

Detection and Identification of Histamine-Producing Bacteria Associated with Harvesting and Processing Mahimahi and Yellowfin Tuna^{†‡}

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ABSTRACT

Histamine poisoning is one of the most common chemically induced seafoodborne illnesses reported in the United States today. The causative agents are biogenic amines, commonly produced by gram-negative bacteria. The purpose of this study was to detect and identify histamine-producing bacteria associated with standard industry practices during the harvesting, receiving, and processing of mahimahi and yellowfin tuna in North Carolina. Twenty-nine composite samples were obtained from 18 mahimahi and 11 yellowfin tuna and analyzed for their histamine content. No sample analyzed exceeded 2 ppm histamine, the lower detection limit. Composite fish muscle and environmental samples were screened ($n = 386$) for the presence of histamine-producing bacteria. Twenty-six percent (145) of 549 isolates selected on the basis of their morphological characteristics tested positive on Niven's media. Sixty-three Niven-positive isolates were gram negative, and 58 were gram positive. Of the 43 isolates tested further, 5 were confirmed as histamine producers, and all 5 produced at low levels (<250 ppm in 48 h at >15°C). Three gram-negative and two gram-positive isolates were identified as *Enterobacter cloacae* and *Staphylococcus kloosii*, respectively. This study revealed that gram-negative bacteria might not be solely responsible for histamine production in at-risk fish. The confirmation of histamine-producing bacteria demonstrates the potential risk for histamine production. However, no detectable levels were found in the composite fish muscle samples analyzed even though 60% of the yellowfin tuna harvested did not meet the U.S. Food and Drug Administration's regulatory hazard analysis critical control point guidelines for temperature reduction. Therefore, no seafood safety risks were found under the standard industry practices observed in this study.

Histamine poisoning is one of the most common chemically induced seafoodborne illnesses reported in the United States today (18). The U.S. Food and Drug Administration (FDA) promulgated industry guidelines in 1994 to establish procedures for the safe processing and importing of fish and fishery products on the basis of the hazard analysis critical control point (HACCP) approach (23). The most recent HACCP guidelines for control of histamine production recommend specific time and temperature limits for potentially hazardous fish on the basis of species, size, and water temperature at harvest (24). The FDA recommends that fish be placed in a cooling medium or be cooled to a specific temperature within a prescribed period of time. Primary processors bear the burden of proof that proper cooling techniques have been used from harvest to receipt of fish and are expected to implement the necessary cooling guidelines that are intended to achieve a core temperature of 4.4°C or

less and maintain this temperature throughout handling, processing, and distribution.

Histamine is a naturally occurring compound found in humans that serves as a cell messenger for regulating vascular and bronchial diameter as well as other normal bodily functions (5). It is chemically produced by decarboxylation of the amino acid histidine. Histamine is produced by one of two types of decarboxylase enzymes, a pyridoxal phosphate-dependent enzyme found in animals as well as gram-negative bacteria and a pyruvoyl-dependant enzyme found in gram-positive bacteria (11). Human illness can occur when a high concentration of histamine is ingested, which is associated with potentially hazardous foods such as meat products, dairy products, wine, beer, vegetables, fruits, nuts, chocolate, and most notably, certain fish, both scombroid (i.e., tunas and mackerels) and nonscombroid (i.e., bluefish) (5, 14, 21). The production of histamine in potentially hazardous fish is generally believed to result from the growth of gram-negative, rod-shaped bacteria capable of producing pyridoxal phosphate-dependent decarboxylase enzymes after time and temperature abuse (7).

The primary concern of the FDA is proper handling and chilling of potentially hazardous fish to reduce growth of spoilage bacteria capable of supporting histamine production. Although the FDA has jurisdiction over primary

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processors, the agency does not have regulatory authority over commercial harvesters other than exerting leverage by controlling receipt of raw material as a required part of a HACCP plan. This presents a challenge for both regulatory and industry members alike who are concerned with product safety and regulatory compliance. The purpose of this study was to detect and identify histamine-producing bacteria associated with standard industry practices during the harvesting, receiving, and processing of mahimahi and yellowfin tuna in North Carolina.

MATERIALS AND METHODS

Fish sampling and harvest conditions. Mahimahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacares*) were harvested aboard North Carolina commercially licensed fishing vessels by hook and line techniques. Five and three 1-day trips were made for collecting mahimahi and yellowfin tuna, respectively. A maximum of four fish were collected on each occasion to observe variations in size, harvest conditions, handling techniques, and icing methods. Mahimahi were sampled during summer 2002 and spring 2003. Yellowfin tuna were sampled in spring and summer 2003 and spring 2004. Chilling rates for each fish were observed with Datatrace (Mesa Laboratories, Lakewood, Colo.) factory-calibrated needle probes inserted into the fish at the dorsal fin area. Probes were inserted just behind the head and slightly anterior to the dorsal fin at depths of 4 to 6 in. Internal fish temperatures were recorded at 5-min intervals from point of harvest to time of processing at a commercial manufacturing establishment. Before processing, fish weight, fork length (from head to tip of tail), and girth were recorded.

Environmental sampling. Environmental samples from fish and fish contact surfaces were obtained onboard the vessel, at receiving on the dock, and at the primary processing facility. Samples were obtained by swabbing 10-cm² areas with sterile calcium alginate-tipped applicators (Hardwood Products Company LLC, Guilford, Maine) and placing them into sterile tubes with 10 ml of sterile neutralizing buffer. Approximately 24 samples were obtained on the vessel, 10 at receiving, and 24 at the processing facility. All environmental samples were placed immediately on ice after collection for transport to the North Carolina State University Seafood Laboratory at Morehead City. Microbiological analyses were initiated within 48 h of sample collection.

Tissue sampling and preparation. Sampling of fish muscle was done at the time of primary processing. Approximately 50 g of muscle tissue were obtained from each of three locations (head, belly, and tail) and placed individually in sterile Whirl pack bags (Seward Medical, London, UK). A total of 12 tissue samples (three per fish) were collected each sampling date, placed immediately on ice, and prepared for analyses within 12 h of collection in the Seafood Laboratory. A composite tissue sample was prepared from each fish by aseptically subsampling 10 g from each location, diluting 1 to 10 (wt/vol) with sterilized water because water is used in the extraction step of the Veratox histamine assay (Neogen Corp., Lansing, Mich.), and stomaching (Laboratory Blender Stomacher 400, Seward Medical) at 230 rpm for three cycles of 1 min each with 30-s intervals between each cycle. Immediately after blending, three 100- μ l samples were obtained with a micropipetter (Oxford Benchmate, St. Louis, Mo.) fitted with a wide-bore tip for use in microbiological analyses.

Histamine determination. Blended composite samples were placed under refrigeration for ca. 15 min to allow the suspensions

to settle. Approximately 5 ml of supernatant was collected and filtered into polyethylene tubes with a glass wool syringe in accordance with instructions in the Neogen Veratox histamine test kit. The filtered samples were processed along with a standard curve, as recommended in the ELISA-based Veratox histamine assay. Samples were read with the use of the Stat Fax (Awareness Technologies Inc., Palm City, Fla.) microtiter well reader.

Microbiological analyses. Fish composite tissue and environmental samples were serially diluted in sterilized normal saline solution, and 100- μ l aliquots were spread plated in duplicate on tryptic soy agar (Beckon Dickinson, Sparks, Md.) supplemented with 2% sodium chloride (TSAN₂; Fisher Scientific, Fair Lawn, N.J.) and incubated at 37°C for 48 h. Representative isolates were selected from TSAN₂ plates on the basis of their morphological differences. Between 1 and 15 isolates were selected from each TSAN₂ plate for each sample location and time of collection. Isolates were assigned four-digit identification numbers according to fish type, trip, location, and time. Isolates were purified by sequential streaking on TSAN₂ plates and incubation at 37°C for 48 h.

The pure cultures were transferred to TSAN₂ slants and incubated at 37°C for 24 h. A sample of each isolate taken from each slant was plated on Niven's agar and incubated at 37°C for 48 h to screen for histamine production (17). Niven-positive cultures were maintained on TSAN₂ slants at 8 to 10°C and were frozen at -20°C in suspension (vol/vol) with tryptic soy broth (2 \times) supplemented with 2% sodium chloride and 40 to 50% glycerol. Niven-positive isolates were Gram stained and examined under oil immersion on a compound microscope (\times 1,000, Eclipse E600, Nikon, Melville, N.Y.). Gram-negative rods were further identified with the API 20E Enterobacteriaceae (bioMérieux Vitek, Inc., Hazelwood, Mo.) and the enteric and nonfermenter Becton Dickinson BBL crystal identification tests. Gram-positive isolates were identified by the gram-positive Becton Dickinson BBL crystal identification test. All confirmed histamine-producing isolates were analyzed by Dianne Bourne at Virginia Polytechnic Institute and State University by a cellular fatty acid identification method as described in Ghanem et al. (10) and Moore et al. (16).

Histamine confirmation. Definitively identified gram-negative and gram-positive isolates were streaked in triplicate on TSAN₂ plates supplemented with 0.1% histidine (histidine monohydrochloride monohydrate, Avocado Research Chemicals Ltd., Heysham, UK) (TSAN₂ plus histidine) and incubated at 37°C for 18 to 24 h. A representative colony from each TSAN₂ plus histidine plate was inoculated into 9 ml of tryptic soy broth supplemented with 2% NaCl, 2% histidine (histidine monohydrochloride monohydrate), and 0.0005% pyridoxal-HCl (pH 5.8) (TSB+) and incubated at 37°C for 24 h. A 1-ml sample of each TSB+ suspension was transferred into a tube of fresh TSB+ media and incubated at 37°C for 48 h. A 3-ml subsample of this final culture was transferred into polypropylene centrifuge tubes and centrifuged at 1,170 \times g for 20 min. Supernatants were diluted 1 to 10 in water, and histamine concentrations were determined with the Neogen Veratox histamine test kit.

Histamine production rates. Positively identified histamine-producing isolates were further characterized for their rate of histamine production at 25 and 37°C. A known histamine producer, *Raoultella planticola* ATCC 43176, was used as a positive control. Histamine-producing isolates and the control were streaked onto TSAN₂ plus histidine plates and incubated at 37°C for 18 to 24 h. A single colony from each TSAN₂ plus histidine plate was

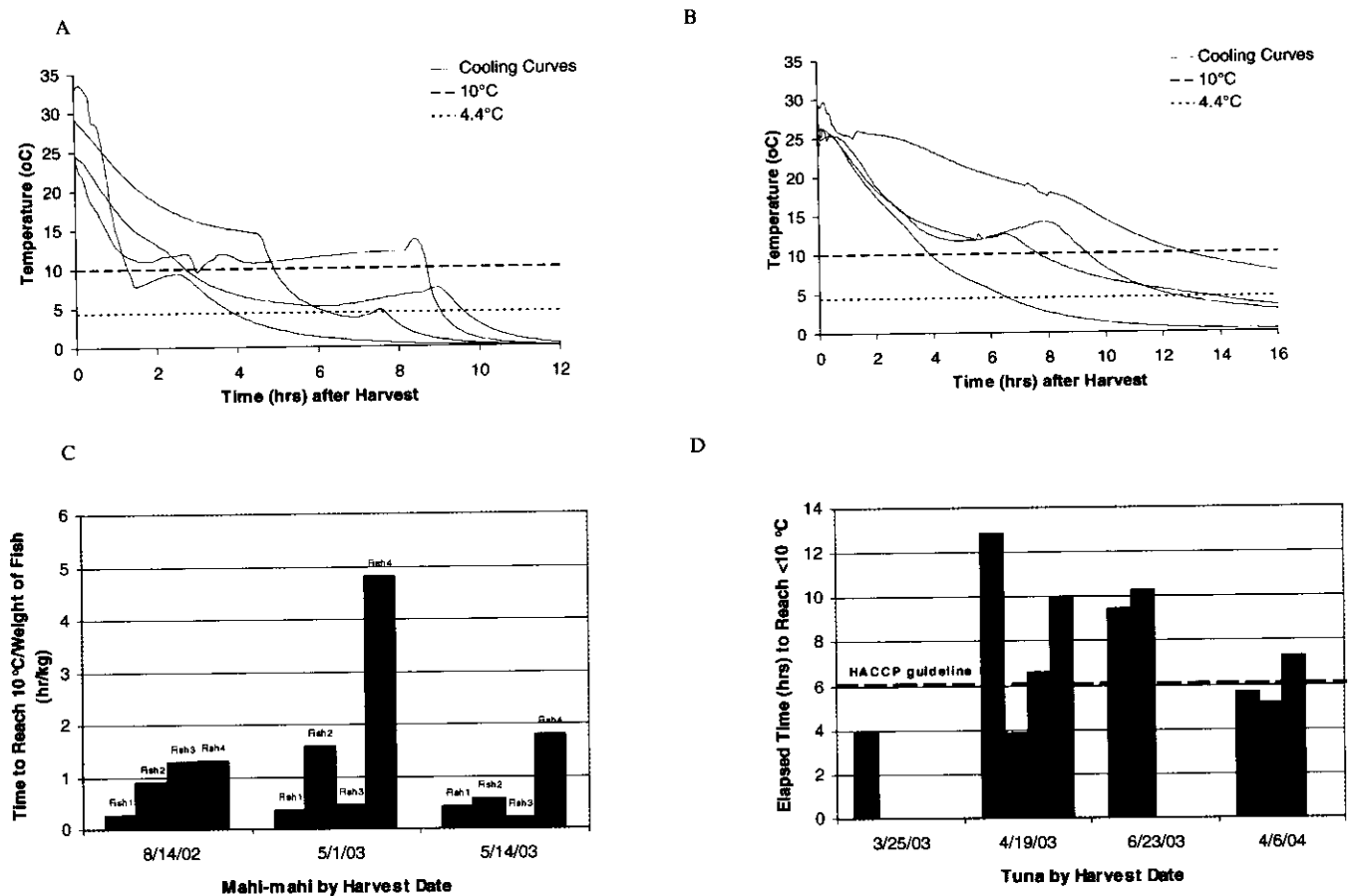


FIGURE 1. Cooling of fish. (A) Representative cooling rate curves of four mahimahi harvested by North Carolina vessels. (B) Representative cooling rate curves of four yellowfin tuna harvested by North Carolina vessels. (C) Time required for mahimahi to reach 10°C from point of harvest. (D) Time required for yellowfin tuna to reach 10°C from point of harvest.

inoculated into 9 ml of TSB+ and incubated at 37°C for 24 h. A negative control (blank 9-ml tube of TSB+) was incubated in triplicate at 37°C for 24 h. Representative 1-ml samples from each TSB+ culture tube were transferred into 9 ml of fresh TSB+ media and incubated at 25 and 37°C for 48 h. Representative 3-ml samples incubated at 25 and 37°C for 24 and 48 h were transferred into polypropylene centrifuge tubes and centrifuged at $1,170 \times g$ for 20 min. Supernatants were diluted 1 to 10 in water, and histamine concentrations were determined with the Neogen Veratox histamine test kit.

RESULTS AND DISCUSSION

None of the 29 composite fish muscle samples analyzed exceeded 2 ppm histamine, the detection threshold for Neogen's ELISA-based Veratox rapid test. This low level of histamine was not unexpected. Fernandez-Salguero and Mackie (8) found similar results in haddock (*Melanogrammus aeglefinus*) and herring (*Clupea harengus*) held for short periods of time on ice. However they found moderate levels of histamine in fish held on ice for a long period of time (≥ 8 days). Ababouch et al. (1) found high levels of histamine in sardines (*Sardina pilchardus*) within a few hours when gross temperature abuse of fish occurred ($\geq 25^\circ\text{C}$). The FDA identified growth of histamine-producing bacteria as the primary cause of histamine production in scombroid fish (24). Factors affecting growth of histamine-producing bacteria include the type and size of fish,

handling techniques, and cooling methods. Our study focused on standard industry practices in harvesting mahimahi and yellowfin tuna, both predominantly hook and line fisheries in North Carolina. There was a relatively short period of time (≤ 36 h) between harvest (death) and primary processing for all fish evaluated in this study. Fish were placed on ice within 30 min of capture and temperature controls (re-icing at ≤ 30 min) were observed at receiving and in handling at the processing facility. The elapsed time between harvest and quantification of histamine in blended fish tissue samples was therefore ≤ 48 h.

During the study, all mahimahi were placed on ice within the HACCP guideline of < 12 h. The majority of yellowfin tuna (60%), however, did not reach the FDA's recommended temperature of $\leq 10^\circ\text{C}$ within 6 h of harvest. Generally, mahimahi cooled faster to $\leq 10^\circ\text{C}$ ($\bar{x} = 2.5$ h, $n = 18$) than yellowfin tuna ($\bar{x} = 7.5$ h, $n = 10$), perhaps because of the relatively smaller size of mahimahi ($\bar{x} = 3.4$ kg, $n = 12$) compared with yellowfin tuna ($\bar{x} = 14.2$ kg, $n = 8$). Another factor could be an insufficient amount of ice used to chill the fish or the inability to chill large fish by the cooling method employed. Craven et al. (6) found that large albacore tuna (9.1 kg) required more time (~ 2 h) to cool to $\leq 10^\circ\text{C}$ than smaller fish (6.8 kg) when placed in seawater and ice slurry conditions. Figures 1A and 1B show representative samples of cooling rates observed for

TABLE 1. A summary of five histamine-producing isolates by type, location, and organism^a

Isolate ID no.	Fish species	Sample time	Sample type	Sample location	Organism (API)	Organism (crystal)	Organism (CFAP)
4077	Mahimahi	Postprocessing	Fish surface	Fish	<i>Citrobacter freundii</i>	<i>C. freundii</i>	<i>Enterobacter cloacae</i>
4083	Mahimahi	Postprocessing	Fish surface	Fish	<i>C. freundii</i>	<i>C. freundii</i>	<i>E. cloacae</i>
4086	Mahimahi	Postprocessing	Environment	Knife	<i>C. freundii</i>	<i>C. freundii</i>	<i>E. cloacae</i>
2015B	Mahimahi	Preharvest	Environment	Fish basket	NA	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus kloosii</i>
5059	Yellowfin tuna	Receiving	Environment	Packing crate	NA	<i>Aerococcus urinae</i>	<i>S. kloosii</i>

^a API, bioMérieux Inc. API 20E Enterobacteriaceae identification system; crystal, Becton Dickinson BBL crystal identification system; CFAP, cellular fatty acid profile identification method.

mahimahi and yellowfin tuna, respectively. Figure 1C shows variations in temperatures of mahimahi harvested on the same day, adjusted for weight of fish. The data reveal that weight alone could not account for the variation in chilling. Other factors might include fish composition and shape, cooling medium, and method of chilling. All vessels observed in this study used ice to chill their catch, with most vessels using less ice than the amount of fish harvested (wt/wt).

The FDA encourages gutting of large tuna ≥ 20 lb (9.1 kg) or requires that strict time and temperature controls (internal temperature of $\leq 10^{\circ}\text{C}$ within 6 h of death) be met if gutting is not performed onboard the vessel. In this study, no vessel harvesting tuna was observed gutting their catch. Under these circumstances, large tuna are expected to be chilled rapidly to achieve the recommended internal temperature of $\leq 10^{\circ}\text{C}$ within 6 h. This recommendation might be difficult to achieve for very large fish because of their mass and shape characteristics. The majority of yellowfin tuna (60%) harvested in this study did not reach the FDA's recommended temperature of $\leq 10^{\circ}\text{C}$ within 6 h (Fig. 1D). After receipt at the processing facility, handling practices were adequate to maintain or further decrease the fish internal temperatures to $\leq 4.4^{\circ}\text{C}$. All fish were held overnight on ice and processed the next day. In general, mahimahi fillets and tuna loins were cut by hand at the fish house and placed back on ice within 30 min of processing, allowing little time for meat temperature to rise and therefore limiting the opportunity for histamine-producing bacteria to propagate.

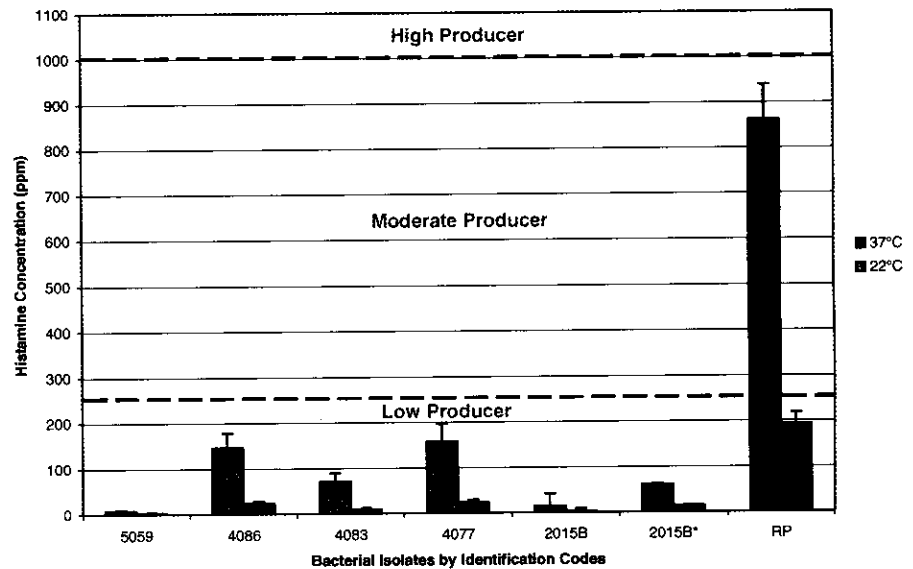
Histamine-producing bacteria. A total of 386 composite fish muscle and environmental samples were screened for the presence of histamine-producing bacteria. A total of 549 unique isolates were selected on the basis of colony morphology, 145 of which were positive on Niven's media. Eighty Niven-positive isolates were associated with mahimahi, and 65 were associated with yellowfin tuna. Sixty-three Niven-positive isolates were identified as gram-negative rods, and 58 were identified as gram-positive rods or cocci. The remaining 24 Niven-positive isolates were gram-variable and not identified. Neither the API 20E test nor BBL crystal methods were able to identify all Niven-positive isolates. The API 20E rapid biochemical test de-

terminatively identified 21 of the gram-negative isolates, whereas the Becton Dickinson BBL crystal method definitively identified 19 gram-negative isolates. In addition, the BBL crystal method identified 44 of the gram-positive isolates. A total of 60 isolates were identified at the $>90\%$ confidence level. Of these, only 43 were viable after freezing in tryptic soy broth (2 \times) supplemented with 2% sodium chloride and 40 to 50% glycerol and hence could be used in subsequent histamine production assays.

Five of the 43 viable isolates were confirmed as histamine producers. Specifically, three were identified as *Citrobacter freundii* by the API and BBL crystal tests, and one each was identified as *Staphylococcus epidermidis* and *Aerococcus urinae* by the BBL crystal test (Table 1). The five histamine-producing isolates were subjected to cellular fatty acid profile (CFAP) identification to confirm their identities (4). The CFAP technique identified the three gram-negative isolates as *Enterobacter cloacae*, which are closely related to *C. freundii*. The CFAP technique identified both gram-positive isolates as *Staphylococcus kloosii*. The CFAP identification might be considered more appropriate for use in environmental testing compared with the API 20E and BBL crystal tests, which were developed for clinical use.

Many researchers have detected and identified histamine-producing bacteria. Gram-negative bacteria are generally thought to be the primary cause of histamine development in scombroid fish (5). Tsai et al. (22) isolated and identified 14 different histamine-producing gram-negative bacteria of the genera *Proteus*, *Enterobacter*, *Aninetobacter*, *Klebsiella*, and *Rahnella*. Frank et al. (9) identified *Morganella morganii*, *Proteus mirabilis*, and *Vibrio alginolyticus* from among 27 different histamine-producing bacteria that they isolated. Omura et al. (19) also isolated histamine-producing *M. morganii*, *Proteus* spp., and *Klebsiella* spp., as well as *Hafnia alvei*, from skipjack tuna and jack mackerel. Other researchers have identified *Citrobacter* spp. as histamine producers (5). However, the CFAP findings in this study might bring into question results from previous studies identifying *Citrobacter* spp. as histamine producers. Kanki et al. (12) recently discovered that several histamine-producing cultures believed to be *Klebsiella pneumoniae* were incorrectly identified strains of *Raoultella planticola*.

FIGURE 2. Histamine production rate of identified bacterial isolates and *Raoultella planticola* incubated at 37°C and 22°C for 48 h.



Histamine confirmation. The 43 viable Niven-positive isolates were tested to verify their ability to produce histamine. Only five (12%) were confirmed as histamine producers. This low percentage is consistent with other studies of Niven-positive isolates in which histamine confirmation by quantification methods confirmed that only 15 to 37% of isolates were actually histamine producers (13, 15, 22). Kim et al. (13) identified a histamine-producing strain of *M. morgani* that gave a false negative when cultured on Niven's media. The potential for false negatives reveals a weakness in the use of Niven's media in the screening of environmental samples for the presence of histamine-producing bacteria. The Niven's method is based on a pH shift in the media that causes a color change from a brown-green to purple (17). It is known that many metabolic processes of microorganisms can result in pH changes in culture media that can lead to false positive results for production of histamine when using Niven's media.

Histamine production rates. The five positively identified histamine-producing isolates were further characterized for their rate of histamine production at 25 and 37°C (Fig. 2). None of the five bacterial isolates identified were high histamine producers, defined as producing >1,000 ppm histamine in 24 h at >15°C (2, 22). *Raoultella planticola* (ATCC 43176), the positive control strain, was a moderate histamine producer (250 to 1,000 ppm in 48 h at $\geq 15^\circ\text{C}$) at the conditions studied. In contrast to our results, Kanki et al. (12) reported that *Raoultella planticola* (ATCC 43176) produced histamine levels of 4,550 ppm with a media volume of 2 ml and an incubation of 18 h at 30°C. Bacteria isolated by Tsai et al. (22) and Ababouch et al. (2) had histamine production rates between 1,000 and 4,000 ppm in 24 h at 37°C. Frank et al. (9) reported that mesophilic histamine-producing bacteria could achieve concentrations of $\geq 10,000$ ppm in 24 h at 32°C. Our five bacterial isolates were characterized as low histamine producers, defined as production of <100 ppm histamine in 24 h at $\geq 15^\circ\text{C}$ or <250 ppm histamine in 48 h at $\geq 15^\circ\text{C}$ (2, 22).

However, isolates 4077 and 4086 did produce >100 ppm histamine at 37°C, but in 48 h rather than 24 h. Tsai et al. (22) reported gram-negative rods that produced only 100 ppm histamine in 24 h at 37°C, and Ababouch et al. (2) found similar levels produced at 35°C. *Streptococcus cremoris* was reported by Babu et al. (3) to produce only 43 ppm histamine in 24 h at 30°C, a concentration lower than any of the gram-negative isolates identified in this study. The majority of bacterial isolates reported by Frank et al. (9) produced histamine concentrations between 10 to 50 ppm histamine, with about a third producing 10,000 ppm or more histamine in 24 h at 32°C.

Few gram-positive bacteria have been studied in association with histamine production in fish. A strain of *Lactobacillus buchneri* was found in Swiss cheese to produce histamine at a rate of 420 ppm in 24 h at 37°C (21). This concentration is more than the histamine levels produced by *S. epidermidis* and *A. urinae* identified in this study. However, the concentration is close to the average concentration found with *S. epidermidis*, if replicates in which no histamine production was found are excluded. Our strain of *S. epidermidis* did not consistently produce histamine, even when conducting multiple replicate assays from the same culture. To ensure that our culture was not mixed, samples were streaked and replated on TSAN₂ several times, and the BBL crystal identification test was performed on both positive and negative histamine-producing cultures. All of the isolates tested were pure cultures and identified by BBL crystal as *S. epidermidis*. The bacterial cultures exhibiting histamine production were retested for histamine production with similar mixed positive and negative results. One explanation for this observation could be a genetic variation within the strain, in which the gene coding for the enzyme responsible for histamine production is lost or its expression is somehow suppressed. This inconsistency in producing histamine could partially explain why gram-positive histamine-producing bacteria have not been studied as much.

There are several possible reasons why bacterial iso-

lates identified in this study were not prolific histamine producers. One reason is that other studies used strains previously confirmed as high histamine producers as models to study growth and histamine production characteristics (1, 3, 13, 21). Others have isolated histamine-producing bacterial strains from thermally or temporally abused fish rather than those strains obtained under near-ideal handling conditions, as observed in our study. Kim et al. (13) and Ababouch et al. (1) studied bacterial isolates from temperature-abused fish that could allow time for a greater number and more prolific histamine-producing bacteria to grow. Another possible reason for low histamine production could be the specific growth media used. We incorporated 2% sodium chloride in the culture media to facilitate the growth and isolation of *Vibrio* sp. that are known to produce histamine. However, Babu et al. (3) found that addition of salt reduced the production of histamine in a strain of *S. cremoris*.

HACCP implications. The FDA's defect action level for histamine in fish is 50 ppm. This study found less than 2 ppm histamine in the 29 composite fish muscle samples analyzed, even though 60% of the yellowfin tuna harvested exceeded the FDA's HACCP temperature guidelines. The results were not unexpected, especially when considering that histamine analysis was performed within 48 h of harvest (death). The isolation of histamine-producing bacteria, however, is a concern for the FDA when mild temperature abuse could predispose fish to greater risk of histamine development by selecting for growth of histamine-producing bacteria (20). Previous studies by a number of authors have shown rapid production of histamine under mild to high temperatures and moderate to long exposure times (1, 8). In addition, it is generally accepted that gram-negative bacteria are the sole cause of histamine production in scombroid fish. This study found two gram-positive histamine-producing isolates on fish contact surfaces where the potential to contaminate product could occur. Gram-negative histamine-producing isolates were obtained from fish surfaces and from a knife used to cut fish (Table 1). The confirmation of histamine-producing bacteria demonstrates the potential risk for contamination of fish with these bacteria. However in this study, which observed standard industry practices, no detectable histamine was found; therefore, no seafood safety risk was demonstrated.

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