Volatile Compounds Produced in Sterile Fish Muscle (*Sebastes melanops*) by *Pseudomonas putrefaciens*, *Pseudomonas fluorescens*, and an Achromobacter Species

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Volatile compounds produced by *Pseudomonas putrefaciens*, *P. fluorescens*, and an *Achromobacter* species in sterile fish muscle (*Sebastes melanops*) were identified by combined gas-liquid chromatography and mass spectrometry. Compounds produced by *P. putrefaciens* included methyl mercaptan, dimethyl disulfide, dimethyl trisulfide, 3-methyl-1-butanol, and trimethylamine. With the exception of dimethyl trisulfide, the same compounds were produced by an *Achromobacter* species. Methyl mercaptan and dimethyl disulfide were the major sulfur-containing compounds produced by *P. fluorescens*.

Tissue enzymes and certain gram-negative bacteria are the main causes of spoilage of fish and fishery products. Although bacterial activity is generally accepted as the primary cause of fish spoilage, the specific functions of the various genera and species involved are not completely resolved.

Bacterial species, such as flavobacteria and micrococi, are generally abundant in fresh fish but are gradually superseded by members of the genera *Achromobacter* and *Pseudomonas* as spoilage develops. Lerke et al. (7) reported that micrococi, flavobacteria, and “coryneforms” (psychrophilic, gram-positive, nonspore-forming rods) were not active spoilage organisms. However, species belonging to the genera *Achromobacter* and *Pseudomonas* were active in spoilage. Shewan et al. (13) and Castell et al. (1, 3, 4) also reported that species of the above genera were the major spoilage bacteria of cod. In addition, Chai et al. (5) observed that the spoilage flora of haddock fillets stored at 1 to 2°C was comprised primarily of fluorescent pseudomonads and *P. putrefaciens*.

This investigation was initiated to identify the volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *P. putrefaciens*, *P. fluorescens*, and an *Achromobacter* species and to assess the spoilage potential of the species examined.

**MATERIALS AND METHODS**

**Sterile muscle tissue.** Sterile fish muscle was obtained from black rockfish (*S. melanops*) by a modified method of Lobben and Lee (8) as described previously (12).

**Bacterial species and cultural conditions.** *P. fluorescens* was isolated from ocean perch and *P. putrefaciens* strain H6 and *Achromobacter* strain H15 were isolated from Dungeness crab. *P. putrefaciens* strains 17 and 19X were obtained from R. E. Levin, Department of Food Science and Technology, University of Massachusetts, Amherst. Tubes of peptone iron agar were inoculated with the above organisms and incubated at 25°C for 3 days to assess H$_2$S production.

Cells of *Achromobacter* strain H15, *P. putrefaciens* strains H6, 17, 19X, and *P. fluorescens*, grown on tryptone-peptone-yeast extract agar with 3% NaCl for 48 h at 25°C, were collected and suspended in sterile, distilled water. Sterile fish muscle (pH 6.4–6.7) was homogenized in an Osterizer blender with sterile, distilled water (1:2, wt/vol) containing sufficient NaCl to obtain a final NaCl concentration of 3%. The homogenates were inoculated with the desired cell suspensions and 10-g quantities were dispensed in sterile, screw-capped vials. The homogenates, containing strains of *P. putrefaciens* and the *Achromobacter* species, were incubated at 1 to 2°C for 12 and 27 days, respectively. Samples inoculated with *P. fluorescens* were incubated at 0°C for 32 days.

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Microbial counts were conducted as described by Lobben and Lee (8) by using tryptone-peptone-yeast extract agar plates supplemented with 3% NaCl. In addition, the contents of each vial were analyzed at selected times throughout the incubation period by combined gas-liquid chromatography and mass spectrometry as described below.

Gas-liquid chromatography. The gas chromatographs, chromatographic columns, and methods of sample preparation and analysis used for the identification of low-boiling compounds, including dimethylamine (DMA) and trimethylamine (TMA), were reported previously (10-12).

Analysis of volatiles collected on Porapak Q. Volatile compounds from inoculated fish homogenates were collected in Porapak Q traps (120 mm by 6 mm inside diameter) for subsequent capillary column, gas chromatographic-mass spectral analysis as described by Miller et al. (12). The capillary column (153.8 m by 0.75 mm inside diameter), coated with 8% Carbowax 20 M and 1% Versamid 900, was operated isothermally at 70°C for 5 min and then temperature programmed to 190°C at one degree per minute. The nitrogen-carrier gas flow rate was 12 ml/min, and the detector and injector port temperatures were 230 and 200°C, respectively.

Mass spectral analysis. An F & M model 810 gas chromatograph was used in conjunction with an Atlas CH-4 mass spectrometer for all mass spectral analyses. The operating conditions for the mass spectrometer were reported previously (11). All identifications were made by comparisons of mass spectra with reference spectra. Relative retention times of authentic compounds, obtained by gas-liquid chromatography, were used to confirm the mass spectral identifications.

RESULTS AND DISCUSSION

A typical flame ionization detector (FID) chromatogram of the volatiles produced by P. putrefaciens strain 17 in sterile fish muscle (10 g) incubated at 1 to 2°C for 15 days is illustrated in Fig. 1. Compounds identified are listed as follows with respective peak numbers: (1) TMA, (2) a silicone contaminant, (3) propionaldehyde, (4) dimethyl disulfide (CH₃-S-S-CH₃), (5) 1-penten-3-ol (tentative identification), (6) 3-methyl-1-butanol, and (7) dimethyl trisulfide (CH₃-S-S-S-CH₃). Methyl mercaptan (CH₃SH) was identified by using a 20% 1,2,3-tris(cyanoethoxy)propane column (11, 12). Although similar chromatographic patterns were observed for P. putrefaciens strains H6 and 19X, slight differences in intensity were evident. The silicone contaminant, peak 2, originated from General Electric anti-foam 60 which was used to control excessive foaming during the entrainment procedure. Peak 5, tentatively identified as 1-penten-3-ol, was considered a product of lipid oxidation. Slight increases in acetaldehyde, propionaldehyde, and acetone were noted in the sterile controls stored at 0°C for 21 days; however, no sulfur-containing compounds were detected.

The alkali flame ionization detector (AFID) and recorder response to volatile compounds produced in sterile muscle tissue by P. putrefaciens strain 17 is shown in Fig. 2. Although the AFID yields a positive response to nitrogen-containing compounds, negative responses may occur with other molecular combinations of functional groups, such as sulfur and chlorine. CH₃-S-S-CH₃, CH₄-S-S-S-CH₃, and several unidentified sulfur-containing compounds gave negative responses and TMA yielded a positive response, as illustrated in Fig. 2. The selectivity of the AFID is clearly demonstrated upon comparison of Fig. 1 and 2. The retention times recorded for the FID analysis (Fig. 1) were slightly shorter than those noted for the AFID analysis (Fig. 2) because of slight differences in program rates between instruments and variations in carrier gas flow rates.

Achromobacter strain H15, a gram-negative coccobacillus, was nonmotile, nonpigmented, and cytochrome oxidase positive. In addition, it did not exhibit oxidative acid production in Hugh Leifson glucose, did not produce H₂S, and

![Fig. 1. FID chromatogram of volatiles produced by P. putrefaciens strain 17 in sterile fish muscle. Column: 8% Carbowax 20 M and 1% Versamid 900 (153.8 m by 0.75 mm inside diameter).](image-url)
was resistant to 2.5 immunizing units of penicillin. At present, the taxonomy of the genus *Achromobacter* is not completely resolved, and several of the characteristics cited above may be applicable to species of *Achromobacter*, *Moraxella*, and *Acinetobacter*. Since an exact generic designation for strain H15 is not possible at this time, it was tentatively identified as an *Achromobacter* species. With the exception of H2S and CH₃-S-S-CH₃, the volatile compounds produced by *Achromobacter* strain H15 were similar to those produced by *P. putrefaciens*. Mass spectral data were not obtained for strain H15; however, the relative retention times observed indicated the presence of CH₃SH, CH₃-S-S-CH₃, 1-penten-3-ol, 3-methyl-1-butanol, and TMA. Strain H15 did not produce detectable amounts of CH₃-S-S-S-CH₃, even after 27 days of incubation at 1 to 2 C.

CH₃SH and CH₃-S-S-CH₃ were the major sulfur-containing compounds produced by *P. fluorescens* in sterile fish muscle stored at 0 C between 7 and 22 days. Although additional components were detected after 32 days at 0 C, mass spectral analyses were not detected because the samples were in such an advanced stage of spoilage.

Since *P. putrefaciens* strains H6, 17, and 19X are active producers of H₂S and are extremely proteolytic, the mechanism of formation of CH₃SH, CH₃-S-S-CH₃, and CH₃-S-S-CH₃ may be the same as that previously suggested for *P. perolens* (12). CH₃SH, which can be derived from cystine, cysteine, methionine, or possibly glutathione (6, 14), may undergo a direct oxidation to CH₃-S-S-CH₃, involving sulfenic acid (CH₃SOH) intermediates. The formation of CH₃-S-S-S-CH₃ may result from the reaction of H₂S with the unstable sulfenic acid, as suggested by Maruyama (9). *Achromobacter* strain H15 and *P. fluorescens* did not produce H₂S, and CH₃-S-S-CH₃ was not detected.

(i) CH₃SH, CH₃-S-S-CH₃, CH₃-S-S-S-CH₃, and H₂S and (ii) CH₃SH and CH₃-S-S-CH₃ were considered to be the major sulfur-containing compounds produced by (i) *P. putrefaciens* and (ii) *P. fluorescens* and *Achromobacter* strain H15, respectively. However, the unidentified sulfur compounds, indicated by negative responses in Fig. 2 and present in trace amounts, may also contribute significantly to the characteristic spoilage odor produced by *P. putrefaciens*.

TMA production in sterile fish muscle by several strains of *P. putrefaciens* and *P. fluorescens* is shown in Fig. 3. The reduction of trimethylamine oxide to TMA by *P. putrefaciens* strains 17, H6, and 19X was pronounced between 4 and 8 days of storage at 1 to 2 C. The microbial counts for the above species increased from 1.4 × 10⁴ cells/g to approximately 1.5 × 10⁴ cells/g during the 12-day incubation period, and a reasonable correlation with the increases in TMA content was observed. The decreases in TMA content that occurred after 7 days of storage (Fig. 3) may be attributed to the extreme volatility of the free amine rather than TMA degradation, since DMA did not increase appreciably. Although not indicated in Fig. 3, an appreciable amount of TMA was produced by *Achromobacter* strain H15 and a noticeable decrease in concentration was also observed after 7 days of incubation at 1 to 2 C. *Achromobacter* strain H15, *P. putrefaciens* strains H6, 17, and 19X, and *P. fluorescens* did not produce significant amounts of DMA, and the latter species did not reduce trimethylamine oxide. The levels of TMA in the sterile controls remained relatively constant throughout the 12-day storage period (Fig. 3).

With the exception of CH₃-S-S-CH₃, the volatile compounds produced in sterile fish muscle by the species examined herein have also been associated with naturally spoiling fish (11). Miller et al. (12) reported that CH₃-S-S-CH₃ was produced in sterile
fish flesh by *P. perolens* but, to the best of our knowledge, the trisulfide has not been identified as a major volatile constituent of spoiling fish. It should be emphasized that the spoilage patterns observed are relevant only to pure bacterial cultures on sterile fish muscle and may be influenced markedly by differences in substrate composition and associative growth of the normal spoilage flora. Nevertheless, the data represent the individual contribution of the selected bacterial species to the overall spoilage pattern.

In an attempt to estimate the fish-spoiling potential of a bacterial culture, one must consider the growth rate and changes produced by the organism when grown at a suitable temperature (1). The proteolytic strains of *P. putrefaciens* and *P. fluorescens* contributed significantly to spoilage in pure culture because of rapid growth rates during low-temperature storage and the production of objectionable odors, as well as pronounced color and textural changes. The off-odors produced by *Achromobacter* strain H15 (nonproteolytic) in sterile muscle tissue were not as intense as those produced by *P. putrefaciens* and *P. fluorescens*, and no marked color or textural changes were observed. Although sterile fish muscle homogenates were used in this study, the characteristic off-odors and flavors associated with naturally spoiling fish were also reproduced in sterile muscle blocks (6) and heat-sterilized fish muscle (2).

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