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Identification of Isobutyronitrile and Isobutyraldoxime *O*-Methyl Ether as Volatile Microbial Catabolites of Valine

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G.l.c.–mass-spectral analysis of headspace above cultures of *Aeromonas* and *Moraxella* spp. indicates the presence of isobutyronitrile, isobutyraldoxime *O*-methyl ether, methacrylonitrile and possibly methacrylaldoxime *O*-methyl ether. Accumulation of these catabolites is maximal under low oxygen concentrations and is enhanced by enrichment of the medium with valine. Isobutyraldoxime *O*-methyl ether is established as the compound observed but not identified in previous studies with other bacterial species involved in spoilage of meat and chicken.

Combined g.l.c. and mass spectrometry has been employed in the analysis of volatile headspace components produced during meat spoilage by a variety of organisms (Gibbs *et al.*, 1979). Several of these organisms, i.e. *Aeromonas* sp. (strain no. 63), *Moraxella* sp. (strain no. 187) and *Alcaligenes* sp. (strain no. 420), produced an unidentified compound of mol.wt. 101 that gave a mass spectrum similar to that described by Freeman *et al.* (1976) for an unidentified volatile metabolite formed by cultures of strains of *Pseudomonas putida* and *Moraxella* sp. grown on irradiation-sterilized chicken. In the present paper we report the chemical identification of this compound and a number of related catabolites produced by two bacterial species when grown on nutrient agar under various conditions and the probable metabolic origin of such compounds.

Materials and Methods

Organisms and cultural conditions

Cultures (18h at 30°C in Oxoid CM 67 nutrient broth, pH7.0) of *Aeromonas* sp. (strain no. 63) and a '*Moraxella*'-like organism (strain no. 187) from previous studies (Patterson & Gibbs, 1977; Gibbs *et al.*, 1979) were transferred to nutrient agar (Oxoid CM) slopes or nutrient agar supplemented with L-valine (0.1%, w/v) in 30g screw-capped bottles. The black rubber liners of the bottles had been replaced by silicone/Teflon discs [Tuf-bond; Pierce and Worrimer (U.K.) Ltd., Chester, Cheshire, U.K.] before sterilization of the media. Various concentrations of O_2 in the gas atmospheres above the cultures (0, 2, 5, 10, 15 and 20%, v/v) were attained by flushing with sterile air/N_2 mixtures for several minutes before the bottles were sealed. Cultures were incubated for 11 days at 15°C before analysis of headspace.

Sampling and analysis of volatile metabolites in headspace

Culture bottles were equilibrated in a water bath at 40°C before being sampled. Volumes of from 0.01 to 2ml were then withdrawn with a Hamilton glass syringe from the headspace above bacterial cultures by insertion through the silicone/Teflon seal. Headspace above pure chemicals was extracted in a similar manner. Samples were injected into a Pye–Unicam 104 (model 74) gas chromatograph coupled via a jet separator inlet to a VG 16F mass spectrometer linked to a VG 2020 data-processing system. The gas chromatograph was equipped with a glass column (1.5m×3mm internal diam.) packed with Tenax GC (60–80 mesh) as stationary phase and operated at a helium gas flow rate of 30ml/min. The temperature of the chromatograph oven was programmed at a rate of 10°C/min from 80°C to 200°C. The mass spectrometer was operated in the electron-impact mode at an ionizing voltage of 70eV and an accelerating voltage of 4kV with the detector amplifier at a setting of 10^{-6}. The instrument was equipped with a strip-chart recorder to record gas chromatograms by monitoring the integrated ion current between *m/e* 35 and 150. Mass spectra of eluted g.l.c. peaks were stored in the data system and printed out textually by using a line printer terminal or in the form of a bar chart by a Bryans XY recorder.

Chemicals

Isobutyraldehyde and isobutyronitrile were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and methacrylonitrile was from Cambrian Chemical Co., Croydon, U.K. Isobutyraldehyde was converted into isobutyraldoxime by treatment with hydroxylamine hydrochloride by using a standard method and the oxime was purified by distillation under reduced pressure [b.p. 116–120°C/39.3kPa (295mmHg)].

The oxime (7g) was converted into the *O*-methyl derivative by refluxing for 24h in dry acetone (200ml) with Ag_2O (9.4g) and iodomethane (5ml). After removal of the acetone under reduced pressure, the residue was taken up in ether (200ml), and the insoluble *N*-methyl derivative (Sidgwick, 1966) was filtered off. The ethereal solution was then shaken with 2% (w/v) Na_2CO_3 (50ml) to extract any unchanged oxime. Ether was removed from the dried solution under reduced pressure, and the resulting oil was fractionally distilled twice in a semi-micro distillation apparatus to give 0.7g of isobutyraldoxime *O*-methyl ether [b.p. 76–78°C/2.3kPa (17mmHg)].

Results and Discussion

Headspace from *Moraxella* cultures grown on nutrient agar in air gave the gas-chromatographic trace shown in Fig. 1. Catabolite A, previously identified in analysis of cultures of this bacterium on meat (Gibbs *et al.*, 1979), is dimethyl sulphide and serves as an internal standard for measurement of retention times. Although catabolites B–E were produced under aerobic conditions, formation was maximal at O_2 concentrations between 0 and 2%. Samples of headspace from cultures of *Aeromonas* sp. grown under aerobic conditions also showed the presence of catabolite D. However, at O_2 concentrations between 2 and 10% catabolites B, C and E were also detected, and all four compounds displayed peaks of height comparable with those of the catabolites produced by *Moraxella* cultures. Relative proportions of the compounds were also broadly similar. Such catabolites were absent from *Aeromonas* cultures grown under completely anaerobic conditions.

Catabolites B and C gave mass spectra that showed a number of similarities. The spectrum of catabolite C exhibited a molecular ion at *m/e* 69 and a base peak at *m/e* 42. Major fragmentation peaks at *m/e* 68 (M^+-H), *m/e* 41 and *m/e* 54 (M^+-CH_3) were strongly suggestive of an α-substituted nitrile. The spectrum was found to be identical with that of an authentic sample of isobutyronitrile.

The spectrum of catabolite B showed a molecular ion at *m/e* 67, a major peak at *m/e* 66 (M^+-H) and a base peak at *m/e* 41, again suggesting a nitrile. A spectrum of an authentic sample of methacrylonitrile was similar in all respects to that of catabolite B.

Catabolite D displayed the spectrum shown in Fig. 2, which is identical with that of an unknown compound found in headspace of cultures of *Moraxella*, *Aeromonas* and *Alcaligenes* spp. on meat (Gibbs *et al.*, 1979). The spectrum was also similar to that described by Freeman *et al.* (1976) for an unidentified headspace component produced by *Pseudomonas putida* and a *Moraxella* sp. cultured on chicken tissue. The latter workers described major peaks in the mass spectrum at *m/e* values of 86, 70, 43, 42, 41 and 27, though they considered the molecular ion to be at *m/e* 102. However, it seems probable that the peak at this *m/e* value is the ^{13}C-isotope peak and that the molecular ion is represented by the peak at *m/e* 101. The loss of methyl and methoxyl fragments giving major fragmentation peaks at *m/e* 86 and *m/e* 70 followed by fragmentation reminiscent of a nitrile with peaks at *m/e* 55, 41, 42 and 43 suggests that catabolite D may in fact be the *O*-methyl ether of an oxime. The *O*-methyl ether of isobutyraldoxime was prepared and its retention time and mass spectrum

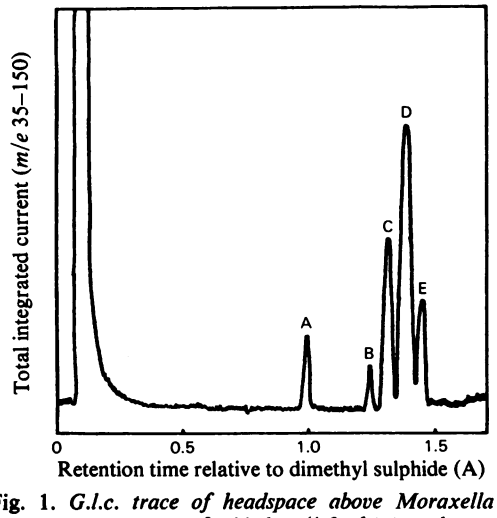

Fig. 1. *G.l.c. trace of headspace above Moraxella sp. grown on nutrient agar for* 11 *days* (1.0ml *injected at arrow*)
For experimental details see the text.

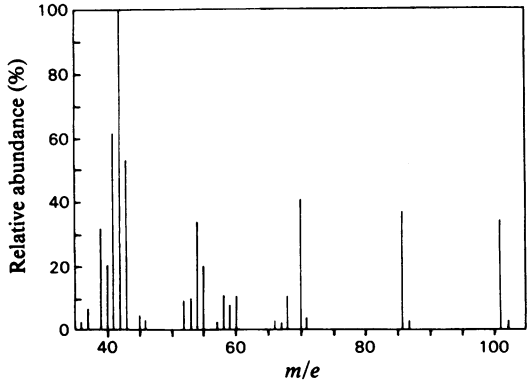

Fig. 2. *Mass spectrum of catabolite D*
For experimental details see the text.

were indeed found to be identical with those of catabolite D.

Though the mass spectrum of catabolite E (Fig. 3) exhibits a somewhat different pattern to that displayed by catabolite D, the presence in headspace of isobutyronitrile in association with isobutyraldoxime *O*-methyl ether suggests by analogy that the presence of methacrylonitrile would be accompanied by that of methacrylaldoxime *O*-methyl ether as catabolite E. In support of this postulate the spectrum of catabolite E shows a molecular ion at *m/e* 99 and a major fragmentation peak at *m/e* 41. However, major fragmentation peaks also occur at *m/e* 72, 71, 57, 40 and 39. Such fragments can be explained in terms of elimination of HCN from the molecular ion by an internal rearrangement involving methoxyl migration leading to the production of the species $CH_3–CH=\overset{+}{C}H–O–CH_3$ which then fragments via loss of H, CH_3, CH_3O or CH_3OH to give peaks at *m/e* 71, 57, 39 and 40 respectively. A rearrangement of this type is not favoured in the molecular ion of the saturated analogue D. Since the instability of preparative intermediates prevented synthesis of methacrylaldoxime *O*-methyl ether, the identification of catabolite E as this compound must remain tentative.

A possible catabolic origin of this group of related compounds is suggested by investigations by Hahlbrock *et al.* (1968) on the biosynthesis of linamarin, the glucoside of acetone cyanohydrin, in flax seedlings. Studies by these workers indicate that isobutyronitrile is an intermediate in the biosynthesis of linamarin from valine. Since valine is not present in high concentration in nutrient agar, the effect of enriching the medium with this amino acid on the formation of nitriles and oximes by cultures of *Moraxella* was determined. Cultures on such a medium showed greatly enhanced accumulation of these catabolites at all oxygen concentrations studied, suggesting that valine was indeed the probable precursor.

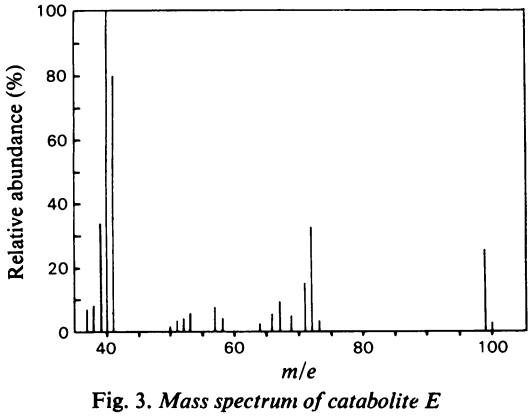

Fig. 3. *Mass spectrum of catabolite E*
For experimental details see the text.

The conversion of this amino acid into isobutyraldoxime *O*-methyl ether and the corresponding nitrile would involve the participation of several intermediates, and could include decarboxylation and *N*-methoxylation to produce the substituted oxime followed by elimination of methanol to form isobutyronitrile. α-Hydroxylation of the substituted oxime or the nitrile would yield compounds that on dehydration would give respectively methacrylaldoxime *O*-methyl ether or methacrylonitrile. This pathway bears similarities to that postulated by Hahlbrock *et al.* (1968), though in the latter case the oxime rather than the *O*-methyl oxime appears to be an intermediate. In none of the experiments described in the present paper was any trace of the unsubstituted oxime found, despite the fact that this compound is appreciably volatile and would have been readily detectable under the conditions employed. This observation suggests that unsubstituted oxime is not a free intermediate in the bacterial catabolic pathway.

Failure to detect the putative α-hydroxylated intermediate can probably be ascribed to the lower vapour pressure of this relatively more polar compound. It is perhaps worth noting that α-hydroxyisobutyronitrile, i.e. acetone cyanohydrin, will readily lose HCN under acid conditions, which could exist in cultures of spoilage bacteria. An elimination of this type might explain the detection of acetone in headspace from cultures of *Moraxella*, *Alcaligenes* and *Aeromonas* spp. on meat (Gibbs *et al.*, 1979), though other catabolic sequences could also give rise to this compound.

The formation of isobutyronitrile and isobutyraldoxime *O*-methyl ether and their unsaturated analogues by bacteria of a number of different genera suggests that this catabolic pathway may be of quite general occurrence among bacteria, particularly under low O_2 concentrations.

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