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Characterization of Hydrogen Sulfide-Producing Bacteria Isolated from Meat and Poultry Plants

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A survey of the types of aerobic organisms able to produce H_2S on peptone iron agar (Levin, 1968), and commonly occurring in meat and poultry plants, revealed that these could be divided into four distinct groups. The ability of representative strains of each type to grow at low temperatures and cause off-odors on chicken muscle was examined. The results are discussed in relation to the role of these organisms in the psychrophilic spoilage of meat and meat products.

Recent work on the bacteriological spoilage of flesh foods has indicated that only a fraction of the total microflora is capable of producing the organoleptic changes associated with spoilage (2, 11). Several workers have emphasized the importance of attack on low-molecular-weight compounds (1, 13) and others indicated that off-odors are evident before extensive proteolysis takes place (18). In particular, the association of sulfide-producing organisms with spoilage has been noted (7, 11, 15, 19, 21).

There is no concise data in the literature about the genera and species of organisms that produce sulfides, and which commonly occur on meat and poultry. This study was undertaken to provide adequate characterization of these organisms as a basis for further detailed studies.

MATERIALS AND METHODS

Origin and isolation of strains. The sources of the strains are given in Table 1. Peptone iron agar (PIA; Difco) developed by Levin (19) was used as a direct diagnostic plating medium for the recovery of hydrogen sulfide-producing strains. These formed black colonies due to the formation of ferrous sulfide.

Plant surfaces were sampled by the swab-rinse technique and poultry carcases by the cut and rinse method described by Patterson (23). Other product samples (fresh and aged beef) were homogenized in saline peptone solution using a Colworth Stomacher (A. J. Seward and Co. Ltd., London) as described by Sharpe and Jackson (25). Serial 10^{-1} dilutions were prepared from this homogenate.

Pour plates of various dilutions were made in PIA and incubated for 3 days at 22 C. Black colonies were removed and purified by streaking on further plates of PIA. The 159 cultures isolated were maintained on PIA slopes at 4 C.

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Morphological features. Media for morphological and cultural examinations were based on nutrient broth (NB) (weight/volume, Lab Lemco [Oxoid L20]), 1.0%; peptone (Oxoid L37), 1.0%; NaCl, 0.5%. Eighteen-hour nutrient agar (NA) slope cultures were gram stained as described by the Society of American Bacteriologists (30) and motility was determined by the hanging drop technique. Size, shape, and cellular arrangement of stained and living preparations were noted. Representative strains of each group of organisms were stained for flagella by the Leifson technique (22). Best results were obtained by allowing a contact time of 30 s after appearance of a fine precipitate on the slide.

Colonial morphology and broth characteristics were described after 3 days at 22 C on NA or in NB (30).

Biochemical tests. All tubes were inoculated with 0.1 ml of a 24-h NB culture, plates and slopes being streaked from a similar source. The temperature of incubation for all tests was 30 C, the period being varied according to the test. Details of the tests employed are shown in Table 2.

Carbohydrate reactions. The ability of strains to produce acid and/or gas from glucose, maltose, sucrose, and lactose was examined in Andrade peptone water. The carbohydrate solutions were sterilized by filtration and added to the basal medium to give a final concentration of 1.0% (wt/vol).

Sole sources of nitrogen. Sulfur-containing nitrogenous compounds known to occur in meats (methionine, cystine, glutathione, taurine) were tested as sole sources of nitrogen. Filter-sterilized solutions of each N source (0.01 M) were added to a basal medium chosen to simulate the carbohydrate and salts composition of meat (16). Solutions used contained the following: solution A, $NaHPO_4/KH_2PO_4$ buffer (0.1 M, pH 6.8); solution B, $MgSO_4 \cdot 7H_2O$ (2.0 g/liter), $CaCl_2 \cdot 2H_2O$ (0.7 g/liter), $ZnSO_4 \cdot 7H_2O$ (0.5 g/liter), $FeSO_4 \cdot 7H_2O$ (0.05 g/liter), $CoSO_4$ (0.05 g/liter); solution C, glucose (0.1 g/liter), glycogen (1.0 g/liter), sodium lactate (18 ml/liter) (50% wt/wt solution).

Solution A was made up in 950 ml of distilled

TABLE 1. *Source and distribution of isolates*

Source	No. of isolates from source	*Pseudomonas putrefaciens*	*Proteus* sp.	*Citrobacter freundii*	Coryneform isolates
Refrigerated steak	26	26			
Beefburger	8			1	7
Frozen broilers	52	12	22	10	8
Trussing area swabs (poultry plant)	36	3	22	8	3
Packing area swabs (poultry plant)	15	1	3	11	
Hand swabs (personnel; meat and poultry plants)	13	6		6	1
Miscellaneous swabs (meat and poultry plants)	9	1	6	2	

TABLE 2. *Description of biochemical tests employed*

Test	Growth medium	Incubation period (days)
Oxidase test (14)	NA	4
Oxidative/fermentative attack on glucose	Hugh and Leifson medium (12)	4
Phosphatase production	Phenolphthalein phosphate agar (3)	4
Indole production	1.0% tryptone water	4
Methyl red and Voges Proskaeur tests	Glucose phosphate broth	4
Citrate utilization	Simmon's citrate agar (28)	7
Nitrate reduction	0.1% (wt/vol) KNO_3 peptone water	4
Phenylalanine deamination	Phenylalanine agar (9)	7
Urease production	Christensen's urea agar (8)	7
Arginine decomposition	Thornley's arginine medium (31)	14
Lecithinase hydrolysis	Egg yolk agar	4
Deoxyribonuclease production	Deoxyribonuclease agar (Difco)	4
Caseolysis	20% (vol/vol) skim milk agar	4
Starch hydrolysis	1% (wt/vol) starch nutrient agar	4
Tributyrin hydrolysis	Tributyrin agar (Oxoid)	4
Tween-80 hydrolysis	Tween-80 agar (27)	7
Gelatin liquefaction	Nutrient gelatin	14
2-Ketogluconate production	1% Na gluconate broth	4

water, and 40 ml of solution C and 10 ml of solution B were added.

Computation of results. The results which were useful for differentiation were resolved into 58 features and presented as a table of strains versus features. The data were analysed by the program CLASP by which pairs of strains in all possible combinations were compared in turn. Similarity was defined as $n_s/(n_s + n_d)$ (n_s = ++, n_d = +−). Negative matches were not counted (29). Strain numbers (123) were included in the computation; the remaining 36 isolates had identical responses with other isolates.

Excision of sterile muscle sections. Sections of sterile muscle were excised from chicken breast (supra coracoid, pectoral proper) using a modification of the technique described by Sharp (24) and Gardner and Carson (10).

Breast skin was carefully removed using sterile instruments and the underlying tissue was painted with aged, saturated solutions of crystal violet and brilliant green which were allowed to dry for 2 h. The muscle was not flamed as this procedure was found to cook into the depth of the tissue. The painted portion was sliced away using sterile instruments and large sections of the underlying muscle were excised and placed in sterile petri dishes. The large sections were cut into smaller portions (2 g) using sterile scissors and were stored in sterile screw-capped bottles. Sections were kept at refrigeration temperatures for at least 14 days and were examined visually and olfactorily before use. Five-milliliter quantities of nutrient broth were added to 10% of the sections and incubated at 22 C as sterility controls.

Ability of representative strains to produce off-odors. The ability of 15 pure cultures representing the various groups of organisms (Table 3) to produce off-odors was tested as follows. Cultures were grown in NB for 2 days at 22 C, harvested by centrifugation, washed, and resuspended in sterile phosphate buffer (0.1 M, pH 6.8) to give a final concentration of approximately 5×10^4 cells/ml. Sterile muscle sections were inoculated with 0.1-ml quantities and incubated at 5 C for 14 days. Sections were examined sensorily at 7 and 14 days for evidence of spoilage.

Growth rates at 5 C. The growth of representative organisms from the major groups at refrigeration

temperatures was measured using a nephelometer (EEL, Evans Electroselemium Ltd., Halstead, Essex). Optical density measurements of NB cultures were recorded at inoculation and on alterate days for 14 days.

RESULTS

The results of the computer analysis (summarized in Table 3) show mean similarities within and between the groups formed. The organisms within each group were recognized as follows: group 1, *Pseudomonas putrefaciens*; group 2, *Proteus* sp. (*Proteus mirabilis* and *Proteus vulgaris*); group 3, *Citrobacter freundii*; group 4, coryneform types. Summary characterizations of the groups are given in Table 4.

In this survey, 48 of the 159 cultures isolated were strains of *P. putrefaciens*. These formed a very distinct group ($\bar{S}$ = 87.0). The distribution of the strains can be seen in Table 1. It is of interest to note that all 26 isolates from spoiling steak stored at 5 C were *P. putrefaciens*. Other sources included frozen and chilled eviscerated chickens, poultry plant personnel, and equipment.

The *P. putrefaciens* strains all grew well at 5 C and much more quickly at this temperature than the other H_2S-producing types isolated. All five representative strains caused a typically sulfide-like spoilage odor when grown on sterile chicken muscle. Off-odors were detectable organoleptically after 7 days storage at 5 C.

The group 2 strains all produced the characteristic swarming colonies of *Proteus* species. The group (53 strains) was distinct with an intragroup mean similarity of 83.2. All but three of the strains were recognized as *P. mirabilis*. The remaining three correspond to the description of *P. vulgaris* (6) and were grouped together at one extreme of the *Proteus* group.

Growth at 5 C was recorded for 29 of 53 *Proteus* strains and four representative isolates developed faster than the *Citrobacter* types at this temperature, although much slower than *P. putrefaciens*. Despite the relatively slow growth rate at low temperatures, all *Proteus* strains tested produced the same characteristic odor. This was not recognized as sulfide-like but was described as "caramel" or "burnt sugar." (These strains liberate H_2S when cultured at 5 C.)

The strains contained in group 3 were recognized as *C. freundii* (36 organisms, $\bar{S}$ = 82.2). Two anaerogenic types were included. Again, in relation to *P. putrefaciens*, these strains grow slowly at 5 C and only slight sulfide-like odors were detectable with two of the four representative strains tested after storage of inoculated chicken muscle for 14 days at 5 C.

The coryneform organisms formed the least

TABLE 3. *Mean similarity within and between groups formed*

Group[a]	1	2	3	4
1	87.0			
2	30.1	83.2		
3	29.6	44.8	82.2	
4	20.4	16.7	26.4	64.1

[a] 1, *Pseudomonas putrefaciens*; 2, *Proteus*; 3, *Citrobacter freundii*; 4, coryneforms.

TABLE 4. *Number of strains in each group giving positive response to tests indicated*[a]

Test	No. of strains in group			
	Group 1 (49)	Group 2 (53)	Group 3 (38)	Group 4 (19)
Gram negative	+	+	+	−
Coryneform arrangement	−	−	−	+
Motile	+	+	+	−
Colony pinpoint	−	−	−	+
Colony circular	+	−	+	+
Colony convex	+	−	+	+
Brown pigmented	+	−	−	−
Swarming growth	−	+	−	−
Growth at 4 C	+	29	33	5
Growth at 37 C	−	+	+	+
NH_4 (sole source N)	+	+	+	−
Methionine (sole source N)	4	47	30	−
Cysteine (sole source N)	−	9	+	−
Glutathione (sole source N)	2	52	35	−
Taurine (sole source N)	1	1	−	−
Oxidase positive	+	−	−	8
Fermentative on glucose	−	+	+	16
Phosphatase positive	+	44	−	3
Indole produced	−	3	−	−
Methyl red positive	1	4	+	14
Voges Proskauer positive	−	22	1	5
Citrate (sole source C)	4	+	+	−
NO_3 reduced	45	+	+	1
Phenylalanine deaminated	−	+	−	−
Urease produced	4	+	31	1
Arginine alkaline	−	3	−	2
Lecithinase produced	19	3	−	1
Deoxyribonuclease produced	43	2	−	3
Caseolysis	46	43	−	3
Tributyrin hydrolysed	46	49	−	2
Tween 80 hydrolysed	46	28	−	1
2-Ketogluconate produced	1	46	−	−
Gelatin liquefied	44	51	9	2
Glucose acid	−	+	+	+
Glucose gas	−	+	+	−
Maltose acid	−	3	+	6
Maltose gas	−	3	+	−
Lactose acid	−	−	9	5
Lactose gas	−	−	9	−
Sucrose acid	−	2	28	14
Sucrose gas	−	2	28	−

[a] Symbols: +, all strains in group positive; −, all strains in group negative; No., number of strain in group positive.

distinct group; 19 strains were included in a group with an intragroup mean similarity of 64.1% S. The strains grew only very slowly if at all at 5 C and representative types did not produce any detectable off-odor after incubation on sterile chicken muscle for 14 days at 5 C.

Seven of the eight strains isolated from beefburgers were coryneform types. It was not, however, possible to test these for spoilage ability against beefburger as sterile sections of this product could not be obtained. In view of their slow growth rate at low temperatures, members of this group are unlikely to be important spoilage agents of refrigerated meat products.

DISCUSSION

The results obtained indicate that a restricted range of bacterial types capable of producing detectable amounts of hydrogen sulfide on PIA occurs in the environs of meat and poultry plants. Of these, *P. putrefaciens* is recognized as potentially the most important spoiler. Both *C. freundii* and coryneform types are discounted as troublesome at 5 C. At higher temperatures, however, it is likely that these might develop much more rapidly with the subsequent onset of sulfide-like odors. *Proteus* strains provide apparently anomalous results; although developing only slowly at refrigeration temperatures, they produce a characteristic spoilage of chicken muscle which was not recognized organoleptically as sulfide-like.

The role of *P. putrefaciens* as a spoilage organism is well documented. A number of workers have noted the presence of this organism and its role in the spoilage of chicken carcases (4, 5). Lea and co-workers (17), however, concluded that *P. putrefaciens* and a pigmented pseudomonad produced changes little greater than those observed during sterile autolysis. All *P. putrefaciens* strains examined in this study produced potent off-odors from chicken muscle. *P. putrefaciens* has also been widely implicated in the spoilage of fish and fishery products (7, 11, 19).

Other *Pseudomonas* strains have been reported in the literature as producers of sulfides and sulfide-like odors. Nichol et al. (21) recorded the formation of sulfmyglobin in prepacked beef by *P. mephitica*, but showed that sulfides were only produced under conditions of low oxygen tension. *P. perolens* was shown to produce a number of volatile sulfides when grown on sterile fish muscle (20) whereas *P. putida*, *Pseudomonas* group 1 (26), *P. fragi*, *Pseudomonas* group II, and *Pseudomonas* group III/IV types possibly similar to *P. putrefaciens* also caused sulfide-like odors in fish muscle (11). In this study, the only sulfide-producing pseudomonad recovered from sources within meat and poultry plants was *P. putrefaciens*.

Members of the *Enterobacteriaceae* have been shown to constitute a considerable portion of the flora of poultry carcases when the temperature is allowed to rise to 10 to 15 C (5). This study confirms that normal refrigeration temperatures permit at most only slow growth of the *Citrobacter* and *Proteus* types isolated. However, the latter are able to cause a characteristic off-odor on chicken muscle at 5 C in pure culture. The inherently slow growth rate, however, probably limits development of *Proteus* species in competition with more psychrotolerant organisms. Studies are being carried out to determine the number of cells of *Proteus* species required to cause off-odors in pure culture. Their development and the nature of spoilage in association with the faster growing *P. putrefaciens* is also of interest. It is possible that the odiferous compounds produced by *Proteus* strains are detectable organoleptically at extremely low levels and that relatively few cells are required to attain these levels.

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LITERATURE CITED

1. Adamcic, M., D. S. Clarke, and M. Yaguchi. 1970. Effect of psychrotolerant bacteria on the amino acid content of chicken skin. J. Food Sci. **35**:272-275.
2. Adams, R., L. Farber, and P. Lerke. 1964. Bacteriology of spoilage of fish muscle. II. Incidence of spoilers during spoilage. Appl. Microbiol. **12**:277-279.
3. Barber, M., and S. W. A. Kuper. 1951. Identification of *Staphylococcus pyogenes* by the phosphatase reaction. J. Path. Bacteriol. **63**:65-68.
4. Barnes, E. M., and C. S. Impey. 1968. Psychrophilic spoilage bacteria of poultry. J. Appl. Bacteriol. **31**:97-107.
5. Barnes, E. M., and M. J. Thornley. 1966. The spoilage flora of eviscerated chickens stored at different temperatures. J. Food Technol. **1**:113-119.
6. Breed, R. S., E. G. D. Murray, and N. R. Smith (ed.). 1957. Bergey's manual of determinative bacteriology, 7th ed. Balliere, Tindall and Cox, London.
7. Chai, T., C. Chen, A. Rosen, and R. E. Levin. 1968. Detection and incidence of specific species of spoilage bacteria on fish. II. Relative incidence of *Pseudomonas putrefaciens* and fluorescent pseudomonas on haddock fillets. Appl. Microbiol. **16**:1738-1741.
8. Christensen, W. E. 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon bacteria from each other and from *Salmonella* and *Shigella* types. J. Bacteriol. **52**:461-466.
9. Ewing, W. H., B. R. Davis, and R. W. Reavis. 1957. Phenylalanine and malonate media and their use in enteric bacteriology. Public Health Lab. **15**:153-167.

10. Gardner, G. A., and A. W. Carson. 1967. Relationship between carbon dioxide production and growth of pure strains of bacteria on porcine muscle. J. Appl. Bacteriol. **30**:500–510.
11. 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.
12. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol. **66**:24–26.
13. Jay, J. M., and K. S. Kontou. 1967. Fate of free amino acids and nucleotides in spoiling beef. Appl. Microbiol. **15**:759–764.
14. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature (London) **178**:703.
15. Kraft, A. A., A. W. Brant, and J. C. Ayres. 1956. Detection of hydrogen sulfide in packaged meats and in broken-out shell eggs. Food Technol. **10**:443–444.
16. Lawrie, R. A. 1966. Meat science, 1st ed., p. 67. Pergamon Press, Oxford.
17. Lea, C. H., B. J. H. Stevens, and M. J. Smith. 1969. Chemical and organoleptic changes in poultry meat resulting from the growth of psychrophilic spoilage bacteria at 1 C. Br. Poult. Sci. **10**:203–217.
18. Lerke, P., L. Farber, and R. Adams. 1967. Bacteriology of spoilage of fish muscle. IV. Role of protein. Appl. Microbiol. **15**:770–776.
19. Levin, R. E. 1968. Detection and incidence of specific species of spoilage bacteria on fish. I. Methodology. Appl. Microbiol. **16**:1734–1737.
20. 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.
21. Nicol, D. J., M. K. Shaw, and D. A. Ledward. 1970. Hydrogen sulfide production by bacteria and sulfmyoglobin formation in prepacked chilled beef. Appl. Microbiol. **19**:937–939.
22. Norris, J. R., and H. Swain. 1971. Staining bacteria, p. 105–134. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5A. Academic Press Inc., London.
23. Patterson, J. T. 1971. Microbiological assessment of surfaces. J. Food Technol. **6**:63–72.
24. Sharp, J. G. 1963. Aseptic autolysis in rabbit and bovine muscle during storage at 37 C. J. Sci. Food Agric. **14**:468–479.
25. Sharpe, A. N., and A. K. Jackson. 1972. Stomaching: a new concept in bacteriological sample preparation. Appl. Microbiol. **24**:175–178.
26. Shewan, J. M., G. Hobbs, and W. Hodgkiss. 1960. A determinative scheme for the identification of certain genera of Gram negative bacteria, with special reference to the *Pseudomonadaceae*. J. Appl. Bacteriol. **23**:379–390.
27. Sierra, G. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations of the influence of contact between cells and fatty acid substances. Antonie van Leeuwenhoek J. Microbiol. Serol. **23**:15–22.
28. Simmons, J. S. 1926. A culture medium for differentiating organisms of the typhoid-colon aerogenes groups and for identification of certain fungi. J. Infect. Dis. **39**:209–214.
29. Sneath, P. H. A. 1957. The application of computers to taxonomy. J. Gen. Microbiol. **17**:201–226.
30. Society of American Bacteriologists. 1957. Manual of microbiological methods, p. 16, 148–149. McGraw-Hill Inc., London.
31. Thornley, M. J. 1960. Differentiation of *Pseudomonas* from other Gram negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. **23**:37–52.