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# Volatile Compounds Produced in Sterile Fish Muscle (*Sebastes melanops*) by *Pseudomonas perolens*[1]

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Volatile compounds produced by *Pseudomonas perolens* ATCC 10757 in sterile fish muscle (*Sebastes melanops*) were identified by combined gas-liquid chromatography and mass spectrometry. Compounds positively identified included methyl mercaptan, dimethyl disulfide, dimethyl trisulfide, 3-methyl-1-butanol, butanone, and 2-methoxy-3-isopropylpyrazine. Compounds tentatively identified included 1-penten-3-ol and 2-methoxy-3-sec-butylpyrazine. The substituted pyrazine derivative 2-methoxy-3-isopropylpyrazine was primarily responsible for the musty, potato-like odor produced by *P. perolens*.

Castell and Greenough (2, 3) and Castell et al. (4) described a musty, potato-like odor that commonly developed in chilled fish muscle during the early stages of spoilage. Although this distinctive odor was observed under commercial conditions on chilled fillets of cod, haddock, and flounder, it was not detected on fresh or spoiling round and eviscerated fish. The causative bacterial species was identified as *Pseudomonas perolens*, a psychrophilic organism which utilizes a variety of substrates for growth and odor production (4).

This investigation was initiated to identify the volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *P. perolens*. Particular emphasis was placed on the identification of the compound or compounds responsible for the characteristic musty, potato-like odor.

## MATERIALS AND METHODS

**Sterile muscle tissue.** Black rockfish (*Sebastes melanops*), line-caught off the coast of Newport, Ore., were immediately killed by a blow on the head, individually wrapped in clean towels to prevent puncture damage to the skin, and packed in ice. Sterile fish flesh was obtained essentially as described by Lobben and Lee (11). The whole fish was washed in water, thoroughly swabbed with ethyl alcohol (70%), and dried under the flow of sterile air in a sterility test cabinet (BioQuest). Portions of skin were aseptically removed, and sections of exposed flesh were excised in the sterile atmosphere of the cabinet. Muscle tissues from several fish were pooled and sterility was assessed as described previously (11).

**Bacterial species and cultural conditions.** Cells of *P. perolens* ATCC 10757, grown on Trypticase soy agar (BBL) plates for 48 hr at 25 C, were collected and suspended in sterile distilled water. Sterile fish muscle was homogenized in an Osterizer blendor with sterile distilled water (1:2, w/v) and inoculated with the concentrated cell suspension. The *p*H of the homogenate was adjusted to 7.3 with sterile NaOH, and 10-g quantities were dispensed in sterile, screw-capped vials (Kimble no. 60957, size no. 1). The inoculated vials were incubated at 5, 15, and 25 C and were examined periodically for odor production. Microbial counts were recorded only for those samples incubated at 15 C. When a distinct, musty, potato-like odor was detected, the contents were analyzed by combined gas-liquid chromatography and mass spectrometry, as described below.

**Gas chromatographs.** Varian Aerograph (series 1200 and 1400) and F & M model 810 gas chromatographs were used throughout this investigation. The Varian Aerograph series 1400 instrument was equipped with an alkali flame ionization detector (AFID) which was routinely tuned for optimal performance as described by the manufacturer (Varian Aerograph, Walnut Creek, Calif.). The remaining instruments were equipped with flame ionization detector (FID) systems.

**Gas chromatographic columns and conditions.** A stainless-steel column (5.5 meters by 3 mm, outer diameter), containing 40 to 60 mesh Graphon (Cabot Corp., Billerica, Mass.) coated with 2% tetraethylenepentamine (TEP), was used in conjunction with the AFID for the selective separation and quantitative determination of dimethylamine and trimethylamine as described previously (13).

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An additional stainless-steel column (3.7 meters by 3 mm, outer diameter), containing acid/base washed Celite 545 (60 to 80 mesh) coated with 20% 1,2,3-tris (2-cyanoethoxy) propane (TCEP), was used with a gas entrainment, on-column trapping procedure (15) for the separation and identification of relatively low-boiling compounds. The method of sample preparation was previously reported (14). The sample size and nitrogen gas purge time were increased when more concentrated samples were required for mass spectral analysis.

The TCEP and Graphon plus TEP columns were operated isothermally at 60 C with a nitrogen gas flow rate of 30 ml/min. The detector and injector port temperatures were 210 and 190 C, respectively.

A capillary column (153.8 meters by 0.75 mm, inner 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 180 C at 1 C/min. The nitrogen carrier gas flow rate was approximately 12 ml/min, and the detector and injector port temperatures were 230 and 200 C, respectively.

**Analysis of volatile compounds collected on Porapak Q.** Volatile compounds from inoculated fish homogenates were collected in Porapak Q traps (102 mm by 6 mm, inner diameter) for subsequent capillary column, gas chromatographic-mass spectral analyses essentially as described by Miller et al. (14). All samples were purged with nitrogen (30 ml/min) at 60 C for 30 min while the Porapak Q traps were heated at 55 C with a thermostatically controlled heat gun. After removal of water for an additional 60 min at 55 C, the traps were reversed and heated at 125 to 130 C; the entrained volatile compounds were transferred for 30 min to an open tubular trap (150 mm by 1.25 mm, inner diameter) immersed in a slurry of dry ice and 2-methoxyethanol. The volatile compounds were then introduced into the capillary column through a modified inlet system (20) while the column was connected to the high vacuum system of the mass spectrometer.

The Porapak Q traps were conditioned as reported previously (14). Subsequent gas chromatographic analyses of conditioned traps revealed no significant quantitative or qualitative changes above the background response.

**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. A splitter, attached to the effluent end of the column, routed the column effluent to the FID and Lewellyn separator through a heated outlet in a ratio of 1:5. The operating conditions for the mass spectrometer were described previously (14), and spectra were scanned from *m/e* 25 to *m/e* 250 in 2.5 sec. A micro-volume switching valve (Carle Instruments Inc., Fullerton, Calif.) was installed between the mass spectrometer and gas chromatograph to prevent air from entering the ion source during sample transfer.

## RESULTS

A musty, potato-like odor was produced by *P. perolens* in sterile fish muscle (*S. melanops*) incubated at 5, 15, and 25 C. As expected, the characteristic odor developed more rapidly at the higher temperatures, and a definite sulfide odor was also noted. When ethyl alcohol (0.2%) was added to the sterile fish flesh, a mild fruity aroma, in addition to the predominant musty odor, was detected. Castell and Greenough (3) also reported the production of a fruity, ester-like odor by *P. perolens* when grown on a medium containing valine, glucose, inorganic salts, and 1% ethyl alcohol. However, no fruity odor was produced in the latter medium when the alcohol was omitted.

A typical FID chromatogram of the volatile compounds produced by *P. perolens* in sterile fish muscle (30 g) incubated at 15 C for 7 days is illustrated in Fig. 1. Compounds identified are listed as follows with respective peak numbers: 1 and 2, silicone contaminants; 3, propionaldehyde; 4, a silicone contaminant; 5, dimethyl disulfide ($CH_3—S—S—CH_3$); 6, a sulfur-containing compound; 7, 1-penten-3-ol (tentative identification); 8, 3-methyl-1-butanol; 9 and 10, sulfur-containing compounds; 11, dimethyl trisulfide ($CH_3—S—S—S—CH_3$); 12, not identified; 13, 2-methoxy-3-isopropylpyrazine; and 14, 2-methoxy-3-sec-butylpyrazine (tentative identification). Methyl mercaptan ($CH_3SH$) and butanone were identified by use of the TCEP column. Slight increases in acetaldehyde, propionaldehyde, and acetone were noted in the sterile muscle controls; however, no sulfur-containing compounds were detected. With few exceptions, all identifications were made by comparisons of mass spectra with reference spectra. In addition, the relative retention times of authentic compounds, obtained by gas-liquid chromatography, were used to confirm the mass spectral identifications. 2-Methoxy-3-sec-butylpyrazine was tentatively identified by comparison of the mass spectrum with reference spectra (1). The relative intensities are given in parentheses with the base peak taken as 100. The molecular ion was 166 (5) and the major ions were 138 (100), 124 (63), 151 (50), 137 (36), 105 (11), 109 (11), and 123 (9).

The silicone contaminants, peaks 1, 2, and 4 in Fig. 1, originated from General Electric Antifoam 60, which was added to all samples to control excessive foaming during the entrainment procedure. Since the mass spectrum for peak 7 was rather weak and inconclusive, 1-penten-3-ol was tentatively identified solely on the basis of the retention time of the authentic compound. The mass spectra and negative AFID responses obtained for peaks 6,

9, and 10 substantiated the presence of sulfur-containing compounds having apparent molecular weights of 104, 132, and 132, respectively. Peaks 9 and 10 appeared to be isomers.

The olfactory evaluation of each component eluting from the capillary column was facilitated by the splitter which was attached to the effluent end of the column. The major volatile component, 2-methoxy-3-isopropylpyrazine, had a pronounced musty odor which was identical to that of the authentic compound.

Figure 2 is a typical AFID response to volatile components produced in sterile muscle tissue by *P. perolens*. The identifications of 2-methoxy-3-isopropylpyrazine and 2-methoxy-2-sec-butylpyrazine (tentative identification) were substantiated by use of the selective nitrogen detector. Although the sensitivity of the AFID to nitrogen-containing compounds is about the same as the standard FID (2 N atoms/molecule), it has a selectivity of response which is clearly demonstrated upon comparison of Fig. 1 and 2. The retention times recorded for the AFID analysis were slightly shorter than those noted for the FID analysis because of slight differences in program rates between instruments and variations in carrier gas flow rates. Although the AFID yields a positive response to phosphorus- or nitrogen-containing compounds, negative responses may occur when other molecular combinations of functional groups, for example, sulfur and chlorine are present in the sample. $CH_3—S—S—CH_3$, $CH_3—S—S—S—CH_3$, and several unidentified sulfur-containing compounds gave negative responses, as indicated in Fig. 2.

The growth of *P. perolens* and trimethylamine-dimethylamine production in sterile fish muscle incubated at 15 C are illustrated in Fig. 3. The microbial count increased from $7.1 \times 10^6$ cells/g at 0 days to $1.4 \times 10^8$ cells/g at 6 days. Trimethylamine oxide was not reduced to trimethylamine, and the level of dimethylamine remained relatively constant between 0 and 21 days at 15 C. No significant amounts of trimethylamine or dimethylamine were produced in the sterile controls during the experimental period.

## DISCUSSION

The substituted pyrazine derivative 2-

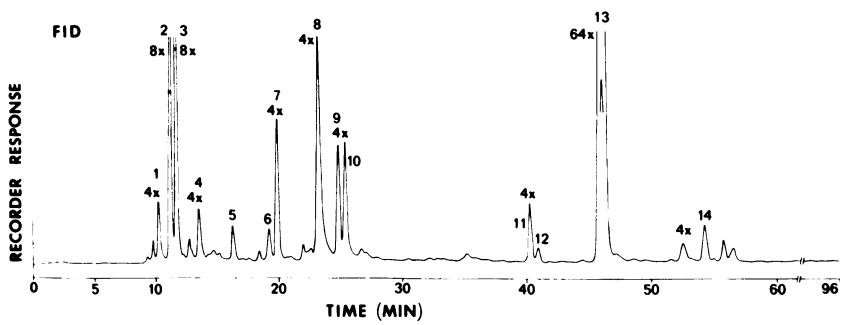


FIG. 1. *Flame ionization detector chromatogram of volatile compounds produced by Pseudomonas perolens in sterile fish muscle. Column: 8% Carbowax 20 M and 1% Versamid 900 (153.8 meters by 0.75 mm, inner diameter).*

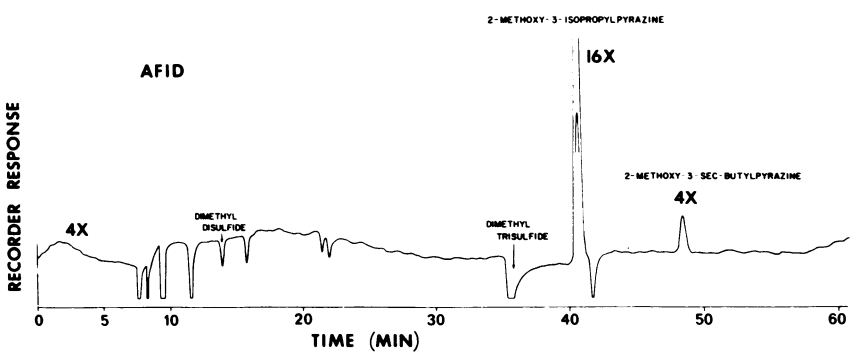


FIG. 2. *Alkali flame ionization detector and recorder response to volatile compounds produced in sterile fish muscle by Pseudomonas perolens. Column: 8% Carbowax 20 M and 1% Versamid 900 (153.8 meters by 0.75 mm, inner diameter).*

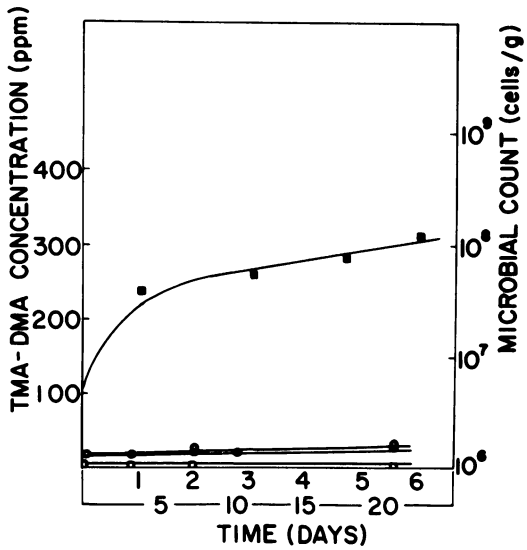


FIG. 3. *Growth of Pseudomonas perolens and trimethylamine-dimethylamine (TMA-DMA) production in sterile fish muscle at 15 C.* (○) *TMA produced by P. perolens;* (●), *TMA in sterile control;* (□), *DMA produced by P. perolens; and* (■), *microbial count.*

methoxy-3-isopropylpyrazine was primarily responsible for the musty, potato-like odor produced by *P. perolens* in sterile fish muscle. It is an extremely potent odorant, having an odor threshold of 2 parts of compound per $10^{12}$ parts of water (21). Although 2-methoxy-3-sec-butylpyrazine was considered a minor constituent of the total volatile fraction, it is also an exceptionally odorous pyrazine derivative (17, 21) which may contribute to the overall musty odor.

*P. perolens*, isolated from fish, appears to be similar to species isolated from musty eggs and chilled meats by Spanswick (22) and Jensen (8), respectively. The latter workers classified their isolates as *Achromobacter perolens*. However, Szybalski (23) stated that, because *A. perolens* produces a green, water-soluble pigment and has a single polar flagellum, it should be classified as *P. perolens*. Olson and Hammer (19, cited by Castell et al. [4]) isolated similar organisms from dairy products, which they described as producing a distinct, potato-like odor. The causal organisms were subsequently identified as *P. graveolens* and *P. mucidolens*.

The compound responsible for the strong, musty aroma produced in sterile milk and Trypticase soy broth by *P. taetrolens*, formerly designated *P. graveolens*, has been identified as 2-methoxy-3-isopropylpyrazine (16). Although 2,5-dimethylpyrazine was also detected, it is a considerably weaker odorant, having a threshold of $1.8 \times 10^6$ parts of compound per $10^{12}$ parts of water (21). *P. perolens* did not produce detectable amounts of 2,5-dimethylpyrazine in sterile fish muscle, skim milk, or Trypticase soy broth.

The formation of several of the sulfur compounds, such as $CH_3SH$, $CH_3—S—S—CH_3$ and $CH_3—S—S—S—CH_3$, can be explained on the basis of available precursors and known reaction sequences. Herbert et al. (7) reported that several sulfide-producing pseudomonads derive $CH_3SH$ from methionine, cystine, or cysteine. Since an important and characteristic reaction of the thiol group is the ready oxidation to disulfide, the following reaction is well documented: $2CH_3SH \xrightarrow{[O]} CH_3—S—S—CH_3$. Any direct oxidation of the resultant disulfide (without cleavage) would yield the thiolsulfinate ester, $CH_3S(O)SCH_3$, and subsequent hydrolysis would give two molecules of the unstable sulfenic acid, $CH_3SOH$ (24). The direct oxidation of thiols may also proceed through sulfenic acid intermediates (10). Since *P. perolens* actively produces $H_2S$ from cystine and methionine (4), and sulfenic acid intermediates are likely to be present, $CH_3—S—S—S—CH_3$ may be formed as follows: $2CH_3SOH + H_2S \rightarrow CH_3—S—S—S—CH_3 + 2H_2O$. The latter reaction was previously reported by Maruyama (12). Preliminary data for *P. fluorescens*, which did not produce $H_2S$ or detectable amounts of $CH_3—S—S—S—CH_3$, appear to substantiate the involvement of $H_2S$ in the synthesis of the trisulfide.

Although several chemical methods for the synthesis of a variety of substituted pyrazines have been reported (5, 9, 18, 21), the biological mechanisms of formation have not been completely resolved. Murray et al. (17) proposed a biosynthetic pathway that might be functional in plants for the synthesis of 2-methoxypyrazines, as well as other substituted pyrazines, from $\alpha$-amino acids and $\alpha,\beta$-dicarbonyls. Green and Elliott (6) demonstrated the enzymatic formation of aminoacetone from threonine by several bacterial species. In the presence of threonine dehydrogenase, threonine can be oxidized to 2-amino acetoacetate, the free acid of which spontaneously decarboxylates to aminoacetone. The substituted pyrazine derivative 2,5-dimethylpyrazine may be formed by the self-condensation of two molecules of aminoacetone. The above mechanism might be functional in cells of *P. taetrolens*, provided an

active threonine dehydrogenase is produced.

Castell et al. (4) demonstrated the production of green potato and musty odors by *P. perolens* grown in a basal medium to which several $\alpha$-amino and $\alpha$-imino acids were added singly. Since *P. perolens* ATCC 10757 is extremely proteolytic, and fish muscle contains a variety of free amino acids and related compounds, the biosynthetic pathway for the synthesis of 2-methoxy-3-isopropylpyrazine, as well as other substituted pyrazines, may indeed involve reactions of amino acids or amino acid derivatives. Further research on the biosynthetic mechanism for the synthesis of 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-sec-butylpyrazine by *P. perolens* is in progress.

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