Coryneform Bacteria Producing Methane Thiol

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(Received 8 December 1975)

INTRODUCTION

Recent work on Cheddar cheese flavour has implicated methane thiol (CH_3SH) as an important constituent of Cheddar aroma (Manning & Robinson, 1973; Manning, 1974). To determine whether this compound might be a metabolite of the bacterial flora of the cheese, isolates from milk and Cheddar cheese were examined for their ability to produce methane thiol. Among such isolates were a number of methane thiol-producing coryneform bacteria. Although methane thiol-producing bacteria have been described previously (Kadota & Ishida, 1972), we are not aware of any reports that coryneform bacteria have this ability. Accordingly these organisms are described in this paper.

METHODS

Isolates. Strains were isolated from raw milk and from homogenates of cheese curd and Cheddar cheese by enrichment using a mineral mix [containing (%): K_2HPO_4 , 0.2; KH_2PO_4 , 0.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01; $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 0.001] plus 0.5 % DL-methionine, or by direct plating on milk agar (skim-milk, 30 %; agar, 2 %). All isolates were tested for the production of methane thiol from L-methionine (see below). Positive strains were examined microscopically, and representative coryneform bacteria were tested physiologically, and examined for the presence or absence of *meso* or LL-diaminopimelic (DAP) acids and of arabinose in the cell wall.

Laboratory strains. Reference strains of *Arthrobacter tumescens* NCIB8914, *Brevibacterium linens* NCIB8546 and non-pigmented grey-white strains of coryneform bacteria, previously isolated from Limburger and Meshanger cheese by Mulder *et al.* (1966) and further described by Crombach (1972, 1974), were included for comparison in many tests.

General culture of strains. Oxoid Nutrient Broth (NB) or Nutrient Agar (NA) were used. Cultures were incubated at 30 °C unless otherwise stated.

Tests for physiological characteristics

Catalase was detected after 2 days incubation in NB, using the method of Harrigan & McCance (1966).

Optimum growth temperatures were determined by measuring extinctions at 580 nm after growth in NB for 4 days at 22, 30, 37, and 40 °C (Lumetron Colorimeter model 401, Photovolt Corp., New York, U.S.A.).

Heat resistance: cultures (0.1 ml) were inoculated into NB, heated to 60 ± 0.1 °C for 30 min, and then plated on NA.

Morphological changes were observed on the EYGA medium described by Cure & Keddie (1972).

Aerobic growth: tested according to the method of Hugh & Leifson (1953).

Anaerobic growth: boiled yeast-glucose broth (YGB) (Naylor & Sharpe, 1958) was inoculated and incubated for 3 days in an anaerobic jar under 90 % hydrogen plus 10 % carbon dioxide.

Oxidase was detected by the method of Kovacs (1956).

Pigmentation: (i) pigment formation on milk agar was observed; (ii) organisms grown in NA were tested for the presence of a carotenoid-like pigment (Jones, Watkins & Erickson, 1973).

Proteolysis was tested using milk agar (clearing zones) and gelatin (liquefaction) as substrates.

Salt tolerance was tested using Owens & Keddie's (1969) Mineral Base E (prepared as described by Cure & Keddie, 1972), supplemented with 0.2 % (w/v) yeast extract, 0.1 % (w/v) glucose and 5, 6, 10, 12 or 15 % (w/v) NaCl.

Carbohydrate utilization: growth in Owens & Keddie's (1969) Mineral Base E, supplemented with 0.02 % (w/v) yeast extract and either 0.5 % (w/v) glucose, lactose, sucrose, glycerol or inulin, or 0.2 % (w/v) citrate, lactate or acetate, was measured as E_{580} .

Nutritional requirements. Owens & Keddie's (1969) Mineral Base E - N (which does not contain an ammonium salt) was supplemented with each of the following (% w/v): $(\text{NH}_4)_2\text{SO}_4$, 0.01; $(\text{NH}_4)_2\text{SO}_4$, 0.01, plus yeast extract, 0.2; methionine, 0.1; $(\text{NH}_4)_2\text{SO}_4$, 0.01, plus glucose, 0.1. Three serial transfers were made in each medium and the presence or absence of growth was observed visually.

Acid end-products from glucose were determined after strains had been growing in YGB for 3 days, by the method of Latham & Legakis (1976) using gas chromatography (GC).

Production of methane thiol from L-methionine. Strains grown on NA were suspended (to give an E_{580} of 10) in 0.05 M-Tris-HCl buffer, pH 8.0, with L-methionine (66 mM) and incubated at 30 °C for 2 to 6 h in a Conway diffusion unit, using the method of Ruiz-Herrera & Starkey (1969). Methane thiol was trapped in 5 % (w/v) mercuric acetate in 10 % (v/v) acetic acid and estimated colorimetrically (Sainsbury & Maw, 1967). To confirm that methane thiol was being produced during incubation, portions of the mercuric acetate were acidified with 2 M-HCl to release the thiol from the mercaptide, and the head space (5 ml) was analysed by GC (Manning, 1974) with authentic methane thiol as a reference.

Cell-wall analysis

Diamino acids. Organisms were grown on NA for 48 h and walls were prepared from whole cells by the rapid method of Schleifer & Kandler (1972). The sample was hydrolysed with 6 M-HCl at 100 °C for 6 h and examined for diamino acids, using the paper chromatographic method of Rhuland *et al.* (1955).

Carbohydrates. Using the method of I. J. Bousfield (personal communication), organisms were grown on NA for 48 h and walls prepared by digestion of washed whole cells with 5 % KOH for 5 min at 120 °C. The residual washed walls were hydrolysed with 2 M-HCl at 100 °C for 3 h and carbohydrates detected by thin-layer chromatography, as described by Lechevalier (1968). Corynebacteria with known wall compositions were included as controls.

RESULTS AND DISCUSSION

Isolates. Four strains producing methane thiol from L-methionine and isolated on different occasions from raw milk (strain R6, NCDO2051), cheese curd (C4, NCDO2050) and Cheddar cheese (CMD1, NCDO2048 and CMD3, NCDO2049) were examined. All were Gram-positive, club-shaped rather irregular rods, catalase positive, oxidase negative, non-motile,

non-sporing and grew well aerobically, but not at all anaerobically. The seven reference strains had these same characteristics.

Morphology. After 2 days, the club-shaped rods had become coccoid in all four strains.

Growth requirements. Only strains CMD1 and CMD3 were tested. They would not grow in the presence of added ammonium salt or L-methionine but grew after three transfers in the presence of ammonium salt plus yeast extract or ammonium salt plus glucose, indicating that exogenous organic growth factors were not required, and that they were nutritionally non-exacting.

Acid end-products from glucose. The only volatile acid produced was acetate. The absence of propionate, profuse aerobic growth and absence of anaerobic growth excluded relationships with propionibacteria.

Table 1 summarizes the characteristics of the isolates and of the reference strains. None of the reference strains produced methane thiol from L-methionine.

Possible classification of isolates

In the coryneform bacteria, the diamino acid present in the wall (*meso*-DAP, LL-DAP, ornithine, diaminobutyric acid or lysine), and the presence or absence of arabinose in walls containing *meso*-DAP, are characteristic for certain species or groups (Schleifer & Kandler, 1972). R. M. Keddle (personal communication) has devised a tentative scheme for the recognition of some taxa within the coryneform bacteria, based on these characteristics and other criteria. Our strains contained *meso*-DAP, but not arabinose in the cell wall (Table 1). Coryneform bacteria known to contain *meso*-DAP in their walls include *Corynebacterium sensu stricto*, the 'rhodochrous' complex (Goodfellow, Fleming & Sackin, 1972) and *B. linens*. The absence of arabinose in our strains excluded a relationship with the genus *Corynebacterium* or the 'rhodochrous' complex and suggested that they be compared with *B. linens*. The lack of relationship with *Corynebacterium* was confirmed by the presence of a growth cycle (rods $\rightarrow$ cocci) and the obligate aerobic nature of the growth in our isolates. In addition, M. Goodfellow (personal communication) has shown that our strains, like *B. linens*, but unlike *Corynebacterium* and the 'rhodochrous' complex, do not contain mycolic acids. Jones (1975) has grouped coryneform and related bacteria into a number of clusters by computer analysis, and there was a general but inconclusive trend for our isolates to show similarities to her clusters F4 and F5 which include strains of *B. linens*. Like *B. linens*, our strains also have a high degree of salt tolerance.

However our methane thiol-producing isolates differ from *B. linens* in that they do not produce the carotenoid-like orange pigment (Jones *et al.*, 1973) found in the latter. They differ from *B. linens* NCIB8546 in being proteolytic, utilizing lactate and sucrose, giving an acid and not an alkaline reaction in Hugh & Leifson medium, in having an optimum growth temperature between 30° and 37° C and in being able to grow at 40 °C. The grey-white cheese strains of Mulder *et al.* (1966) also differ from ours in cell-wall composition, optimum growth temperature, pigmentation and stimulatory effect of salt. Among these cheese strains, Crombach (1972, 1974) has distinguished two different groups by DNA homology and by physiological characteristics (strains AC253 and EC20 falling into one group, and AC256, AC263 and EC16 into the other) and these groups are confirmed here by their different cell-wall compositions, as Keddle has also noted (personal communication). Our strains also appear to be different from the four groups of coryneform bacteria isolated from milk and cheese by Jayne-Williams & Skerman (1966).

Although the methane thiol-producing strains are similar to *B. linens* in cell-wall composition, they differ in other respects. Bousfield (1972) points out that the taxonomy of the

Table 1. *Physiological characteristics and some wall constituents of methane thiol-producing coryneform bacteria and of reference strains*

	Strains isolated at NIRD from milk and cheese				<i>Arthro- bacter tumescens</i> NCIB8914	<i>Brevibac- terium linens</i> NCIB8546	Strains isolated by Mulder from cheese				
	R6	C4	CMD1	CMD3			AC253	EC20	AC256	AC263	EC16
Morphology: rods → cocci	+	+	+	+	+	+	+	+	+	+	+
Growth ($E_{580} \times 100$)											
at 22 °C	14	30	35	5	10	20	36	35	20	20	33
at 30 °C	80	60	88	85	15	5	43	40	19	30	33
at 37 °C†	68	80	76	76	0	0	0	0	0	0	0
at 40 °C‡	62	56	58	70	0	0	0	0	0	0	0
Salt tolerance											
highest % tolerated	15	15	15	15	< 5	12	10	10	12	12	12
highest % stimulatory	12	12	12	12	< 5	10	5	5	< 5	5	8
Proteolysis											
Milk	+	+	+	+	+	—	—	—	—	—	—
Gelatin	+	+	+	+	+	—	+	+	—	—	—
Heat resistance											
% surviving 30 min at 60 °C	7.5	6.2	0.9	0.5	0	0	0	0	0	0	0
Pigmentation											
Colour*	B	B	B	B	F	Or	G	G	G	G	G
Carotenoid-like pigment	—	—	—	—	—	+	—	—	—	—	—
Carbohydrate utilization											
Sucrose	+	+	+	+	+	—	+	+	—	—	—
Lactose	—	—	—	+	+	—	—	—	—	—	—
Lactate	+	+	+	+	—	—	+	+	+	+	+
Starch hydrolysis	—	—	—	—	—	—	+	+	—	—	—
Growth on Hugh & Leifson medium†	a	a	a	a	a	alk	alk	a	a	a	a
Methane thiol from methionine	+	+	+	+	—	—	—	—	—	—	—
Cell-wall analysis											
Type of DAP	meso	meso	meso	meso	LL	meso	—	—	meso	meso	meso
Presence of arabinose	—	—	—	—	—	—	—	—	+	+	+

All strains utilized glucose and acetate. None utilized inulin or citrate.

* B, pale brown, water soluble; F, fawn; Or, orange; G, grey.

† a, aerobic growth acid; alk, aerobic growth alkaline.

‡ 0, no growth

genus *Brevibacterium* is unsatisfactory, and both he and *Bergey's Manual of Determinative Bacteriology* (1974) consider the genus to be a mere repository for a variety of Gram-positive organisms. In *Bergey's Manual of Determinative Bacteriology* (1974), the genus is regarded as a *genus incertae sedis*. Our strains must therefore remain as an unclassified member of the 'Coryneform Group of Bacteria' (*Bergey's Manual of Determinative Bacteriology*, 1974).

The production of methane thiol by these coryneform bacteria makes them of interest in flavour studies, although their natural habitat is unlikely to be milk. Their high growth temperatures suggest a human or animal origin, and, because of their halophilic nature, possibly skin.

Characteristics of methane thiol-producing coryneform bacteria

They possess a growth cycle (rods → cocci); achieve optimum growth between 30 and 37 °C; are strict aerobes; are stimulated by as much as 12 % NaCl; are proteolytic; slowly produce a pale brown water soluble pigment; utilize acetate and lactate; produce some acetic acid from glucose; and their cell walls contain *meso*-DAP but no arabinose.

The four strains described here are deposited with the National Collection of Dairy Organisms.

The authors are greatly indebted to Dr R. M. Keddie for much helpful advice and discussion and for reference strains. They also wish to thank Dr M. Goodfellow for examining strains for mycolic acids, Dr M. J. Latham for performing gas chromatography, Dr I. J. Bousfield for methods of cell-wall analysis, Dr W. H. J. Crombach for reference strains, and Mrs W. E. G. Krzanowski for technical assistance.

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