate (PLP) as a cofactor and is not inhibited by the feedback inhibition of the product, resulting in accumulation of high levels of histamine (>2,000 ppm) (Kamath et al., 1991). Histamine accumulated rapidly in fish muscle with the prevalence of *M. morganii* during storage at ambient temperature (25°C), which is the optimum temperature for histamine accumulation.

Histamine-producing bacteria including *M. morganii* are traditionally isolated using the conventional culture method (Stratton and Taylor, 1991). In general, colonies on differential or selective media are isolated by their morphological characteristics. Since most frequently isolated histamine formers in fish are enteric bacteria, Niven's differential medium, capable of detecting amino acid decarboxylation in enteric bacteria, is widely used to enumerate histamine formers in fish (Niven et al., 1981). However, the use of Niven's medium generates high rates of false positives and false negatives, presumably due to the presence of competing non-histamine formers (Kim et al., 2001a). By screening histamine formers on selective medium prior to plating on differential media, the detection rate of true histamine formers is substantially increased (Kim et al., 2001a). However, this process still requires a lengthy assay time and intensive labor (Kim et al., 2001b). In addition, the procedure is not suitable for the detection of specific species due to its low sensitivity, requiring a bacterial population of $>10^2$ CFU/g in samples.

The use of oligonucleotides as hybridization probes or amplification primers has become an integral part of the methodology for microbial identification (Gendel, 1996).

PCR has been widely used for rapid detection of pathogens in food. However, molecular techniques have not been widely used for the detection of histamine formers in food. Only universal primers were developed for the detection of histamine-producing lactic acid bacteria, such as *Lactobacillus* 30A, *Clostridium perfringens*, *Leuconostoc œnos*, *Lactobacillus buchneri*, and *Micrococcus* spp. in cheese and dairy products (Le Jeune et al., 1995). Since alignment of the *hdc* (histidine decarboxylase) gene sequences of these bacteria showed a high degree of similarity, the primers were derived from conserved sequences in the *hdc* gene. Gram-negative histamine formers are the dominant isolates from fish. However, among gram-negative species, the *hdc* gene sequences are only known for *M. morganii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Vibrio anguillarum* (Kamath et al., 1991). No species-specific primer has been reported for their detection. Selection of a proper target gene is the most critical factor to design a unique primer (Klein and Juneja, 1997). For the detection of *Vibrio parahaemolyticus*, primers have frequently been derived from the nucleotide sequence of the *tdh* (thermostable direct hemolysin) gene; however, few environmental and seafood isolates of *V. parahaemolyticus* contain the *tdh* gene (Lee et al., 1995). Oligonucleotide primers specific for *tdh* were also shown to react positively with *tdh*-negative and several non-*parahaemolyticus* species, such as *V. hollicase*, *V. mimicus*, and *V. cholerae* non-O1 (Bej et al., 1999).

The 16S ribosomal RNA gene (rDNA) has been widely used for studying phylogenetic relationships and identifying bacterial species because it is present in high copy numbers in all bacteria, functionally constant, and composed of highly conserved as well as variable domains (Vandamme et al., 1996). Therefore, the objective of this study

was to develop a PCR assay based on 16S rDNA target primers for rapid and sensitive detection of *M. morganii* in fish. The 16S rDNA of *M. morganii* was sequenced to design the PCR primers. The primer specificity was confirmed by testing the primers with various ATCC strains and isolates. The sensitivity of the PCR assay was evaluated in artificially contaminated albacore homogenate with *M. morganii*.

Materials and Methods

Bacterial strains

Morganella morganii OSL36 was used for sequencing 16S rDNA and testing primer specificity and sensitivity of PCR assay. *M. morganii* OSL36 was originally isolated from albacore tuna during storage at 25°C (Kim et al., 2000). Its biochemical and antibacterial characteristics as well as histamine and biogenic amine production are well characterized.

To test specificity of primers designed in this study, enteric bacterial strains were obtained from American Type Culture Collection (ATCC, Manassas, VA). Strains tested were *M. morganii* ATCC 28550, *Proteus mirabilis* ATCC 7002, *Proteus vulgaris* ATCC 6380, *Providencia stuartii* ATCC 33672, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 23355, *Klebsiella pneumoniae* ATCC 4211, *Serratia fonticola* ATCC 29848, *Hafnia alvei* ATCC 13337, *Citrobacter freundii* ATCC 8090, *Yersinia enterocolitica* ATCC 9610, and *Escherichia coli* ATCC 25922.

The primer specificity of Mm208F and Mm1017R was confirmed by testing with various species of histamine formers and marine bacteria. Bacteria were previously

isolated using selective media such as violet red bile glucose (VRBG) and thiosulfate citrate bile salts sucrose (TCBS) agars from albacore and mackerel during the seasons from 1998 to 1999. The isolates were identified by the Vitek identification system (bioMérieux, Vitek system, Inc., Hazelwood, MI) following the manufacturer's instruction. The species tested and their isolation sources are listed in Table 8.3. In this study, strains were cultured overnight in LB broth at 25 or 37°C and used for further tests.

Sequencing of 16S rDNA for *Morganella morganii*

The 16S rDNA of *M. morganii* was amplified by universal PCR primers. The primers used were: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Dojka et al., 1998). Primers were synthesized by Integrated DNA Technologies, INC (Coralville, IA). Each 50 μl PCR mixture contained 10× PCR buffer (Qiagen, Valencia, CA), each deoxynucleoside triphosphate (Qiagen) at a concentration of 100 μM, each forward and reverse primer at a concentration of 1 μM, and 1.25 U of *Taq* polymerase (Qiagen). Amplification was performed with a thermal minicycler (MJ research, Watertown, MA) using the following conditions: 30 cycles consisting of 94°C for 1 min, 50°C for 45 s, and 72°C for 2 min, followed by a final 12 min extension at 72°C.

Pooled PCR product was confirmed in a 1% agarose gel by comparing DNA size with a DNA mass ladder (Bioline, Springfield, NJ). The PCR product was purified from the gel with a Gel Extraction Kit (Qiagen) and cloned by using a TA cloning kit (Invitrogen, Carlsbad, CA) as recommended by the manufacturers. Plasmid DNA from

overnight culture was prepared by using a Qiaprep spin miniprep kit (Qiagen). Bidirectional sequence was obtained by using T4 and M13 priming sites on either side of the insert (Davis Sequencing, Davis, CA).

Primer specificity analysis

To determine phylogenetic relationships, the 16S rDNA sequence of *M. morganii* was analyzed by using the DNA sequence editor in GCG v. 10 (Genetics Computer Group, Madison, WI). The 16S rDNA sequence of *M. morganii* was aligned with those of closely related enteric bacteria obtained from the Nucleotide Sequence Database (GenBank). Similarities were calculated using the distance function in GCG with the Kimura 2-parameter correction. A phylogenetic tree was inferred with the neighbor-joining algorithm (Saiton and Nei, 1987). Primers were designed based on four variable regions observed from the alignments of 16S rDNA sequences (Table 8.1).

Primer specificity was tested with DNA extracts from *M. morganii* and other species. Three sets of primers designed in this study were tested with ATCC strains. One-half μl of overnight cell culture of each was used as PCR template. PCRs were carried out in duplicate as described above, except for the annealing temperatures. The annealing temperature used for each primer set is listed in Table 1. Unique primers found for *M. morganii* were tested with several different species or isolates to confirm primer specificity.

DNA extraction from fish homogenates

For sensitivity analysis, 25 g of dorsal muscles from fresh albacore were blended with 75 mL of peptone water (0.1%) in a sterile blend jar, and an aliquot of 10 ml was dispensed into a test tube. Serially diluted *Morganella morganii* culture with peptone water (0.1%) was inoculated into the fish homogenate. Final inoculation levels were adjusted at 10^1 to 10^8 CFU/ml, and inoculation levels were enumerated by the standard pour plate method (FDA, 1992). For the enrichment step, each 1 ml of fish homogenate was inoculated into 9 ml of trypticase soy broth (TSB) supplemented with 0.5 % NaCl and incubated at 37°C for 6 hr with shaking.

Total DNAs in seeded and non-seeded fish homogenates were extracted by using three different extraction methods, described below, and QIAamp® DNA mini kit (Qiagen) following the manufacturer's instruction. Phenol-chloroform extractions for DNA were carried out by the Bej method (1999) as follows. One-half ml aliquots were treated with 0.2 ml of a lysis buffer consisting of 10 mM EDTA, 100 mM Tris Cl (pH 8.0), 5 mg proteinase K/ml, and 50 mg sarkosyl/ml at 56°C for 20 min. After lysis, 100 μl of ice-cold 3M NaOAc (pH 4.6) was added, followed by an equal volume (800 μl) of phenol-chloroform-isoamyl alcohol mixture (25:24:1, v/v/v). Samples were centrifuged at 10,000 × *g* for 10 min, and clear supernatants were removed. Total DNAs were precipitated with 0.8 ml of ice-cold isopropanol and centrifuged at 12,000 × *g* for 10 min. The pellets were washed with 0.5 ml of ice-cold 70% alcohol, resuspended in 10 μl of TE buffer (pH 8.0), and incubated at 65°C for 30 min before PCR amplification. One μl was taken from each and used as a template.

DNA in albacore homogenate was also extracted by the Chelex and guanidine isothiocyanate methods (Kimura et al., 2001). For the Chelex method, one ml of fish homogenate was centrifuged for 5 min at 15,000 × g. The pellet was resuspended with 200 μl of 5% Chelex 100 (Bio-Rad Laboratories, Hercules, CA) solution (w/v). After vortexing, the bacterial suspension was boiled for 10 min, cooled down in ice, and centrifuged for 5 min at 14,000 × *g*. Five μl of the supernatant was used as a template. For the guanidine isothiocyanate method, 1 ml of sample was centrifuged for 5 min at 14,000 × *g*. The pellet was resuspended in 500 μl of 4 M guanidine isothiocyanate solution (Gibco BRL., Gaintherburg, Md.) containing Tween 20 (2%, w/v). The suspension was boiled for 10 min, placed on ice, and centrifuged for 10 min at 14,000 × g. The supernatant (400 μl) was transferred to a tube containing 400 μl of 100% isopropanol and centrifuged for 10 min at 14,000 × *g*. The pellet was rinsed with 75% isopropanol and dissolved in 160 μl of distilled water followed by heating for 3 min at 70°C. The DNA was centrifuged for 5 min at 14,000 × *g*, and 5 μl of the supernatant was taken for PCR.

Results

16S rDNA targeted PCR primers for *Morganella morganii*

The full length (1,503 bp) 16S rDNA of *Morganella morganii* was sequenced and reported for the first time here (Fig. 8.1). In general, the 16S rDNA sequence showed 94 to 95% identity to those of enteric bacteria, i.e., *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Serratia* spp., *Yersinia enterocolitica*, *Salmonella enterica*, *Escherichia coli*, *Erwinia carotovora*, *Edwardsiella ictaluri*, *Hafnia alvei*, *Proteus* spp.,

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   1 AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG
  51 CAAGTCGGGC GGTAACAGGG AGAAGCTTGC TTCTCTGCTG ACGAGCGGCG
 101 GACGGGTGAG TAATGTATGG GGATCTGCCT GATGGAGGGG GATAACTACT
 151 GGAAACGGTA GCTAATACCG CATAATGTCC ACGGACCAAA GCGGGGGACC
 201 TTCGGGCCTC GCACCATCAG ATGAACCCAT ATGGGATTAG CTTGTANGTG
 251 AGGTACCGGC TCACCTAGGC GACGATCCCT AGTTGGTCTG AGAGGATGAT
 301 CAGCCACACT TGGGACTGAG ACACGGCCCA GACTCNTACG GGAGGCAGCA
 351 GTGGGGAATA TTGCACAATG GGCGCAAGCC TGATGCAGCC ATGCCGCGTG
 401 TATGAAGAAG GCCTTCGGGT TGTAAAGTAC TTTCAGTCGG GAGGAAGGTG
 451 TTAAGGTTAA TAACCTTGGC AATTGACGTT ACCGACAGAA GAAGCACCGG
 501 CTAACTCCGT GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC
 551 GGAATTACTG GGCGTAAAGC GCACGCAGGC GGTTGGTTGA GTCAGATGTG
 601 AAATCCCCGG GCTTAACCCG GGAATTGCAT CTGATACTGG TCAGCTAGAG
 651 TCTTGTAGAG GGGGGTAGAA TTCCATGTGT AGCGGTGAAA TGCGTAGAGA
 701 TGTGGAGGAA TACCGGTGGC GAAGGCGGCC CCCTGGACAA AGACTGACGC
 751 TCAGGTGCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC
 801 ACGCTGTAAA CGATGTCGAC TTGGAGGTTG TGCCCTTGAG GCGTGGCTTC
 851 CGGAGCTAAC GCGTTAAGTC GACCGCCTGG GGAGTACGGC CGCAAGGTTA
 901 AAACTCAAAT GAATTGACGG GGGCCCGCAC AACGGTGGAG CATGGGTTTA
 951 ATTCGATGCA ACGCGAAGAA CCTTACCTAC TCTTGACATC CAGAGAACTT
1001 AGCAGAGATG CTTTGGTGCC TTCGGGAACT CTGAGACAGG TGCTGCATGG
1051 CTGTCGTCAG CTCGTGTTGT GAAATGTTGG GTTAAGTCCC GCAACGAGCG
1101 CAACCCTTAT CCTTTGTTGC CAGCGCGTGA TGGCGGGAAC TCAAAGGAGA
1151 CTGCCGGTGA TAAACCGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGG
1201 CCCTTACGAG TAGGGCTACA CACGTGCTAC AATGGCGTAT ACAAAGGGAA
1251 GCGACCCTGC GAAGGCAAGC GGAACTCATA AAGTACGTCG TAGTCCGGAT
1301 TGGAGTCTGC AACTCGACTC CATGAAGTCG GAGTCGCTAG TAATCGTAGA
1351 TCAGAATGCT ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC
1401 ACACCATGGG AGTGGGTTGC AAAAGAAGTA GGTAGCTTAA CCTTCGGGAG
1451 GGCGCTTACC ACTTTGTGAT TCATGACTGG GGTGAAGTCG TAACAAGGTA
1501 ACC
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Fig. 8.1. The 16S rDNA sequence of *Morganella morganii*.

and *Providencia* spp (data not shown). When a phylogenetic tree was inferred from the 16S rDNA sequences of *M. morganii* and other enteric bacteria, *M. morganii* was most closely related to *Providencia stuartii* followed by *Proteus vulgaris* and *P. mirabilis* (Fig. 8.2).

To differentiate *M. morganii* from other enteric bacteria, PCR primers were designed based on the variable regions of 16S rDNA sequences (Table 8.1). In general, each primer showed a few base mismatches with 16S rDNA sequences of other enteric bacteria. However, the sequence of each primer showed great differences from those of marine bacteria, such as *Vibrio fluvialis* and *Photobacterium damsela.* The sequences of the primer, Mm208F, and its correspondent to other enteric bacteria are shown in Table 8.2. When two sets of primers, Mm449F and Mm1017R; and Mm208F and Mm1136R, were tested with 12 species of enteric bacteria, they showed all positive reactions with DNA of *M. morganii* (data not shown). However, these primer pairs also amplified DNAs of several enteric bacteria. Mm449F and Mm1017R amplified *Enterobacter cloacae* and *Citrobacter freundii*; and Mm208F and Mm1136R amplified *Proteus* spp., *Enterobacter* spp., and *Klebsiella pneumoniae.*

Table 8.1. Primers used in this study

Primer set	Sequence (5'-3')	Annealing Temperature	Amplicon Size (bp)
Mm449F	TGTTAAGGTTAATAACCTTGGCAATTGA	58°C	568
Mm1017R	CAAAGCATCTCTGCTAAGTTCTCTGGATG		
Mm208F	CTCGCACCATCAGATGAACCCATAT	60°C	928
Mm1136R	GCCATCACGCGCTGGCAACAAAGGATAA		
Mm208F	CTCGCACCATCAGATGAACCCATAT	60°C	809
Mm1017R	CAAAGCATCTCTGCTAAGTTCTCTGGATG		

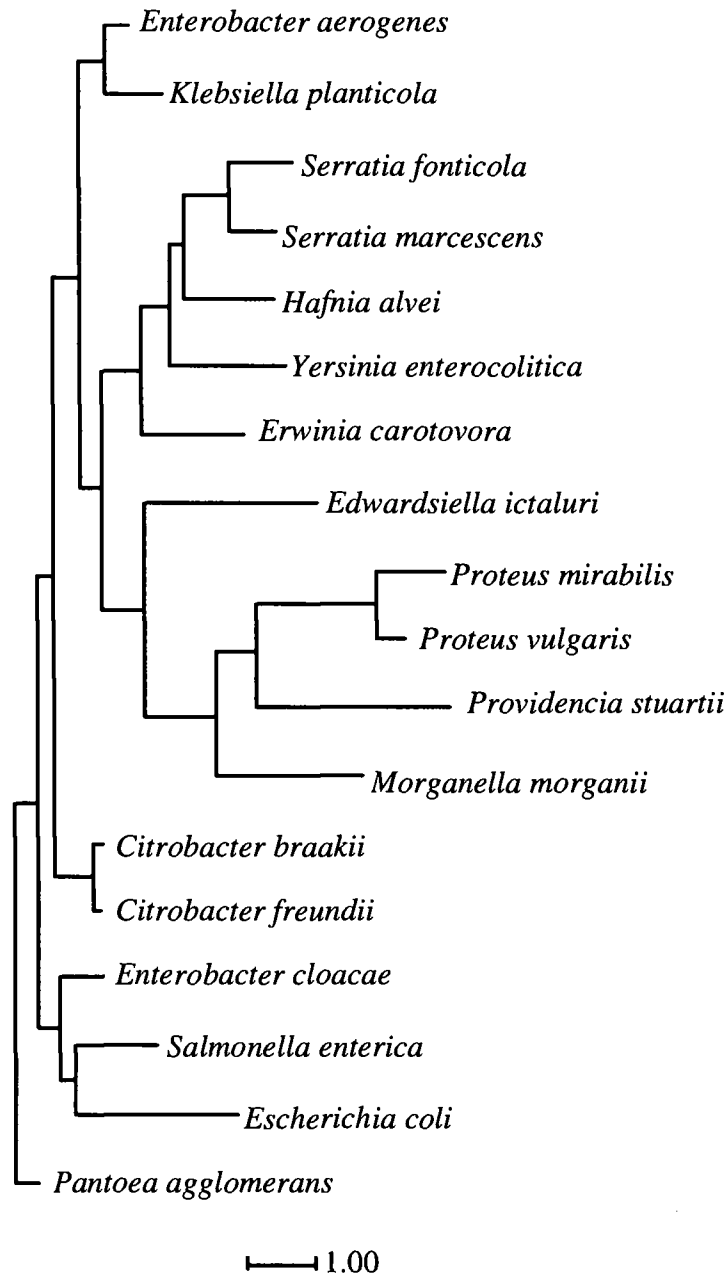

Fig. 8.2. Phylogenetic relationships among 16S rDNA sequences of *Morganella morganii* and other enteric bacteria. The tree was using the neighbor-joining algorithm with the Kimura two-parameter distance correction.

Table 8.2. Alignments of forward primer, Mm208F with the 16S rDNA of *Morganella morganii* and other enteric bacteria

Species	Gene sequence (5'-3')	[a]GenBank Accession No.
Morganella morganii	CTCGCACCATCAGATGAACCCATAT	[b]AY043168
Proteus vulgaris	CT**T**GC**G**C**T**ATC**G**GATGAACCCATAT[†]	AJ233425
Proteus mirabilis	CT**T**GCAC**T**ATC**G**GATGAACCCATAT[†]	AF008582
Providencia stuartii	CT**T**GC**G**C**TG**TCGGATGAACCCATAT[†]	AF008581
Escherichia coli	CTC**TTG**CCATC**G**GATG**TG**CCCA**G**AT	Z83205
Klebsiella planticola	CTC**ATG**CCATCAGATG**TG**CCCA**G**AT	AF181574
Enterobacter cloacae	CTC**TTG**CCATC**G**GATG**TG**CCCA**G**AT	AJ251469
Enterobacter aerogenes	CTC**ATG**CCATCAGATG**TG**CCCA**G**AT	AJ251468
Citrobacter freundii	CTC**TTG**CCATC**G**GATG**TG**CCCA**G**AT	AJ233408
Hafnia alvei	CTC**A**C**G**CCATCAGATG**TG**CCCA**G**AT	M59155
Serratia fonticola	CTC**A**C**G**CCATCAGATG**TG**CCCA**G**AT	AJ233429
Yersinia enterocolitica	CTC**A**C**G**CCATC**G**GATG**TG**CCCA**G**AT	X68672
Edwardsiella ictaluri	CTC**ATG**CCATCAGATGAACCCA**G**AT	AF310622
Vibrio fluvialis	CT**TCGGGCCTCTCGCGTCAGG**ATAT	X76335
Photobacterium damsela	CT**TCGGGCCTCTCGCGTCAGG**AT**TA**	Y18496

[a]The 16S rDNA sequences for alignment of enteric bacteria were obtained from GenBank and each accession numbers were listed.
[b]The 16S rDNA sequence of *Morganella morganii* was submitted to GenBank.

The unique primer pair only amplified *M. morganii* was Mm208F (5'-CTC GCA CCA TCA GAT GAA CCC ATA T-3') and Mm1017R (5'-CAA AGC ATC TCT GCT AAG TTC TCT GGA TG-3') (Fig. 8.3). The melting temperatures of Mm208F and Mm1017R were 60.4 and 61°C, respectively. The primers generated PCR products of the predicted size, 809 bp, only with 16S rDNA of *M. morganii*: ATCC 28550, *M. morganii* OSL36, and a stain of *M. morganii* isolated from decomposed mackerel. No

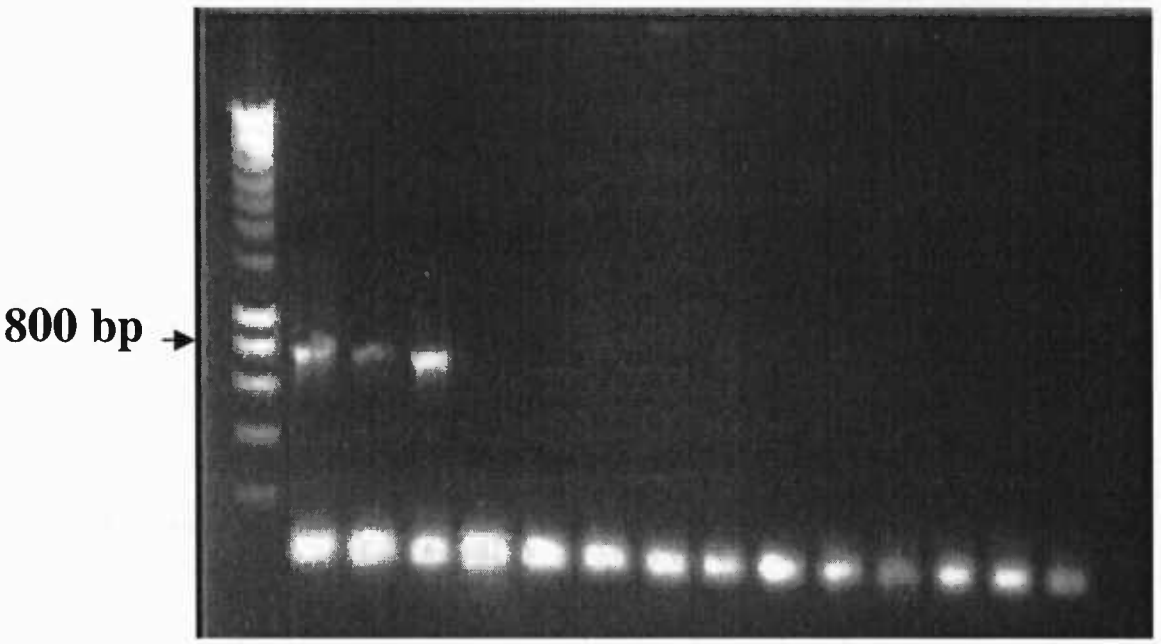

Fig. 8.3. Specificity of PCR assay with the primers Mm208F and Mm1017R for detection of *Morganella morganii*. Primer specificity was tested with DNA of *M. morganii* and other enteric bacterial ATCC strains. Lanes: 1. Molecular weight marker; 2. *M. morganii* OSL36; 3. *M. morganii* ATCC 28550; 4. *M. morganii* isolate; 5. *Proteus vulgaris* ATCC 6380; 6. *Enterobacter cloacae* ATCC 23355; 7. *Enterobacter aerogenes* ATCC 13048; 8. *Klebsiella pneumoniae* ATCC 4211; 9. *Escherichia coli* ATCC 25922; 10. *Citrobacter freundii* ATCC 8090; 11. *Serratia fonticola* ATCC 29848; 12. *Yersinia enterocolitica* ATCC 9610; 13. *Hafnia alvei* ATCC 13337; 14. *Providencia stuartii* ATCC 33672; 15. *Edwardsiella ictaluri* ATCC 3268.

positive amplification was shown with DNA from any other ATCC enteric bacterial strains tested. Primer specificity was also evaluated by testing primers at several annealing temperatures ranging from 60 to 65°C. The primers consistently showed positive PCR results only with DNA from *M. morganii* at all the annealing temperatures tested (data not shown). No positive PCR amplification was observed with DNAs from other bacterial species. The optimum annealing temperature for PCR was 62°C, showing the brightest DNA band among the detected DNA bands. Thus, this annealing temperature was used to test PCR specificity with isolates and sensitivity of PCR assay.

Specificity of primers Mm208F and Mm1017R

The specificity of the PCR assay was evaluated with 18 different isolates. The primers showed positive reactions with DNAs of all the *M. morganii* strains isolated from the temperature-abused muscles of albacore and mackerel (Fig. 8.4 and Table 8.3). The primers showed negative amplifications with DNA of all non-target species regardless of their isolation sources. Enteric histamine formers tested were *Proteus vulgaris*, *Providencia alkalifaciens*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter braakii*, *Hafnia alvei*, and *Serratia fonticola* isolated from the muscle, intestine, or gill of fish. In addition, no PCR product was detected after amplification with DNAs from marine bacteria, such as *Vibrio* spp., *Pseudomonas* spp., *Aeromonas hydrophila*, and *Photobacterium damsela* isolated from the intestine, gill, skin, or muscle of fish.

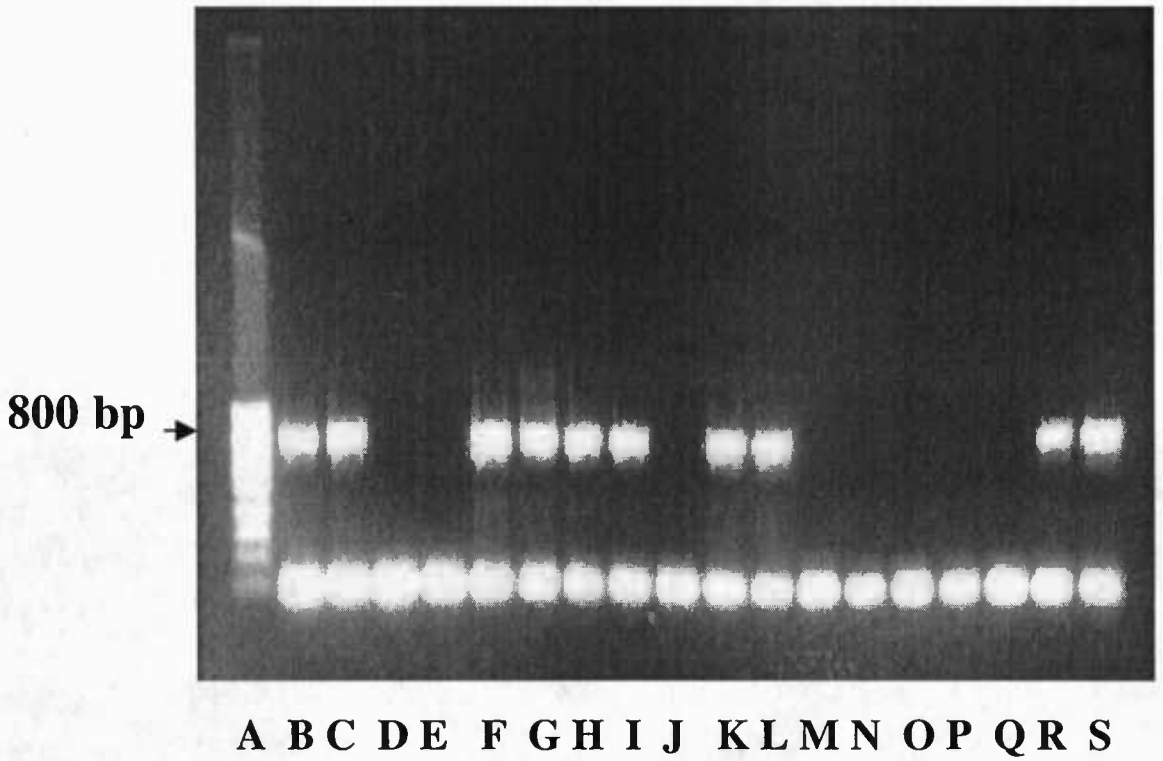

Fig. 8.4. Specificity of PCR assay with the primers Mm208F and Mm1017R for detection of *Morganella morganii*. Primer specificity was tested with DNA of *M. morganii* and other bacterial species isolated from fish. Lanes: A. Molecular weight marker; B-C. *M. morganii*; D. *Pseudomonas putida*; E. *Enterobacter aerogenes*; F-I. *M. morganii*; J. *Proteus vulgaris*; K-L. *M. morganii*; M. *Vibrio alginolyticus*; N. *Vibrio parahaemolyticus*; O-P. *Hafnia alvei*; Q. *Photobacterium damsela*; and R-S. *M. morganii*.

Table 8.3. Result of PCR with isolates used to detect PCR specificity

Strain	Source	Year of isolation	Number of strain	PCR Result
Morganella morganii	Albacore (muscle)	1998	2	+
Morganella morganii	Albacore (muscle)	1999	4	+
Morganella morganii	Mackerel (muscle)	1999	7	+
Proteus vulgaris	Mackerel (muscle)	1999	1	-
Proteus vulgaris	Albacore (intestine)	1999	3	-
Providencia alkalifaciens	Albacore (intestine)	1999	2	-
Enterobacter aerogenes	Albacore (muscle)	1998	3	-
Enterobacter cloacae	Albacore (intestine)	1999	4	-
Klebsiella pneumoniae	Albacore (muscle)	1998	3	-
Klebsiella oxytoca	Albacore (muscle)	1998	3	-
Citrobacter braakii	Albacore (muscle)	1998	2	-
Hafnia alvei	Albacore (muscle)	1998	6	-
Hafnia alvei	Albacore (muscle)	1999	2	-
Serratia fonticola	Albacore (muscle)	1998	2	-
Photobacterium damsela	Mackerel (muscle)	1999	1	-
Photobacterium damsela	Albacore (intestine)	1999	1	-
Pseudomonas fluorescens	Albacore (muscle)	1998	2	-
Pseudomonas putida	Albacore (muscle)	1998	1	-
Vibrio parahaemolyticus	Mackerel (gill)	1999	1	-
Vibrio parahaemolyticus	Albacore (intestine)	1999	1	-
Vibrio alginolyticus	Albacore (gill)	1999	2	-
Vibrio alginolyticus	Albacore (intestine)	1999	1	-
Vibrio alginolyticus	Mackerel (gill)	1999	1	-
Vibrio fluvialis	Mackerel (gill)	1999	1	-
Pasteurella multocida	Albacore (intestine)	1999	1	-
Aeromonas hydrophila	Albacore (intestine)	1999	2	-

Sensitivity of PCR assay

To screen a suitable DNA extraction method for albacore homogenates, DNAs were extracted with several different methods and tested with the primers Mm208F and Mm1017R. The phenol-chloroform extraction method showed the best result among the three tested methods. DNA extracted by guanidine isothiocyanate showed a pale DNA band after PCR amplification (data not shown). No DNA amplification was detected in samples extracted with Chelex 100.

DNAs in albacore homogenates were extracted by the phenol-chloroform method to test sensitivity of the PCR assay. Originally, *M. morganii* ranging from 10^6 to 10^8 CFU/ml in fish homogenate was detected after the PCR amplification (Fig. 8.5a). The sensitivity of PCR assay, however, was greatly improved with the enrichment of samples in TSB. After a 6-h enrichment, the PCR product was detected in albacore homogenate originally inoculated with 9 CFU/ml of *M. morganii* (Fig. 8.5b). Total DNA extracted from non-seeded albacore homogenate did not give any signal to PCR amplification even after enrichment. Consistent result was observed when DNAs were extracted by using the purification kit (Fig. 8.5c). After the PCR amplification, all DNA bands from the enriched samples could be clearly seen in a 1% agarose gel.

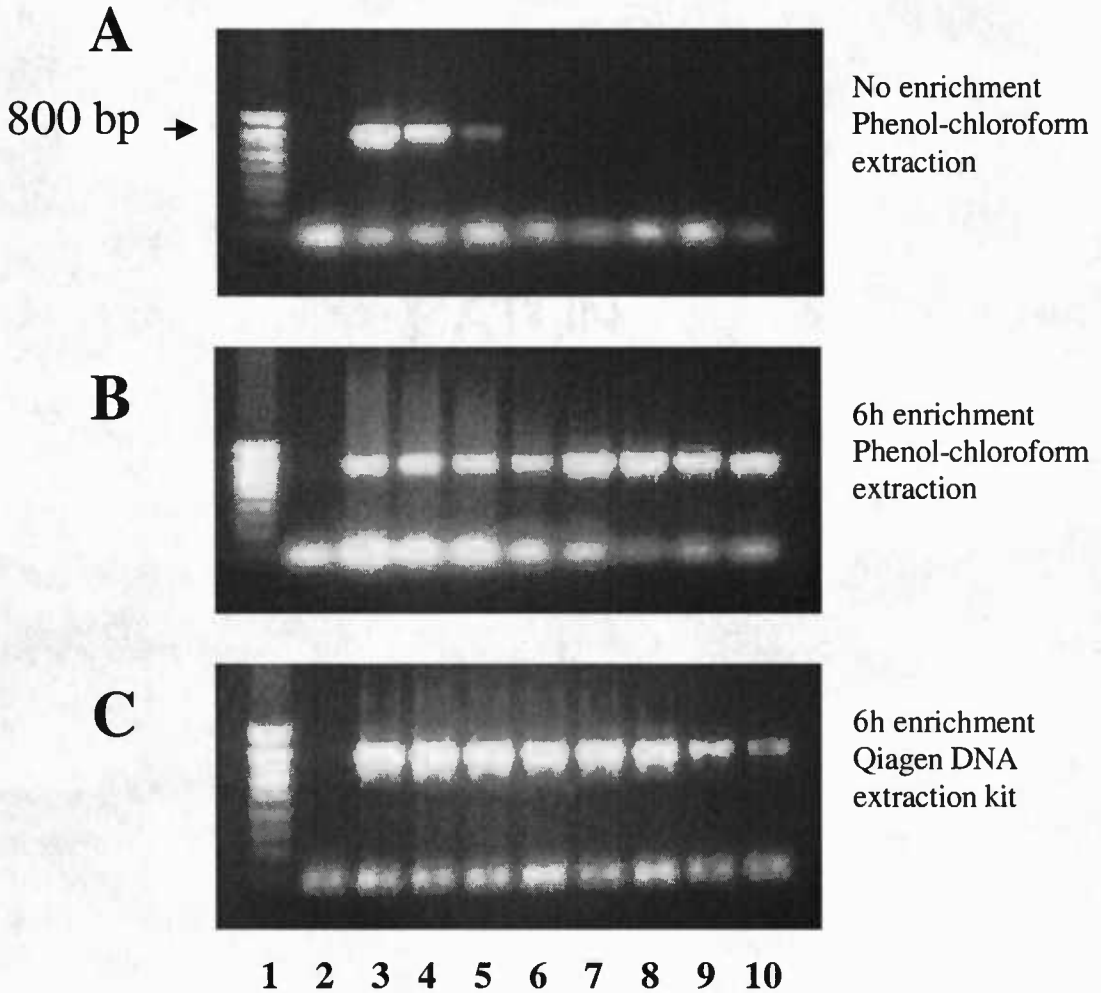

Fig. 8.5. Sensitivity of PCR assay for detection of *Morganella morganii* in fish homogenate. A: Serially diluted *M. morganii* culture was inoculated into fish homogenates. Total DNAs were extracted by using the phenol-chloroform extraction and used as PCR templates.
B: Fish homogenates were enriched in TSB at 37°C for 6 h. Total DNA was extracted by using the phenol-chloroform extraction and used as PCR templates.
C: Total DNA in 6 hr enriched fish homogenate was extracted by using QIAamp® DNA mini kit (Qiagen) and used as PCR templates.
Lanes: 1. Molecular weight marker; 2. Non-seeded fish muscle homogenate; 3. 9×10^7 CFU/ml; 4. 9×10^6 CFU/ml; 5. 9×10^5 CFU/ml; 6. 9×10^4 CFU/ml; 7. 9×10^3 CFU/ml; 8. 9×10^2 CFU/ml; 9. 9×10 CFU/ml; 10. 9 CFU/ml.

Discussion

The 16S rDNA is a very good target for studying phylogenetic relationships of microorganism, because regions within rDNA evolve at different rates, resulting in both conserved and variable nucleotide regions (Kolbert and Persing, 1999). Conserved sequences in 16S rDNA allow the selection of universal oligonucleotide primers for PCR amplification of most microorganisms. Thus, sequence analysis of 1,540 nucleotides has become rapid and inexpensive, and complete sequences allow phylogenetic comparison with the available complete sequences in the database, such as GenBank and the Ribosomal Database Project (RDP) (Stackebrandt and Goebel, 1994). The comparison of 16S rDNA sequences has been used as a reliable method for the classification and identification of several bacterial species (Matsuki et al., 1998). Therefore, 16S rDNA-targeted primers have been widely developed for monitoring microbial populations to determine the distribution of bacterial species in their natural habitats and to assess the diversity of microorganisms in natural communities (Rappé et al., 1998).

Analysis of 16S rDNA sequences data is advantageous for bacterial identification. Genus-specific primers targeting highly conserved 16S rDNA sequence have been developed for the detection of a closely related phylogenetic cluster, *Bifidobacterium*, in food and fecal samples (Kaufmann et al., 1997; Matsuki et al., 1998). For the detection of *Escherichia coli* from water and milk, PCR primers, based on the partial gene sequence of 16S rRNA, successfully differentiated *E. coli* from closely related *Shigella* spp. (Tsen et al., 1998; Lin and Tsen, 1999). Primers deriving from species-specific regions in the 16S rDNA have been tested for several species of *Rhodococcus* (Bell et al.,

1999). They showed positive results with all or most strains of the target species but did not cross-react with other species. However, some studies have shown that the taxonomic resolution of this molecular target may be limited when addressing phylogenetic relationships of closely related bacterial species, particularly those containing sequence homology of 97% or greater (Stackebrandt and Goebel, 1994).

The genus *Morganella* belongs to the tribe *Proteese* of the family *Enterobacteriaceae* along with two other genera, *Proteus* and *Providencia* (O'Hara et al., 2000). Most members of the *Proteus-Providencia-Morganella* group share a number of common biochemical characteristics, including methyl red and urease positive reactions, growth in KCN broth, motility, and production of phenylalanine deaminase (Janda et al., 1996). *M. morganii* was originally named as *Proteus morganii* due to its urease production, but transferred to the genus *Morganella* as the sole species in the genus (Pignato et al., 1999). This species shows little phenotypical strain-to-strain variations in its biochemical characteristics and is divided into two groups on the basis of the ability to ferment trehalose (Janda et al., 1996; Jensen et al., 1992).

Since the 16S rDNA sequence of *M. morganii* has not been reported, the gene was amplified by using bacterial primer 27F and 1492R, generating 1,503 bp of sequence. The close relationship of *M. morganii* to the *Proteus-Providencia* group was confirmed when the phylogenetic relationship was inferred from the 16S rDNA of enteric bacteria. *Proteus vulgaris* and *Providencia* spp. are prolific histamine formers together with *M. morganii* (Kim et al., 2001b). Other closely related enteric bacteria such as *Hafnia alvei*, *Klebsiella* spp., and *Enterobacter* spp. are also histamine formers, producing 200 to 1,000 ppm histamine in culture broth. However, use of the species specific 16S rDNA-targeted

primers successfully distinguished *M. morganii* from other enteric bacteria as well as marine bacteria. The primers showed specificity at the broad annealing temperature ranges tested. The most suitable temperature for PCR was found at temperature higher than the melting temperatures of each primer calculated according to the method of Allawi and SantaLucia (1997). It has been reported that the stringency of primers is improved by increasing the annealing temperature (Bell et al., 1999). *E. coli* was specifically discriminated from other enteric bacteria when the annealing temperature was elevated to 72°C, which is 10°C higher than the melting temperature of the primers (Sabat et al., 2000).

PCR shows high sensitivity in detecting target bacteria. However, the detection of bacterial species is complicated in foods, because of low numbers of the organism of interest in foods, complex and varying microbial flora, and the diverse physical and chemical compositions of foods and environment (Wolcott, 1991). Common inhibitors reported in PCR amplification include various components of body fluids and reagents encountered in clinical specimens (hemoglobin, urea, and heparin), food constituents (organic and phenolic compounds, glycogen, fat, and Ca^{2+}), and environmental compounds (phenolic compounds, humic acids, and heavy metals) (Wilson, 1997). Thus, various approaches, such as enrichment of samples and a sensitive DNA extraction procedure, have been made to improve the sensitivity. Removal of humic compounds in soil was accomplished by CTAB treatment of samples (Bell et al., 1999). In soil, 10^4 CFU/g of *Rhodococcus* spp. was detected, and the use of a secondary PCR allowed the detection of 1 CFU/g. A 6-h enrichment of samples in TSB at 42°C allowed the detection of 1.4 CFU/g of *E. coli* in ground beef (Gannon et al., 1992). An initial

inoculum level of 9.3 CFU/g of *V. parahaemolyticus* was detected in oyster after enrichment in TSB supplemented with 3% NaCl for 3 h at 35°C (Lee et al., 1995). *Clostridium botulinum* was detected in foods contaminated with 10 CFU/g after an 18-h enrichment under anaerobic conditions (Fach et al., 1995). In general, high levels of background bacterial DNA in enriched samples were not inhibitory to specific amplification, and 10^2 to 10^4 CFU/g of target could be detected in several food types (Dickinson et al., 1995). The detection limit of *V. parahaemolyticus* was not reduced when 10^4 to 10^5 *E. coli* cells were present in the PCR mixtures (Lee et al., 1995). High levels of other contaminating bacteria in chicken products did not affect the specificity of PCR for the detection of *Campylobacter* spp. (Giesendorf et al., 1992). In this study, 10^6 CFU/ml of *M. morganii* was required for specific detection in albacore homogenate; however, its detection was improved 100-thousand-fold by a 6-hr enrichment of samples in TSB.

Monitoring the presence of *M. morganii* in the marine environment and freshly caught fish is important to address the contamination source of *M. morganii* and prevent histamine formation in fish. On-board handling is the most critical step to prevent histamine formation, since any delay of proper storage of fish leads to histamine formation in fish muscles (FDA, 1998; Kim et al., 2001b). However, detection of *M. morganii* has been hampered due to the lack of reliable isolation methods as well as low sensitivity of the conventional culture method. When *M. morganii* is isolated by using selective or differential media, utilization of a substrate to indicate its presence on the media is often inhibited by the competition with other bacteria (Kim et al., 2001a). Rapid and sensitive detection of *M. morganii* is also required in clinical microbiology. This

species is an opportunistic human pathogen causing a variety of nosocomial illnesses including urinary tract disease, septicemia, and wound infection (Janda et al., 1996). Detection of the bacteria has been based on serotyping, bacteriocin, bacteriophage typing, and antibiotics resistance patterns (Pignato et al., 1999). The PCR assay developed in this study could be applied for direct detection of *M. morganii* in various types of food and environmental samples without the need for isolation of pure culture.

Conclusion

The 16S rDNA sequence of *M. morganii* showed a considerable homology with those of enteric bacteria. Specifically designed primers based on the limited variable regions of 16S rDNA sequence detected 9 CFU/ml of *M. morganii* in albacore homogenate with a 6-h enrichment. The PCR assay developed in this study should be useful to identify the contamination route of *M. morganii* from the marine environment and routinely monitor the presence of *M. morganii* during handling and storage of fish to assist the implement of HACCP in the fishery industry.

Acknowledgments

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References

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C.L., Jones, D.D., and Kaysner, C.A. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh*, and *trh*. J. Microbiol. Methods 36: 215-225.

Bell, K.S., Kuyukina, M.S., Heidbrink, S., Philp, J.C., Aw, D.W.J., Ivshina, I.B., and Christofi, N. 1999. Identification and environmental detection of *Rhodococcus* species by 16S rDNA-targeted PCR. J. Appl. Microbiol. 87: 472-480.

Centers for Disease Control and Prevention. 2000. Surveillance for foodborne-disease outbreaks-United States, 1993-1997. *In* CDC surveillance summaries, MMWR, 49: 1-72.

Dickinson, J.H., Kroll, R.G., and Grant, K.A. 1995. The direct application of the polymerase chain reaction of DNA extracted from foods. Lett. Appl. Microbiol. 20: 212-216.

Dojka, M.A., Hugenholtz, P., Haack S.K., and Pace N.R. 1998. Microbial diversity in a hydrocarbon and chlorinated-solvent contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64: 3869-3877.

Fach, P., Gibert, M., Griffais, R., Guillou, J.P., and Popoff, M.R. 1995. PCR and gene probe identification of Botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium* spp. and evaluation in food samples. Appl. Environ. Microbiol. 61: 389-392.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th ed. Arlington, VA.

Food and Drug Administration. 1998. Ch.7. Scombrotoxin (histamine) formation. *In* Fish and Fishery Products Hazards and Controls Guide. 2nd ed., p. 73-90. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Food and Drug Administration. 2000. FDA's evaluation of the seafood HACCP program for 1998/1999. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Gannon, V.P.J., King, R.K., Kim, J.Y., and Thomas, E.J.G. 1992. Rapid and sensitive method for detection of shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol. 58: 3809-3815.

Gendel, S.M. 1996. Computational analysis of the specificity of 16S rRNA-derived signature sequences for identifying food-related microbes. Food Microbiol. 13: 1-15.

Giesendorf, B.A.J., Quint, W.G.V., Henkens, M.H.C., Stegeman, H., Huf, F.A., Niesters, H.G.M. 1992. Rapid and sensitive detection of *Campylobacter* spp. in chicken products by using the polymerase chain reaction. Appl. Environ. Microbiol. 58: 3804-3808.

Janda, J.M., Abbott, S.L., Khashe, S., and Robin, T. 1996. Biochemical investigations of biogroups and subspecies of *Morganella morganii*. J. Clin. Microbiol. 34: 108-113.

Jensen, K.T., Frederiksen, W., Hickman-Brenner, F.W., Steigerwalt, A.G., Riddle, C.F., and Brenner, D.J. 1992. Recognition of *Morganella* subspecies, with proposal of *Morganella morganii* subs. *morganii* subs. nov. and *Morganella morganii* subs. s*ibonii* subs. nov. Int. J. Syst. Bacteriol. 42: 613-620.

Kamath, A.V., Vaaler, G.L., and Snell, E.E. 1991. Pyridoxal phosphate-dependent histidine decarboxylase: cloning, sequencing, and expression of genes from *Klebsiella planticola* and *Enterobacter aerogenes* and properties of the overexpressed enzymes. J. Biol. Chem. 266: 9432-9437.

Kaufmann, P., Pfefferkorn, A., Teuber, M., and Meile, L. 1997. Identification and quantification of *Bifidobacterium* species isolated form food with genus-specific 16S rRNA target probes by colony hybridization and PCR. Appl. Environ. Microbiol. 63: 1268-1273.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Kim, S.H., Field, K.G., Morrissey, M.T., Price, R.J., Wei, C.I., and An, H. 2001a. Source and identification of histamine-producing bacteria from fresh and temperature-abused albacore. J. Food Prot. 64: 1035-1044.

Kim, S.H., Field, K.G., Chang, D.S., Wei, C.I., and An, H. 2001b. Identification of bacteria crucial to histamine accumulation in Pacific mackerel during storage. J. Food Prot. (in press).

Kimura, B., Kawasaki, S., Nakano, H., and Fujii, T. 2001. Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. Appl. Environ, Microbiol. 67: 206-216.

Klein, P.G. and Juneja, V.K. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. Appl. Environ. Microbiol. 63: 4441-4448.

Kolbert, C.P. and Persing, D.H. 1999. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr. Opin. Microbiol. 2: 299-305.

Le Jeune, C., Lonvaud-Funel, A., Brink, B.T., Hofstra, H., and van der Vossen, J.M.B.M. 1995. Development of detection system for histidine decarboxylating lactic acid bacterial based on DNA probes, PCR and activity test. J. Appl. Bacteriol. 78: 316-326.

Lee, C.Y., Pan, S.F., and Chen, C.H. 1995. Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with PCR. Appl. Environ. Microbiol. 61: 1311-1317.

Lin, C.K. and Tsen, H.Y. 1999. Comparison of the partial 16S rRNA gene sequences and development of oligonucleotide probes for the detection of *Escherichia coli* cells in water and milk. Food Microbiol. 16: 551-562.

Matsuki, T., Watanabe, K., Tanaka, R., Oyaizu, H. 1998. Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. FEMS Microbiol. Lett. 167: 113-121.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53:1024-1029.

Morii, H., Cann, D.C., and Taylor, L.Y. 1988. Histamine formation by luminous bacteria in mackerel stored at low temperatures. Nippon Suisan Gakkaishi 54: 299-305.

Niven, C.F., Jeffrey, M.B., and Corlett, D.A. 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. Appl. Environ. Microbiol. 41: 321-322.

O'Hara, C.M., Brenner, F.W., and Miller, J.M. 2000. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clin. Microbiol. Rev. 13: 534-546.

Pignato, S., Giammanco, G.M., Grimont, F., Grimont, P.A.D., and Giammanco, G. 1999. Molecular characterization of the Genera *Proteus*, *Morganella*, and *Providencia* by ribotyping. J. Clin. Microbiol. 37: 2840-2847.

Rappé, M.S., Suzuki, M.T., Vergin, K.L., and Giovannoni, S.J. 1998. Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environment nucleic acid samples from the Pacific and Atlantic Coasts of the Unites States. Appl. Environ. Microbiol. 64: 294-303.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish and shellfish. Adv. Food Nutr. Res. 39: 329-364.

Sabat, G., Rose, P., Hickey, W.J., and Harkin, J.M. 2000. Selective and sensitive methods for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. Appl. Environ. Microbiol. 66: 844-849.

Saiton, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Stackebrandt, E. and Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequences analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846-849.

Stratton, J.E. and Taylor, S.L. 1991. Scombroid poisoning. p.331-351. *In* Microbiology of Marine Food Products. van Nostrand Reinhold, N.Y.

Tsen, H.Y., Lin, C.K., and Chi, W.R. 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. J. Appl. Microbiol. 85: 554-560.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60: 407-438.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63: 3741-3751.

Wolcott, M.J. 1991. DNA-based rapid methods for the detection of foodborne pathogens. J. Food Prot. 54: 387-401.

Chapter 9

Summary

Proper handling and storage of fish after catch are required in controlling histamine formation. The highest level of histamine formed in the muscles during storage of fish at ambient temperature (25°C). Toxicological level of histamine (>50 mg/100 g) was accumulated after the fish became spoiled and unsuitable for human consumption with gross bacterial growth. Histamine formation was effectively controlled during storage of fish at 0°C. It was also necessary to chill fish rapidly and maintain temperature of fish at 0°C even after evisceration and degilling of fish.

Histamine-producing bacteria were rarely isolated from fresh fish due to the low bacterial population loaded in fresh fish and mainly found in the gill and skin. However, storage temperature greatly influenced the growth of various types of bacterial floras and, consequently, histamine accumulation in fish. Most frequently isolated prolific histamine formers were mesophilic enteric bacteria; thus, the ambient temperature was the most favorable condition to support their growth and histamine formation. Among them, *Morganella morganii* was the most prolific histamine former and played the major role in histamine accumulation during storage of fish. In mackerel, *M. morganii* was the most prevalent species, and histamine rapidly accumulated in the muscles with their growth. However, *M. morganii* was hardly isolated even from the spoiled muscles of albacore. Their low population in albacore is consistent with the observation of the low histamine accumulation during storage of albacore. This result supports the common observation that albacore has rarely been involved in scombroid poisoning. It was also shown that the

isolation rate of *M. morganii* decreased as the storage temperature decreased, regardless of the length of storage. *M. morganii* was not isolated at 4°C or below, coinciding with the low levels of histamine found in fish muscle at the tmeperatures.

M. morganii formed significant amounts of histamine (>200 mg/100 g) in artificially contaminated mackerel, albacore, and mahi-mahi, when the fish were improperly stored at 25°C. Mackerel was the most susceptible fish species due to its high free histidine content and proteolysis activity in the muscle. Histamine formation by *M. morganii* was controlled during frozen storage; however, the bacteria survived during frozen storage, recovered its activity, and produced toxicological levels of histamine in the muscles when incubated at 25°C after thawing. It indicates the importance of handling and storage conditions after thawing of fish in restaurant and seafood industry. Therefore, avoiding contamination of prolific histamine formers from the marine environment and preventing their proliferation during handling and distribution of fish would be indispensable to prevent histamine formation and, subsequently, outbreaks of scombroid poisoning.

To monitor the presence of *M. morganii* in various food and environment, direct detection of *M. morganii* was achieved by developing the PCR assay without the need for isolation of pure culture. Specifically designed primers based in the limited variable regions of 16S rDNA sequence detected 9 CFU/ml of *M. morganii* in albacore homogenate with a 6-h enrichment. The PCR assay developed in this study should be useful to identify contamination route of *M. morganii* from the marine environment and routinely monitor the presence of *M. morganii* during handling and storage of fish to assist the implement of HACCP in the fishery industry.

Bibliography

Ababouch, L., Afilal, M.E., Rhafiri, S., and Busta, F.F. 1991. Identification of histamine-producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperatures (25°C). Food Microbiol. 8: 127-136.

Ababouch, L., Afilal, M.E., Benabdeljelil, H., and Busta. F.F. 1991. Quantitative changes in bacteria, amino acids and biogenic amines in sardine (*Sardina pilchardus*) stored at ambient temperature (25-28°C) and in ice. Int. J. Food Sci. Tech. 26: 297-306.

Abe, H. 1983. Distribution of free L-histidine and its related compounds in marine fishes. Bull. Jpn. Soc. Sci. Fish. 49: 1683-1687.

Ahmed, F.E (ed.). 1991. Ch. 4. Naturally occurring fish and shellfish poisons. p.87-110. *In* Seafood Safety. National Academy Press, Washington, D.C.

Antoine, F.R., Wei, C.I., Littell, R.C., and Marshall, M.R. 1999. HPLC method for analysis of free amino acids in fish using *o*-phthaldialdehyde precolumn derivatization. J. Agric. Food Chem. 47: 5100-5107.

AOAC. 1995. Histamine in seafood: Fluorometric method. Sec. 35.1.32, Method 977.13. *In* Official Methods of Analysis of AOAC International. 16th ed. p. 6-17. AOAC International, Gaithersburg, MD.

Arnold, S.H., Price, R.J., and Brown, W.D. 1980. Histamine formation by bacteria isolated from skipjack tuna, *Katsuwonus pelamis*. Bull. Jpn Soc. Sci. Fish. 46: 991-995.

Babu, S., Chander, H., Batish, V.K., and Bhatis, K.L. 1986. Factors affecting amine production in *Streptococcus ceremoris*. Food Microbiol. 3: 359-362.

Baldrati, G., Fornari, M.B., Spotti, E., and Incerti, I. 1980. Effect of temperature on histamine formation in fish rich in histidine. Industr. Con. 55: 114-122.

Barancin, C.E., Smoot, J.C., Findlay, R.H., and Actis, L.A. 1998. Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen *Vibrio anguillarum*. Plasmid. 39: 235-244.

Baranowski, J.D., Brust, P.A., and Frank, H.A. 1985. Growth of *Klebsiella pneumoniae* UH-2 and properties of its histidine decarboxylase system in resting cells. J. Food Biochem. 9: 349-360.

Baranowski, J.D., Frank, M.A., Brust, P.A., Chongsiriwatana, M., and Premaratne, R.J. 1990. Decomposition and histamine content in mahimahi (*Coryphaena Hippurus*). J. Food Prot. 53: 217-222.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., and Turc, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-496.

Bean, N.H. and Griffin, P.M. 1990. Foodborne disease outbreaks in the United States, 1973-1987. J. Food Prot. 53: 804-817.

Becker, K., Southwick, K., Reardon, J., Berg, R., MacCormack, J.N. 2001. Histamine poisoning associated with eating tuna burgers. JAMA. 285: 1327-1330.

Behling, A.R. and Taylor, S.L. 1982. Bacterial histamine production as a function of temperature and time of incubation. J. Food Sci. 47: 1311-1314, 1317.

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C.L., Jones, D.D., and Kaysner, C.A. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh*, and *trh*. J. Microbiol. Methods 36: 215-225.

Bell, K.S., Kuyukina, M.S., Heidbrink, S., Philp, J.C., Aw, D.W.J., Ivshina, I.B., and Christofi, N. 1999. Identification and environmental detection of *Rhodococcus* species by 16S rDNA-targeted PCR. J. Appl. Microbiol. 87: 472-480.

Ben-Gigirey, B., Craven, C., and An, H. 1998. Histamine formation in albacore muscle analyzed by AOAC and enzymatic methods. J. Food Sci. 63: 210-214.

Ben-Gigirey, B., Vieites-Baptista de Sousa, J.M., Villa, T.G., and Barros-Velazquez, J. 1999. Histamine and cadaverine production by bacteria isolated from fresh and frozen albacore (*Thunnus alalunga*). J. Food Prot. 62: 933-939.

Bennour, M., El Marrakchi, A., Bouchriti, N., Hamama, A., and El Ouadaa, M. 1991. Chemical and microbiological assessments of Mackerel (*Scomber scombrus*) stored in ice. J. Food Prot. 54: 784, 789-792.

Benson, H.J. (ed.). 1992. Microbiological applications: a laboratory manual in general microbiology. WCB, Dubuque, IA.

Bremer, P.J., Osborne C.M., Kemp R.A., van Veghel, P. and Fletcher, G.C. 1998. Thermal death times of *Hafnia alvei* cells in a model suspension and in artificially contaminated hot-smoked kahawai (*Arripis trutta*). J. Food Prot. 61: 1047-1051.

Cappuccino, J.G. and Sherman, N. (ed.). 1992. Microbiology. Third ed. The Benjamin/Cummings Publishing Company, Inc., Redwood City, CA.

Centers for Disease Control and Prevention. 1986. Epidemiologic notes and reports restaurant-associated scombroid poisoning fish poisoning-Alabama, Tennessee. MMWR. 35: 264-265.

Centers for Disease Control and Prevention. 1989. Epidemiologic notes and reports scombroid fish poisoning-Illinois, South Carolina. MMWR. 38: 140-142, 147.

Centers for Disease Control and Prevention. 1996. Surveillance for foodborne-disease outbreaks-United States, 1988-1992. In *CDC surveillance summaries*, MMWR, 45: 1-72.

Centers for Disease Control and Prevention. 2000. Surveillance for foodborne-disease outbreaks-United States, 1993-1997. In *CDC surveillance summaries*, MMWR, 49: 1-72.

Centers for Disease Control and Prevention. 2000. Limitations of field investigations: Etiology and food vehicle not determined for a substantial proportion of food borne outbreaks in food net, 1998-1999.

Centers for Disease Control and Prevention. 2000. Scombroid fish poisoning-Pennsylvania, 1998. MMWR, 49: 398-400.

Chen, C.M., Wei, C.I., Koburger, J.A., and Marshall, M.R. 1989. Comparison of four agar media for detection of histamine-producing bacteria in tuna. J. Food Prot. 52: 808-813.

Clifford, M.N., Walker, R., and Wright, J. 1989. Studies with volunteers on the role of histamine in suspected scombrotoxicosis. J. Sci. Food Agric. 47: 365-375.

Craven, C., Hilderbrand, K., Kolbe, E., Sylvia, G., Daeschel, M., Gloria, B., and An, H. 1995. Understanding and controlling histamine formation in troll-caught albacore tuna: A review of preliminary findings from the 1994 season. Oregon Sea Grant (ORESU-T-95-00). Corvallis, OR.

Dickinson, J.H., Kroll, R.G., and Grant, K.A. 1995. The direct application of the polymerase chain reaction of DNA extracted from foods. Lett. Appl. Microbiol. 20: 212-216.

Difco. 1985. Difco manual: dehydrated culture media and reagents for microbiology, 10th ed., Difco laboratories, Detroit, MI.

Dojka, M.A., Hugenholtz, P., Haack S.K., and Pace N.R. 1998. Microbial diversity in a hydrocarbon and chlorinated-solvent contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64: 3869-3877.

Eerola, S., Hinkkanean, R., Lindfords, E., and Hirvi, T. 1993. Liquid chromatographic determination of biogenic amines in dry sausages. J. AOAC Int. 76: 575-577.

Eitenmiller, R.R., Wallis, J.W., Orr, J.H., and Phillips, R.D. 1981. Production of histidine decarboxylase and histamine by *Proteus morganii*. J. Food Prot. 44: 815-820.

El Marrakchi, A., Bennour, M., Bouchritt, N., Hamama, A., and Tagafait, H. 1990. Sensory, chemical, and microbiological assessments of Moroccan sardines (*Sardina pilchardus*) stored in ice. J. Food Prot. 53: 600-605.

Fach, P., Gibert, M., Griffais, R., Guillou, J.P., and Popoff, M.R. 1995. PCR and gene probe identification of Botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium* spp. and evaluation in food samples. Appl. Environ. Microbiol. 61: 389-392.

Fletcher, G.C., Summers, G., Winchester, R.V., and Wong, R.J. 1995. Histamine and histidine in New Zealand marine fish and shellfish species particularly kahawai (*Arripis trutta*). J. Aquatic Food Prod. 4: 53-74.

Fletcher, G.C., Summers, G., and van Veghel, P.W.G. 1998. Levels of histamine and histamine-producing bacteria in smoked fish from New Zealand markets. J. Food. Prot. 61: 1064-1070.

Food and Drug Administration. 1992. Aerobic plate count. p. 17-26. *In* FDA Bacteriological Analytical Manual. 7th ed. Arlington, VA.

Food and Drug Administration. 1994. Proposed rules. Fed. Reg. 59: 4149.

Food and Drug Administration. 1995. Procedures for the safe and sanitary processing and importing of fish and fishery products. Fed. Reg. 60: 65096-65202.

Food and Drug Administration. 1996. Decomposition and histamine raw, frozen tuna and mahi-mahi; canned tuna; and related species. CPG 7108.240, Sec. 540.525.

Food and Drug Administration. 1998. Ch.7 Scombrotoxin (histamine) formation. p. 73-90. *In* Fish and Fishery Products Hazards and Controls Guide. 2nd ed., Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Food and Drug Administration. 2000. FDA's evaluation of the seafood HACCP program for 1998/1999. Department of Health and Human Services, Public Health Service, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, D.C.

Fouz, B., Toranzo, A.E., Milán, M., and Amaro, C. 2000. Evidence that water transmits the disease caused by the fish pathogen *photobacterium damselae* subsp. *damselae*. J. Appl. Microbiol. 88:531-535.

Frank, H.A. and Yoshinaga D.H. 1984. Histamine formation in tuna. Seafood Toxins. ACS Symposium Series. No. 262. p. 443-451. American Chem. Soc., Washington, D.C.

Frank, H.A., Baranowski, J.D., Chongsiriwatana, M., Brust, P.A., and Premaratne, R.J. 1985. Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (*Coryphaena hippurus*) after incubation at 0 and 32°C. Int. J. Food Microbiol. 2: 331-340.

Gannon, V.P.J., King, R.K., Kim, J.Y., and Thomas, E.J.G. 1992. Rapid and sensitive method for detection of shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol. 58: 3809-3815.

Gennari, M., Tomaselli, S., and Cotrona, V. 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) sea and stored in ice. Food Microbiol. 16: 15-28.

Giesendorf, B.A.J., Quint, W.G.V., Henkens, M.H.C., Stegeman, H., Huf, F.A., Niesters, H.G.M. 1992. Rapid and sensitive detection of *Campylobacter* spp. in chicken products by using the polymerase chain reaction. Appl. Environ. Microbiol. 58: 3804-3808.

Gram, L., Trolle, G., and Huss, H.H. 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4: 65-72.

Haaland, H., Arnesen, E., and Njaa, L.R. 1990. Amino acid composition of whole mackerel (*Scomber scombrus*) stored anaerobically at 20°C and at 2°C. Int. J. Food Sci. Technol. 25: 82-87.

Hernández-Herrero, M.M., Roig-Sagués, A.X., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1999. Halotolent and halophilic histamine-forming bacteria isolated during the ripening of salted anchovies (*Engraulis encrasicholus*). J. Food Prot. 62: 509-514.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. 1994. Bergey's manual of determinative bacteriology, 9th ed. Williams and Wilkins Co., Baltimore.

Hui, J.Y. and Taylor, S.L. 1985. Inhibition of in vivo histamine metabolism in rats by foodborne and pharmacologic inhibitors of diamine oxidase, histamine N-methyltransferase, and monoamine oxidase. Toxicol. Appl. Pharmacol. 8: 241-249.

Hungerford, Y.M. and Arefyev, A.A. 1992. Flow-injection assay of enzyme inhibition in fish using immobilized diamine oxidase. Anal. Chimica. Acta. 261: 351-359.

Hwang, D.F., Chang, S.H., Shiau, C.Y., and Cheng, C.C. 1995. Biogenic amines in the flesh of sailfish (*Istiophorus platypterus*) responsible for scombroid poisoning. J. Food Sci. 60: 926-928.

ICMSF. 1986. Microorganisms in Foods 2. Sampling for microbiological analysis: principles and specific application, 2nd ed. University of Toronto Press, Buffalo, NY.

Jacoby, D.R. 1987. On-board Handling and preservation of frozen troll-caught albacore, *Thunnus alalunga*. Mar. Fish. Rev. 49: 46-50.

Janda, J.M., Abbott, S.L., Khashe, S., and Robin, T. 1996. Biochemical investigation of biogroups and species of *Morganella morganii*. J. Clin. Microbiol. 34: 108-113.

Jay, J.M. (ed.). 1996. Ch. 15. Low temperature food preservation and characteristics of psychrotrophic microorganisms. p. 330-342. *In* Modern Food Microbiology. Fifth Ed., ITP. New York, NY.

Jensen, K.T., Frederiksen, W., Hickman-Brenner, F.W., Steigerwalt, A.G., Riddle, C.F., and Brenner, D.J. 1992. Recognition of *Morganella* subspecies, with proposal of *Morganella morganii* subs. *morganii* subs. nov. and *Morganella morganii* subs. s*ibonii* subs. nov. Int. J. Syst. Bacteriol. 42: 613-620.

Jhaveri, S.N., Leu, S., and Constantinides, S.M. 1982. Atlantic mackerel (*Scomber scombus*, L): Shelf life in ice. J. Food Sci. 47: 1808-1810.

Joosten, H.M.L.J. and Northolt, M.D. 1989. Detection, growth and amine producing capacity of lactobacilly in cheese. Appl. Environ. Microbiol. 55: 2356-2359.

Jørgensen, L.V., Dalgaard, P., and Huss, H.H. 2000. Multiple compound quality index for cold-smoked salmon (*Salmo salar*) developed by multivariate regression of biogenic amines and pH. J. Agric. Food Chem. 48: 2448-2453.

Kamath, A.V., Vaaler, G.L., and Snell, E.E. 1991. Pyridoxal phosphate-dependent histidine decarboxylase: cloning, sequencing, and expression of genes from *Klebsiella planticola* and *Enterobacter aerogenes* and properties of the overexpressed enzymes. J. Biol. Chem. 266: 9432-9437.

Kaufmann, P., Pfefferkorn, A., Teuber, M., and Meile, L. 1997. Identification and quantification of *Bifidobacterium* species isolated form food with genus-specific 16S rRNA target probes by colony hybridization and PCR. Appl. Environ. Microbiol. 63: 1268-1273.

Kim, I.S. and Bjeldanes, L.F. 1979. Amines content of toxic and wholesome canned tuna fish. J. Food Sci. 44: 922-923.

Kim, S.H., An, H., and Price, R.J. 1999. Histamine formation and bacterial spoilage of albacore harvested off the U.S. Northwest coast. J. Food Sci. 64: 340-344.

Kim, S.H., Ben-Gigirey, B., Barros-Velazquez, J., Price, R.J., and An, H. 2000. Histamine and biogenic amine production by *Morganella morganii* isolated from temperature-abused albacore. J. Food Prot. 63: 244-251.

Kim, S.H., Field, K.G., Morrissey, M.T., Price, R.J., Wei, C.I., and An, H. 2001. Source and identification of histamine-producing bacteria from temperature-abused albacore. J. Food Prot. 64: 1035-1044.

Kim, S.H., Field, K.G., Chang, D.S., Wei, C.I., and An, H. 2001. Identification of bacteria crucial to histamine accumulation in Pacific mackerel during storage. J. Food Prot. (in press).

Kimura, B., Kawasaki, S., Nakano, H., and Fujii, T. 2001. Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. Appl. Environ, Microbiol. 67: 206-216.

Klausen, N.K. and Huss, H.H. 1987. Growth and histamine production by *Morganella morganii* under various temperature conditions. Int. J. Food Microbiol. 5: 147-156.

Klein, P.G. and Juneja, V.K. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. Appl. Environ. Microbiol. 63: 4441-4448.

Kolbert, C.P. and Persing, D.H. 1999. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr. Opin. Microbiol. 2: 299-305.

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C., and Winn, W.C. Jr. (ed.). 1997. Diagnostic microbiology. 5th Ed. Lippincott. Philadelphia, PA.

Kurihara, K., Wagatuma, Y., Fujii, T., and Okuzumi, M. 1993. Effect of reaction conditions on L-histidine decarboxylation activity of halophilic histamine-producing bacteria. Nippon Suisan Gakkaishi 59: 1745-1748.

Kvenberg. J.E (ed.). 1991. Microbiology of marine food products. AVI, New York, NY.

Lehane, L. and Olley, J. 2000. Histamine fish poisoning revised. Int. J. Food Microbiol. 58: 1-37.

Le Jeune, C., Lonvaud-Funel, A., Brink, B.T., Hofstra, H., and van der Vossen, J.M.B.M. 1995. Development of detection system for histidine decarboxylating lactic acid bacterial based on DNA probes, PCR and activity test. J. Appl. Bacteriol. 78: 316-326.

Lee, C.Y., Pan, S.F., and Chen, C.H. 1995. Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with PCR. Appl. Environ. Microbiol. 61: 1311-1317.

Lin, C.K. and Tsen, H.Y. 1999. Comparison of the partial 16S rRNA gene sequences and development of oligonucleotide probes for the detection of *Escherichia coli* cells in water and milk. Food Microbiology, 16: 551-562.

Lindberg, A.M., Ljungh, A., Ahrné, S., Löfdahl, S., and Molin, G. 1998. *Enterobacteriaceae* found in high numbers in fish, minced meat and pasteurized milk or cream and the presence of toxin encoding genes. Int. J. Food Microbiol. 39: 11-17.

Llano, D.G., Cuesta, P., and Rodríguez, A. 1998. Biogenic amine production by wild lactococcal and leuconostoc strains. Letters Appl. Microbiol. 26: 270-274.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Roig-Sagués, A.X., and Mora-Ventura, M.A.T. 1994. Bacteriological quality of tuna fish (*Thunnus thynnus*) destined for canning: Effect of tuna handling on presence of histidine decarboxylase bacteria and histamine level. J. Food Prot. 57: 318-323.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernádez-Herrero, M., and Mora-Ventura, M.A.T. 1994. Evaluation of histidine decarboxylase activity of bacteria isolated from sardine (*Sardina pilchardus*) by an enzymatic method. Lett. Appl. Microbiol. 19: 70-75.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., Roig-Sagués, A.X., and Mora-Ventura, M.T. 1996. Sensory quality and histamine forming during controlled decomposition of tuna (*Thunnus thynnus*). J. Food Prot. 59: 167-174.

López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., and Mora-Ventura, M.T. 1996. Incidence of histamine-forming bacteria and histamine content in scombroid fish species from retail markets in the Barcelona area. Int. J. Food Microbiol. 28: 411-418.

Maijara, R.L. 1993. Formation of histamine and tyramine by some lactic acid bacteria in MRS-broth and modified decarboxylation agar. Lett. Appl. Microbiol. 17: 40-43.

Matsuki, T., Watanabe, K., Tanaka, R., Oyaizu, H. 1998. Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. FEMS Microbiol. Lett. 167: 113-121.

Middlebrooks, B.L., Toom, P.M., Douglas, W.L., Harrison, R.E., and McDowell, S. 1988. Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus maculatus*). J. Food Sci. 53: 1024-1029.

Miriam, A., Griffiths, S.G., Lovely, J.E., and Lynch, W.H. 1997. PCR and probe-PCR assay to monitor broodstock Atlantic salmon (*Salmo salar* L.) ovarian fluid and kidney tissue for presence of DNA of the fish pathogen *Renibacterium salmoninarum*. J. Clin. Microbiol. 35: 1322-1326.

Morii, H., Cann, D.C., and Taylor, L.Y. 1988. Histamine formation by luminous bacteria in mackerel stored at low temperatures. Nippon Suisan Gakkaishi 54: 299-305.

Morrow, J.D., Margolies, G.R., Rowland, J., and Roberts, L.J. 1991. Evidence that histamine is the causative toxin of scombroid-fish poisoning. N. Engl. J. Med. 324: 716-720.

Niven, C.F., Jeffrey, M.B., and Corlett, D.A. 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. Appl. Environ. Microbiol. 41: 321-322.

Okuzumi, M., Hiraishi, A., Kobayashi, T., and Fujii, T. 1994. *Photobacterium histaminum* sp. nov., a histamine-producing marine bacterium. Int. J. Syst. Bacteriol. 44: 631-636.

Oliver, J.D. and Kaper, J.B. 1997. Ch. 13. *Vibrio* species. *In* Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, D.C.

Omura, Y., Price, R.J., and Olcott, H.S. 1978. Histamine-forming bacteria isolated from spoiled skipjack tuna and jack mackerel. J. Food Sci. 43: 1779-1781.

Pacheco-Aguilar, R., Lugo-Sánchez, M.E., and Robles-Burgueño, M.R. 2000. Postmortem biochemical and functional characteristic of Monterey sardine muscle stored at 0°C. J. Food Sci. 65: 40-47.

Pérez-Villarreal, B. and Pozo, R. 1990. Chemical composition and ice spoilage of albacore (*Thunnus alalunga*). J. Food Sci. 55: 678-682.

Pignato, S., Giammanco, G.M., Grimont, F., Grimont, P.A.D., and Giammanco, G. 1999. Molecular characterization of the Genera *Proteus*, *Morganella*, and *Providencia* by ribotyping. J. Clin. Microbiol. 37: 2840-2847.

Price, R.J., Melvin, E.F., and Bell, J.W. 1991. Postmortem changes in chilled round, bled and dressed albacore. J. Food Sci. 56: 318-321.

Price, R.J. and Melvin, E.F. 1994. Recommendations for on board handling of albacore tuna. Sea Grant Extension Program, Food Science and Technology Extension, University of California, Davis.

Rappé, M.S., Suzuki, M.T., Vergin, K.L., and Giovannoni, S.J. 1998. Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environment nucleic acid samples from the Pacific and Atlantic Coasts of the Unites States. Appl. Environ. Microbiol. 64: 294-303.

Rawles, D.D., Flick, G.J., and Martin, R.E. 1996. Biogenic amines in fish and shellfish. Adv. Food Nutr. Res. 39: 329-364.

Ridell, J. and Korkeala, H. 1997. Minimum growth temperatures of *Hafnia alvei* and other *Enterobacteriaceae* isolated from refrigerated meat determined with a temperature gradient incubator. Int. J. Food Microbiol. 35: 287-292.

Rodríguez-Jerez, J.J., López-Sabater, E.I., Hernández-Herrero, M.M., and Mora-Ventura, M.T. 1994. Histamine, putrescine and cadaverine formation in Spanish semipreserved anchovies as affected by time/temperature. J. Food Sci. 59: 993-997.

Rodríguez-Jerez, J.J., Mora-Ventura, M.T., López-Sabater, E.I., and Hernández-Herrero, M. 1994. Histidine, lysine, and orinithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. J. Food Prot. 57: 784-787, 791.

Roig-Sagués, A.X., Hernández-Herrero, M., López-Sabater, E.I., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1996. Histidine decarboxylase activity of bacteria isolated from raw and ripened *Salchichón*, a Spanish cured sausage. J. Food Prot. 59: 516-520.

Roig-Sagués, A.X., Hernández-Herrero, M., López-Sabater, E.I., Rodríguez-Jerez, J.J., and Mora-Ventura, M.T. 1997. Evaluation of three decarboxylating agar media to detect histamine and tyramine-producing bacteria in ripened sausages. Lett. Appl. Microbiol. 25: 309-312.

Russell, F.E. and Maretic, Z. 1986. Scombroid poisoning: Mini-review with case histories. Toxicon. 24: 967-973.

Ryder, J.M., Buisson, D.H., Scott, D.N., and Fletcher, G.C. 1984. Storage of New Zealand jack mackerel (*Trachurus novaezelandiae*) in ice: Chemical, microbiological and sensory assessment. J. Food Sci. 49: 1453-1456, 1477.

Ryser, E.T., Marth, E.H., and Taylor, S.L. 1984. Histamine production by psychrotrophic pseudomonads isolated from tuna fish. J. Food Prot. 47: 378-380.

Sabat, G., Rose, P., Hickey, W.J., and Harkin, J.M. 2000. Selective and sensitive methods for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. Appl. Environ. Microbiol. 66: 844-849.

Saiton, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Shelef, L.A., Surtani, A., Kanagapandian, K., and Tan, W. 1998. Automated detection of amino acid decarboxylation in salmonellae and other *Enterobacteriaceae*. Food Microbiol. 15: 199-205.

Stackebrandt, E. and Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequences analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846-849.

Stratton, J.E. and Taylor, S.L. 1991. Scombroid poisoning. p. 331-351. *In* Microbiology of Marine Food Products. Van Nostrand Reinhold, N.Y.

Stratton, J.E., Hutkins, R.W., and Taylor, S.L. 1991. Biogenic amines in cheese and other fermented foods: a review. J. Food Prot. 54: 460-470.

Sumner, S. and Taylor, S.L. 1989. Detection method for histamine-producing, dairy-related bacteria using diamine oxidase and leucocrystal violet. J. Food Prot. 52: 105-108.

Suzuki, S., Matsui, Y., and Takama, K. 1988. Profiles of polyamine composition in putrefactive Pseudomonas type III/IV. Microbios. Lett. 38: 105-109.

Taylor, S.L. 1978. Histamine production by food-borne bacterial species. J. Food Safety. 1: 173-187.

Tanase, S., Guirard, B.M., and Snell, E.E. 1985. Purification and properties of a pyridoxal 5'-phosphate-dependent histidine decarboxylase from *Morganella morganii* AM-15. J. Biol. Chem. 260: 6738-6746.

Taylor, S.L. and Lieber, E.R. 1979. *In vivo* inhibition of rat intestinal histamine-matabolizing enzymes. Food Cosmet. Toxicol. 17: 237-240.

Taylor, S.L. and Speckhard, M.W. 1983. Isolation of histamine-producing bacteria from frozen tuna. Mar. Fish. Rev. 45: 35-39.

Taylor, S.L. 1986. Histamine food poisoning: toxicology and clinical aspects. CDC. 17: 91-128.

Taylor, S.L., Stratton, J.E., and Nordlee, J.A. 1989. Histamine poisoning (Scombroid fish poisoning): An allergy-like intoxication. Clin. Toxicol. 27: 225-240.

Tolmasky, M.E., Actis, L.A., and Crosa, J.H. 1995. A histidine decarboxylase gene encoded by the *Vibrio anguillarum* plasmid Pjm1 is essential for virulence: histamine is a precursor in the biosynthesis of anguibactin. Mol. Microbiol. 15: 87-95.

Tsen, H.Y., Lin, C.K., and Chi, W.R. 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. J. Appl. Microbiol. 85: 554-560.

Vaaler, G.L., Brasch, M.A., and Snell, E.E. 1986. Pyridoxal 5'-phosphate-dependent histidine decarboxylase. J. Biol. Chem. 261: 11010-11014.

Van Poelje, P.D. and Snell, E.E. 1990. Pyruvoyl-dependent enzymes. Annu. Rev. Biochem. 59: 29-59.

Veciana-Nogués, M.T., Vidal-Carou, M.C., Mariné-Font, A. 1989. Histamine and tyramine in preserved and semi-preserved fish products. J. Food Sci. 54: 1653-1655.

Velázquez, L.C., Escudero, M.E., and Guzmán, A.M.S. 2001. Antibacterial effects of different food-related phosphates using *Aeromonas hydrophila*. J. Food Prot. 64: 195-200.

Venugopan, V. and Shahidi, F. 1994. Thermostable water dispersions of Myofibrillar proteins from Atlantic mackerel (*Scomber scombus*). J. Food Sci. 59: 265-268, 276.

Wang, C. and Silva, J.L. 1999. Prevalence and characteristics of *Aeromonas* species isolated from processed channel catfish. J. Food Prot. 62: 30-34.

Wei, C.I., Chen, C.M., Koburger, J.A., Otwell, W.S., and Marshall, M.R. 1990. Bacterial growth and histamine production on vacuum packaged tuna. J. Food Sci. 55: 59-63.

Wendakoon, C.N., Murata, M., and Sakaguchi, M. 1990. Comparison of non-volatile amine formation between the dark and white muscle during storage. Nippon Suisan Gakkaishi. 56: 809-818.

Wendakoon, C.N. and Sakaguchi, M. 1993. Combined effect of sodium chloride and clove on growth and biogenic amine formation of *Enterobacter aerogenes* in mackerel muscle extract. J. Food Prot. 56: 410-413.

Widjojoatmodjo, M.N., Fluit, A.D., and Verhoef, J. 1995. Molecular identification of bacteria by fluorescence-based PCR-single strand conformation polymorphism analysis of the rRNA gene. J. Clin. Microbiol. 33: 2601-2606.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63: 3741-3751.

Wolcott, M.J. 1991. DNA-based rapid methods for the detection of foodborne pathogens. J. Food Prot. 54: 387-401.

Yatsunami, K. and Echigo, T. 1993. Changes in the number of halotolerant histamine-forming bacteria and contents of non-volatile amines in sardine meat with addition of NaCl. Nippon Suisan Gakkashi 59: 123-127.

Yoshinaga, D.H. and Frank, H.A. 1982. Histamine-producing bacteria in decomposing skipjack tuna (*Katsuwonus pelamis*). Appl. Environ. Microbiol. 44: 447-452.