APPLIED MICROBIOLOGY, June 1973, p. 952-955
Vol. 25, No. 6

Identification of the Volatile Compounds Produced in Sterile Fish Muscle (*Sebastes melanops*) by *Pseudomonas fragi*[1]

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Received for publication 19 January 1973

Volatile compounds produced by *Pseudomonas fragi* strain 18 in sterile fish muscle (*Sebastes melanops*) were identified by combined gas-liquid chromatography and mass spectrometry. Compounds positively identified included dimethyl sulfide, acetaldehyde, ethyl acetate, ethyl alcohol, and dimethyl disulfide. Methyl mercaptan, ethyl butyrate, ethyl hexanoate, and butanone were tentatively identified by relative retention times of the authentic compounds. The fruity odor that developed in fish muscle during incipient spoilage was attributed to a synergistic flavor interaction involving the ethyl esters of acetate, butyrate, and hexanoate.

Castell and Greenough (3) described a fruity or ester-like odor that commonly developed in chilled fish muscle during the early stages of spoilage. This distinctive odor, encountered more often on commercially prepared fillets rather than round or eviscerated fish, was reproduced in sterile fish tissue and fish media by bacterial cultures isolated from fish. The causative bacterial species was identified as *Pseudomonas fragi*, a psychrophilic organism which utilizes a variety of amino acids for odor production (4, 5).

This study was initiated to identify the volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *P. fragi*. Particular emphasis was placed on the identification of the compounds responsible for the characteristic fruity odor.

MATERIALS AND METHODS

Sterile muscle tissue. Sterile fish muscle was obtained from black rockfish (*S. melanops*) by a modified method of Lobben and Lee (10) as described by Miller et al. (13).

Cultures and cultural conditions. *Pseudomonas* type III no. 18, obtained from J. M. Shewan at the Torry Research Station, Aberdeen, Scotland, was reclassified and designated a type II pseudomonad, *P. fragi* strain 18. An additional strain of *P. fragi* was obtained from the stock culture collection, Department of Microbiology, Oregon State University. Cells of each strain, grown on Trypticase soy agar (BBL) plates for 48 h at 25 C, were collected and suspended in sterile, distilled water. Sterile muscle tissue (pH 6.4-6.7) was homogenized, inoculated, and dispensed in screw-capped vials as reported previously (13). An additional muscle homogenate, adjusted to pH 7.3 with sterile NaOH, was treated as described above. Duplicate samples (pH 6.4-6.7 and pH 7.3), supplemented with 0.2% ethyl alcohol, were also prepared. Sterile homogenized milk and milk fortified with 0.2% ethyl alcohol were prepared by autoclaving at 121 C for 10 min.

All sample vials and appropriate controls were incubated at 5, 15, and 25 C and checked periodically for odor production and microbial counts. When the fruity odor was pronounced, the contents of selected vials were analyzed by combined gas-liquid chromatography and mass spectrometry.

Gas-liquid chromatography and mass spectral analyses. The gas chromatographs, mass spectrometer, chromatographic columns, and methods of sample preparation and analysis used for the separation and identification of aliphatic methylamines and other low-boiling compounds were reported previously (11-13).

RESULTS AND DISCUSSION

An ester-like or fruity odor was produced by *P. fragi* strain 18 in sterile fish muscle (*S. melanops*) incubated at 5, 15, and 25 C. The characteristic odor developed more rapidly at the higher temperatures and was gradually superceded by a distinct sulfide odor. Since strain 18 did not reduce trimethylamine oxide to trimethylamine, no typical amine odor was apparent.

[1] Technical paper no. 3506, Oregon Agricultural Experiment Station, Oregon State University, Corvallis, Ore. 97331.

Preliminary gas chromatographic analyses of the volatiles produced by strain 18 in sterile muscle tissue at pH 6.4 to 6.7 and pH 7.3 indicated limited concentrations of several components that were suggestive of ethyl esters on the basis of past experience. In an attempt to enhance ester production, the sterile fish homogenates were supplemented with 0.2% ethyl alcohol, and the volatile compounds produced by strain 18 were identified by gas-liquid chromatography and mass spectrometry after 4, 8, and 12 days incubation at 15 C. Compounds positively identified and listed in Table 1 included dimethyl sulfide, acetaldehyde, ethyl acetate, ethyl alcohol, and dimethyl disulfide. Methyl mercaptan, ethyl butyrate, ethyl hexanoate, and butanone were tentatively identified by relative retention times of the authentic compounds. Limited amounts of acetone were detected in the distilled water used for all analyses, and methylene chloride was considered a persistent contaminant in the atmosphere in which the samples were prepared and analyzed. The microbial count in fish homogenates (pH 6.4–6.7), supplemented with 0.2% ethyl alcohol, increased from 4.0×10^6 cells/g at 0 days to 1.1×10^{10} cells/g at 8 days.

The olfactory evaluation of each component, eluting from the column, was facilitated by a splitter which was attached to the effluent end of the column (13). The compound that eluted with a retention time almost identical to that of ethyl butyrate had a strong, fruity odor but, because of the limited concentration, the mass spectrum was weak. Although the parent ion for ethyl butyrate, *m/e* 116, was not discernible, the relative intensities observed for *m/e* 29, 60, 71, and 88 were strongly suggestive of an ethyl ester. Ethyl hexanoate was tentatively identified by relative retention time. A retention time of 99.7 cm for the authentic compound compared reasonably well with the retention time of 98.6 cm recorded for the peak in question.

The fruity aroma produced by strain 18 in fish during the early stages of spoilage or incubation was attributed to a synergistic flavor interaction involving ethyl acetate, ethyl butyrate, and ethyl hexanoate (17). The strong sulfide odor that persisted during continued incubation was the result of marked increases in methyl mercaptan (tentative identification), dimethyl sulfide, and dimethyl disulfide.

Castell et al. (4) reported that lipolytic and nonlipolytic strains of *P. fragi* isolated from fish produced fruity odors primarily from monoamino monocarboxylic acids. It was suggested that the fatty acids were produced by a number of different reactions involving deamination, whereas decarboxylation could result in the formation of the necessary primary alcohol. Dimethyl sulfide may be derived from methionine (6) or dimethyl-β-propiothetin (1, 8) and methyl mercaptan and dimethyl disulfide could be formed as reported previously (13).

TABLE 1. *Volatile compounds produced by P. fragi strain 18 in sterile fish muscle (S. melanops) incubated at 15 C*

Compound	Method of Identification
Methyl mercaptan	RT
Dimethyl sulfide	RT-MS[a]
Acetaldehyde	RT-MS
Ethyl acetate	RT-MS
Ethyl alcohol	RT-MS
Ethyl butyrate	RT
Butanone	RT
Dimethyl disulfide	RT-MS
Ethyl hexanoate	RT

[a] RT, retention time; MS, mass spectrum. Column (3.7 m by 3 mm, outer diameter) containing Celite 545 (60–80 mesh) coated with 20% 1,2,3,-tris(cyanoethyoxy)propane.

Repeated attempts to produce fruity odors in sterile fish muscle inoculated with the strain of *P. fragi* obtained from the Department of Microbiology at this institution were unsuccessful. Apparently, this strain lost its ability to produce esters. Strong, fruity odors are characteristic of new isolates of *P. fragi*, but the ability to form esters is easily lost by continued subculturing of the organism under laboratory conditions. However, in some cases, ester production can be restored by growing the organism on a medium containing the necessary or suitable substrates.

Since authentic strains of *P. fragi* characteristically produce a fruity aroma in many dairy products (7, 14, 17), *P. fragi* strain 18 was also cultured in sterile, homogenized milk supplemented with 0.2% ethyl alcohol. A typical flame-ionization detector chromatogram of the volatiles produced by strain 18 in milk incubated at 15 C for 4 days is illustrated in Fig. 1. Compounds identified are listed as follows with respective peak numbers: (1) methyl mercaptan (tentative identification), (2) dimethyl sulfide, (3) acetaldehyde, (4) ethyl acetate and acetone, (5) ethyl alcohol, (6) not identified, (7) ethyl butyrate, (8) dimethyl disulfide, (9) not identified, (10) ethyl hexanoate, and (11) heptanone. The small peak immediately after peak 6 had a retention time identical to that recorded for 2-butanone. Bills and Day (2) reported that acetaldehyde, dimethyl sulfide, butanone, and acetone are usually present in milk. Although

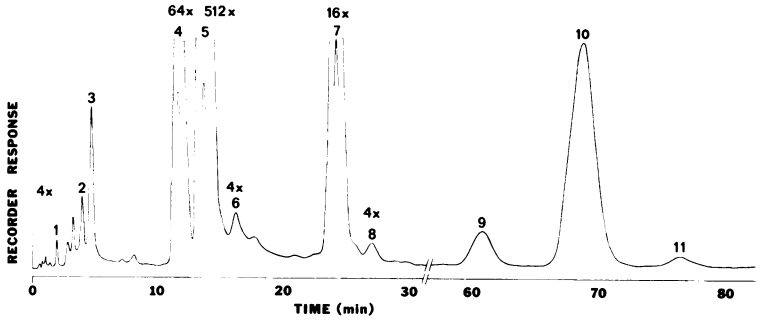

FIG. 1. *Flame-ionization detector chromatogram of the volatiles produced by P. fragi strain 18 in homogenized milk incubated at 15 C for 4 days. Column: 20% 1,2,3,-tris(cyanoethyoxy)propane (3.7 m by 3 mm, outer diameter).*

the concentrations of the latter three compounds remained relatively constant throughout the 4-day incubation period, acetaldehyde increased between 12 to 24 h and then decreased substantially with continued incubation. An increase and subsequent decrease in acetaldehyde content during the early phases of incubation were also observed in fish homogenates inoculated with strain 18. Keenan et al. (9) previously observed a very active reduction of acetaldehyde to ethyl alcohol by several pseudomonads, including *P. fragi*. Marked increases in the concentrations of ethyl acetate, ethyl butyrate, and ethyl hexanoate were noted between 1 and 4 days of incubation at 15 C, and the ratio of peak areas of the esters at 4 days was approximately 4:2:1, respectively (Fig. 1). The microbial count in homogenized milk increased from 4.9×10^5 cells/g at 0 days to 2.4×10^8 cells/g at 5 days, and a reasonable correlation with the production of esters was observed. In addition, there was no evidence of bacterial growth or ester formation in the uninoculated controls. The fruity aroma produced by *P. fragi* strain 18 in milk was due primarily to the production of ethyl butyrate and ethyl hexanoate. These results correlated well with data previously reported for recognized strains of *P. fragi* cultured in milk (16) and, therefore, further substantiated the reclassification of *Pseudomonas* type III no. 18 to a type II pseudomonad, *P. fragi*.

Although *P. fragi* strain 18 produced a fruity aroma in homogenized milk and sterile fish muscle, quantitative differences in the resultant ethyl esters were quite apparent. Appreciable amounts of ethyl acetate, ethyl butyrate, and ethyl hexanoate were produced in homogenized milk (Fig. 1). In contrast, ethyl acetate was the major ester produced in sterile fish muscle, and only limited concentrations of ethyl butyrate and ethyl hexanoate were detected. Therefore, it is quite apparent that ester production can be influenced considerably by the medium or available substrates (15).

The data presented above indicate that *P. fragi*, the cause of the fruity defect in dairy products, apparently plays a similar role in the spoilage of chilled fish muscle. Although sterile fish muscle homogenates were used in this investigation, the fruity and sulfide odors produced by *P. fragi* have been associated with naturally spoiling fish (6) and were also reproduced in sterile muscle blocks (6) as well as on heat-sterilized muscle and autoclaved fish media (3).

ACKNOWLEDGMENTS

This investigation was supported by a National Oceanic and Atmospheric Administration (maintained by the U.S. Department of Commerce) Institutional Sea Grant 04-3-158-4.

LITERATURE CITED

1. Ackman, R. G., J. Hingley, and K. T. MacKay. 1972. Dimethyl sulfide as an odor component in Nova Scotia fall mackerel. J. Fish. Res. Bd. Can. **29**:1085–1088.
2. Bills, D. D., and E. A. Day. 1966. Dehydrogenase activity of lactic streptococci. J. Dairy Sci. **49**:1473–1477.
3. Castell, C. H., and M. F. Greenough. 1957. The action of *Pseudomonas* on fish muscle. I. Organisms responsible for odours produced during incipient spoilage of chilled fish muscle. J. Fish. Res. Bd. Can. **14**:617–625.
4. Castell, C. H., and M. F. Greenough. 1959. The action of *Pseudomonas* on fish muscle. IV. Relation between substrate concentration and the development of odours by *Pseudomonas fragi*. J. Fish. Res. Bd. Can. **16**:21–31.
5. Castell, C. H., M. F. Greenough, and J. Dale. 1959. The action of *Pseudomonas* on fish muscle. III. Identification of organisms producing fruity and oniony odours. J. Fish. Res. Bd. Can. **16**:13–19.
6. Herbert, R. A., M. S. Hendrie, D. M. Gibson, and J. M Shewan. 1971. Bacteria active in the spoilage of certain seafoods. J. Appl. Bacteriol. **34**:41–50.
7. Hussong, R. V., H. F. Long, and B. W. Hammer. 1937 Iowa Agr. Expt. Sta. Res. Bull. No. 225, 119–136. Cited in J. Fish. Res. Bd. Can. **14**:617–625.
8. Kadota, H., and Y. Ishida. 1972. Production of volatile sulfur compounds by microorganisms. Annu. Rev Microbiol. **26**:127–138.

9. Keenan, T. W., D. D. Bills, and R. C. Lindsay. 1967. Dehydrogenase activity of *Pseudomonas* species. Appl. Microbiol. **15**:1216–1218.
10. Lobben, J. C., and J. S. Lee. 1968. Roles of microorganisms in the deterioration of rockfish. Appl. Microbiol. **16**:1320–1325.
11. Miller, A. III, R. A. Scanlan, J. S. Lee, and L. M. Libbey. 1972. Quantitative and selective gas chromatographic analysis of dimethyl- and trimethylamine in fish. J. Agr. Food Chem. **20**:709–711.
12. Miller, A. III, R. A. Scanlan, J. S. Lee, and L. M. Libbey. 1972. Volatile compounds produced in ground muscle tissue of canary rockfish (*Sebastes pinniger*) stored on ice. J. Fish. Res. Bd. Can. **29**:1125–1129.
13. Miller, A. III, R. A. Scanlan, J. S. Lee, L. M. Libbey, and M. E. Morgan. 1973. Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas perolens*. Appl. Microbiol. **25**:257–261.
14. Pereira, J. N., and M. E. Morgan. 1958. Identity of esters produced in milk cultures of *Pseudomonas fragi*. J. Dairy Sci. **41**:1201–1205.
15. Reddy, M. C., D. D. Bills, and R. C. Lindsay. 1969. Ester production by *Pseudomonas fragi*. II. Factors influencing ester levels in milk cultures. Appl. Microbiol. **17**:779–782.
16. Reddy, M. C., D. D. Bills, R. C. Lindsay, L. M. Libbey, A. Miller III, and M. E. Morgan. 1968. Ester production by *Pseudomonas fragi*. I. Identification and quantification of some esters produced in milk cultures. J. Dairy Sci. **51**:656–659.
17. Reddy, M. C., R. C. Lindsay, and D. D. Bills. 1969. Ester production by *Pseudomonas fragi*. III. Synergistic flavor interaction of esters at subthreshold concentrations. J. Dairy Sci. **52**:1198–1201.