Isolation of Histamine-Producing Bacteria From Frozen Tuna

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Introduction

The bacterial spoilage of tuna and certain other scombroid and non-scombroid fishes is sometimes accompanied by the formation of high levels of histamine in the edible tissues of the fish (Hillig, 1956; Tomiyasu and Zenitani, 1957; Arnold and Brown, 1978; Frank et al., 1981). The formation of histamine is particularly noteworthy because the presence of high levels of histamine in spoiled fish has been associated with outbreaks of food poisoning also known as scombroid fish poisoning (Merson et al., 1974; Arnold and Brown, 1978; Lerke et al., 1978; Kim, 1979). Fresh scombroid fish possess virtually no histamine (Hardy and Smith, 1976; Frank et al., 1981). However, the scombroid fish have high levels of free histidine in their muscle tissues (Lukton and Olcott, 1958). Histamine is generated from histidine during spoilage by bacteria that possess the requisite enzyme, histidine decarboxylase (Arnold and Brown, 1978; Omura et al., 1978; Yoshinaga and Frank, 1982).

Many different bacterial species are known to possess histidine decarboxylase (Arnold and Brown, 1978; Taylor et al., 1978; Taylor et al., 1979; Yoshinaga and Frank, 1982). However, only *Proteus morganii* (Kawabata et al., 1956; Sakabe, 1973), *Klebsiella pneumoniae* (Taylor et al., 1979), and *Hafnia alvei* (Havelka, 1967) have been isolated from fish incriminated in scombroid fish poisoning incidents. In some species, i.e., *K. pneumoniae*, the ability to produce histamine is limited to only certain strains (Taylor et al., 1979). Behling and Taylor (1982) indicated that the histamine-producing bacteria could be divided into two categories: Those species capable of producing large quantities of histamine (>100 mg/100 ml) in tuna fish infusion broth (TFIB) during a short incubation period (<24 hours) at temperatures above 15 °C and those species capable of producing somewhat lesser quantities of histamine (>25 mg/100 ml) in TFIB after prolonged incubation (≥48 hours) at temperatures of 30 °C or above. *P. morganii*, *K. pneumoniae*, and *E. aerogenes* appear to belong to the category of prolific histamine producers, while the tested strains of *H. alvei*, *Citrobacter freundii*, and *Escherichia coli* were slow producers of histamine (Taylor et al., 1978; Behling and Taylor, 1982). Certainly, other bacteria may also be prolific histamine producers. The recent isolation of *Clostridium perfringens* from decomposing skipjack tuna and its identification as a prolific histamine producer (Yoshinaga and Frank, 1982) would seem to underscore this point.

Histamine-producing bacteria have been isolated from the skin, gills, intestines, and muscle tissues of spoiling fish (Kimata, 1961; Lerke et al., 1978; Omura et al., 1978; Yoshinaga and Frank, 1982), and are often considered part of the normal microflora of fish (Yoshinaga and Frank, 1982). However, the circumstances under which tuna would acquire bacteria such as *P. morganii*, *K. pneumoniae*, and *C. perfringens* which are not part of the normal microflora of seawater has never been adequately explained.

The microflora of fish is often a reflection of the microflora of their aquatic environment (Shewan and Hobbs, 1967). *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Vibrio*, *Micrococcus*, *Bacillus*, and coryneforms comprise the typical microflora of freshly caught marine fish (Shewan and Hobbs, 1967; Shewan, 1971; Sera et al., 1974), although the microflora of freshly caught tuna or other scombroid fish has not been studied. *Enterobacteriaceae* such as *P. morganii* and *K. pneumoniae* are rarely if ever found on freshly caught marine fish (Shewan and Hobbs, 1967). *C. perfringens* has been found in marine sediments from Puget Sound (Matches et al., 1974) and from fish on several occasions (Matches et al., 1974; Yoshinaga and Frank, 1982). However, the possibility remains that histamine-producing bacteria may not be part of the normal microflora of scombroid fish but may instead represent post-catching contaminants. *P.*

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ABSTRACT—A method was developed for the isolation of histamine-producing bacteria from frozen tuna. The method was equally effective for the recovery of Proteus morganii, Klebsiella pneumoniae, *and* Enterobacter aerogenes, *and was effective in recovering these bacteria when fewer than* 10^3 *organisms/g were present in the frozen tuna. The key feature of the isolation method was a 24-hour incubation in trypticase-soy broth which allowed recovery of freeze-injured bacteria.*

Histamine-producing bacteria were isolated from three of ten frozen tuna by use of this isolation technique. Gills, intestines, and muscle tissues were surveyed. A P. morganii *strain capable of producing 535 mg of histamine/100 ml within 24 hours at 37°C in tuna fish infusion broth (TFIB) was isolated from one sample of gills. Two other gill samples yielded* Citrobacter freundii *isolates capable of producing 24-35 mg of histamine/100 ml in 24 hours at 37°C in TFIB. No histamine-producing isolates were obtained from any of the intestine or muscle samples.*

morganii has been isolated from canned tuna associated with episodes of scombroid fish poisoning in West Germany, and the contamination almost certainly occurred during restaurant handling procedures (Yamani et al., 1981). A similar situation may have occurred in an outbreak of scombroid fish poisoning involving consumption of tuna sashimi in San Francisco where *K. pneumoniae* was isolated from kitchen scraps obtained from a Japanese restaurant (Lerke et al., 1978).

If the occurrence of histamine-producing bacteria in scombroid fish is the result of post-catching contamination, then the incidence of recovery of such bacteria from fish off-loaded at canneries should be sporadic and dependent on the level of sanitation on the boats. If histamine-producing bacteria are part of the normal microflora of tuna, then such bacteria should be isolated frequently from tuna if an adequate isolation procedure was employed. Studies on the incidence of recovery of histamine-producing bacteria from such fish have not been reported. Fish usually arrive frozen, and studies would perhaps be compromised by freeze injury of the histamine-producing bacteria. This study was undertaken to develop an effective method for the recovery of histamine-producing bacteria from frozen tuna and to apply that technique in assessing the incidence of recovery of histamine-producing bacteria from tuna.

Materials and Methods

Fish

Frozen skipjack tuna, *Katsuwonus pelamis*, each weighing between 2.2 and 3.8 kg (5 and 8.5 pounds) were obtained from a major tuna packer in the western United States. The tuna were originally obtained from catches off the coasts of South America, the western United States, Japan, the Philippines, and Nauru. The tuna were shipped on dry ice to Madison, Wis., and were held frozen until analysis. All tuna arrived frozen and without overt signs of decomposition.

Bacterial Media

Trypticase soy broth (TSB) and trypticase soy agar (TSA) were prepared according to label directions. Niven agar (NA) was prepared according to the procedure of Niven et al. (1981). The preparation of TFIB has been described previously (Omura et al., 1978). Trypticase soy broth-histidine medium (TSBH) was TSB with 0.1 percent histidine added (Taylor and Woychik, 1982).

Recovery, Isolation, and Identification of Histamine-Producing Bacteria

The tuna were thawed, and samples of muscle (from an area adjacent to the intestinal cavity and near the gills), gills, and intestine (the entire intestine was used) were aseptically removed. The samples were blended in an Osterizer[1] blender without any addition of liquid. Portions (1 g) of the blended muscle and gill samples and portions (1 ml) of the homogenized intestinal samples were placed in 9 ml of TSB. After incubation at 35 °C for 24 hours, serial dilutions of the TSB were plated onto TSA or NA. The Niven agar plates were overlayed with additional NA. The plates were incubated aerobically at 35 °C for 24 hours in the case of the TSA plates and up to 5 days in the case of the NA plates. The NA plates were checked daily for the presence of purple colonies or colonies with purple halos indicative of histamine-producing bacteria (Niven et al., 1981). These colonies were picked and streaked onto TSA to obtain isolates. These isolates were then restreaked on NA plates to confirm that they produced purple colonies. Additional isolates were randomly picked from the TSA plates. These isolates were streaked onto NA plates, and any isolates producing purple colonies were confirmed as before and retained.

The histamine-producing capabilities of the presumptive histamine-producing isolates were determined in TFIB as previously described (Behling and Taylor, 1982). Samples of the culture fluid were withdrawn after 0, 6, 24, and 48 hours of incubation at 37 °C. The level of histamine in the samples was determined by a modification of the AOAC method (AOAC, 1980) as described previously (Behling and Taylor, 1982). Aerobic plate counts (APC) were also conducted on these samples (Behling and Taylor, 1982).

Isolates producing in excess of 20 mg of histamine/100 ml in 48 hours in TFIB were identified using the API 20 E *Enterobacteriaceae* system (Guthertz et al., 1976; Taylor et al., 1979).

Evaluation of Recovery Procedure

To determine if the recovery procedure was adequate for the isolation of low numbers of histamine-producing bacteria from frozen tuna, three known histamine-producing bacterial strains were inoculated into tuna. The three histamine-producing bacterial strains, *Klebsiella pneumoniae* T2, *Proteus morganii* 110SC2, and *Enterobacter aerogenes* 42 were obtained in earlier studies (Lerke et al., 1978; Taylor et al., 1978). The isolates were transferred from TSA slants into 5 ml of TSBH and allowed to incubate for 24 hours at 37 °C; 0.2 ml of each culture was transferred to another 5 ml of TSBH and incubated for 18 hours at 37 °C. The 18-hour culture was diluted with saline until the density was comparable to McFarland standard #2 (McFarland, 1907). This diluted culture was inoculated at a rate of 0.1 ml of culture/g of tissue into blended samples of either intestines, gills, or muscle tissue obtained from a single skipjack tuna. The sample was blended further to insure homogeneous distribution of the bacteria. An uninoculated control was also included. The inoculated tissues were separated into 10 g samples and stored in a freezer at −15 °C for up to 14 days. Samples were removed from the freezer at 1-, 7-, and 14-day intervals. Inoculated control samples that were not subjected to frozen storage were obtained

[1]Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1.—Isolation of histamine-producing bacteria from frozen skipjack tuna.

Fish no.	No. of tentative histamine-producing isolates		No. of confirmed histamine-producing isolates	
	Niven agar plates	TSA plates	Niven agar plates	TSA plates
1	4-M, 3-G[1]	1-G	0	0
2	8-G	0	8-G	0
3	0	0	0	0
4	2-G	0	0	0
5	3-G	2-M, 5-I, 3-G	1-G	1-G
6	7-M, 3-I, 3-G	4-M, 2-G	0	1-G
7	0	0	0	0
8	4-M	3-M	0	0
9	5-I, 3-G	7-I, 5-G	0	0
10	1-M, 1-G	3-G	0	0
Total	47	35	9	2

[1]M = muscle, G = gills, I = intestines.

Table 2.—Histamine production by the confirmed histamine-producing isolates in TFIB.

Bacterial species	Source of isolation	Log increase in APC[1]	Histamine concentration (mg/100 ml)			
			0 h	6 h	24 h	48 h
Proteus morganii	Tuna #2 Gills	1.09	6.3	476	535	665
Citrobacter freundii	Tuna #5 Gills	0.88	ND[2]	0.4	4.1	23.9
Citrobacter freundii	Tuna #6 Gills	1.14	ND	0.2	7.3	35.3

[1]Logarithmic increase in aerobic plate count after 24 hours of incubation in TFIB at 35°C with an initial inoculum level of more than 10^7 bacteria/ml.
[2]ND = not detectable.

immediately after inoculation for each organism and type of tissue. The 10 g frozen samples were transferred aseptically to 90 ml of TSB and allowed to incubate at 35 °C for 24 hours. This culture fluid was serially diluted, plated on TSA and NA, and incubated for 24 hours (TSA) or 48 hours (NA) at 35 °C. For inoculated control samples not subjected to freezing, the procedure was similar except that the samples were not given the 24-hour recovery period in TSB. The histamine-producing isolates were picked from the NA plates and their identities checked using the API 20 E *Enterobacteriaceae* system.

Results

Confirmed histamine-producing bacteria were isolated from only three of ten frozen skipjack tuna (Table 1). In all three cases, the histamine-producing isolates were obtained from the gills. No histamine-producing bacteria were isolated from any of the muscle or intestinal samples. A total of 82 tentative histamine-producing isolates were initially obtained from the NA and TSA plates. However, only 11 of these tentative isolates were confirmed as histamine producers by their ability to produce in excess of 20 mg of histamine/100 ml in TFIB within 48 hours at 37 °C. Most of the remaining 71 isolates did not produce characteristic purple colonies on Niven agar plates. Instead, the colonies were often red or pink. The inclusion of these isolates as tentative histamine producers represented a conservative approach in the use and interpretation of Niven agar plates along with a desire to insure that no histamine producers would be missed in the recovery procedure.

Of the eleven histamine-producing isolates obtained from the gills of frozen tuna, eight were identified as *Proteus morganii* and three were identified as *Citrobacter freundii*. The eight isolates of *P. morganii* were obtained from the Niven agar plates of the gill sample from tuna #2 indicating the possible presence of relatively high numbers of this organism in that particular sample. The *C. freundii* strain from tuna #5 was isolated once each from the TSA and NA plates, while the *C. freundii* strain from tuna #6 was isolated only once from a TSA plate.

The *P. morganii* strain obtained from tuna #2 was by far the most prolific histamine producer among the three strains compared in Table 2. This strain was able to produce in excess of 535 mg of histamine/100 ml in TFIB within 24 hours at 37 °C. By comparison, the two *C. freundii* isolates produced only 3-5 percent as much histamine as the 48-hour sampling period. The *C. freundii* isolates also showed a more pronounced lag period in terms of histamine production by comparison to the *P. morganii* strain. Large inoculum levels were used in these experiments to increase the likelihood of bacterial histamine production and eliminate any possible problems associated with failure to grow rapidly in TFIB. All three bacterial strains grew in TFIB increasing about 1 log in numbers within 24 hours. The high initial histamine level with the *P. morganii* strain represents carryover from the 18-hour TSBH cultures.

The evaluation of this procedure for the recovery of histamine-producing bacteria from frozen tuna tissues indicated that the method worked quite well. When between 10^2 and 10^3 organisms/g were inoculated into muscle, gill, and intestine samples from skipjack tuna and the samples were frozen, histamine-producing bacteria were consistently recovered from the NA plates. The recovery procedure using 24 hours incubation in TSB worked equally well for *P. morganii* 110SC2, *K. pneumoniae* T2, and *E. aerogenes* 42, and for all three tuna tissues studied. Histamine-producing isolates were obtained just as easily from samples stored frozen for 1, 7, and 14 days. Usually, 48-hour incubations were necessary before purple colonies appeared on the NA plates. The purple colonies were confirmed to be identical in biochemical profiles to the known histamine-producing bacteria with which the sample was originally inoculated. Failure to use the TSB recovery step usually resulted in a lack of recovery of histamine-producing bacteria from frozen tuna samples, while the histamine producers were consistently recovered from nonfrozen samples without the recovery step.

Discussion

A suitable method was developed for the recovery of histamine-producing bacteria from frozen tuna. This method was effective for recovering rather low levels ($<10^3$ organisms/g) of histamine-producing bacteria from frozen tuna. Without the 24-hour recovery period in TSB, the histamine-producing bacteria could not be isolated successfully from frozen tuna indicating that freezing sublethally injures these bacteria. This method was shown to be effective for the recovery of *K. pneumoniae*, *P. morganii*, and *E. aerogenes*. Its effectiveness for the recovery of other histamine-producing bacteria such as *C. perfringens* and the recently described psychrophilic, halophilic N-group bacteria (Okuzumi et al., 1981) cannot be evaluated since these bacteria were not included in this study.

The use of Niven agar aided considerably in the isolation and identification of the histamine-producing bacteria, although the low pH of this medium would be predicted to discourage growth by some histamine-producing bacteria, especially the anaerobes. The histamine-producing bacteria isolated from frozen tuna grew well on NA following the TSB recovery step. Although only 11 of 82 tentative histamine producers were confirmed to be histamine producers in TFIB, this should not be taken as an indication that NA yields a high number of false positives. Only eight isolates gave typical purple colonies on NA plates, and these were the *P. morganii* isolates from the gills of tuna #2. The remaining 74 isolates would have been classified as questionable or negative reactions in most situations. However, we wanted to be certain that no histamine-producing isolates were missed, and thus many of the colonies were picked and assayed to insure that no atypical reactions would be found among histamine producers on NA plates. The results indicate that NA will be very useful in future studies, and that only typical purple colonies should be retained for further investigation.

Despite the use of these effective recovery, isolation, and identification methods, only one of ten frozen tuna yielded a high-level histamine producer. Apparently, histamine-producing bacteria are not common components of the natural microflora of tuna. If histamine-producing *Enterobacteriaceae* are present, the level of contamination must be below 10^3 organisms/g. If such low level contamination occurs in tuna, other bacteria would likely overgrow the histamine producers in temperature abuse situations. Although 10 tuna constitute a fairly small sample size, the tuna came from a variety of fishing locales and fishing boats.

While this study provides evidence that the histamine-producing *Enterobacteriaceae* are not part of the normal microflora of tuna, the possible presence of other types of histamine-producing bacteria cannot be ignored. *C. perfringens* and the psychrophilic, halophilic N-group bacteria (Yoshinaga and Frank, 1982; Okuzumi et al., 1981) would not likely have been detected in these experiments due to the choice of aerobic and mesophilic incubation conditions and the use of Niven agar.

P. morganii, *K. pneumoniae*, and *H. alvei* are the only species of histamine-producing bacteria that have been isolated from tuna implicated in outbreaks of scombroid fish poisoning (Kawabata et al., 1956; Havelka, 1967; Sakabe, 1973; Taylor et al., 1979). Yet, these species were not isolated from nine of the ten tuna included in the survey. The isolation of enteric bacteria, including one strain of *P. morganii*, from gill samples of several tuna may not reflect the occasional presence of these bacteria as part of the normal microflora of the gills. Tuna are often moved using the gills as convenient "hand-holds." The possibility of contamination of gill samples during post-catch handling cannot be discounted. In fact, post-catching contamination should be considered as the likely source of histamine-producing bacteria in many outbreaks of scombroid fish poisoning. Many outbreaks involve raw fish that have been handled in restaurants or homes increasing the likelihood for contamination with histamine-producing enteric bacteria. Certainly, Yamani et al. (1981) provide strong evidence to indicate that restaurants have been involved as sites of contamination in some outbreaks.

The isolation of histamine-producing enteric bacteria from the gills emphasizes the possible importance of the gills as a reservoir for these bacteria. In several studies on the distribution of histamine in spoiled tuna (Lerke et al., 1978; Frank et al., 1981), the muscle tissue adjacent to the gills had the highest level of histamine. The bacteria responsible for histamine formation in these studies (Lerke et al., 1978; Frank et al., 1981) could have arisen from either the gills or the anterior portion of the intestinal tract. As mentioned above, the gills are much more likely to be contaminated with histamine-producing bacteria during handling than are the intestines or muscle tissues. Invasion of histamine-producing bacteria into the muscle tissues should certainly be considered a secondary event.

This study provides the first clear evidence that histamine-producing *Enterobacteriaceae* are not part of the normal microflora of tuna. Unfortunately, the isolation scheme was not oriented toward the possible isolation of *C. perfringens* and the N-group bacteria. Future studies will have to take these bacteria into consideration. Also, the isolation of *P. morganii* from the gills of one tuna could have been due to either its presence as a part of the normal microflora or to post-catching contamination. The only way to determine if *P. morganii* and other histamine-producing enteric bacteria exist occasionally as constituents of the normal microflora of tuna is to repeat our isolation studies on fresh-caught tuna on a tuna boat before any chance occurs for contamination via handling.

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