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A Study of the Cider-sickness Bacillus—a New Variety of *Zymomonas anaerobia*

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SUMMARY: Cider sickness is a disorder of sweet low-acid ciders and perries. The causal organism was isolated after anaerobic incubation of the centrifuged deposit from infected cider on a medium which contained apple juice, 1 % (w/v) yeast extract and 10 µg. Actidione/ml., at pH 4.5. The causal organism found was a Gram-negative motile rod for which the name *Zymomonas anaerobia* var. *pomaceae* is proposed on the basis of a comparison of its morphology, cultural and biochemical characters with those of *Termobacterium mobile* Lindner (synonyms: *Pseudomonas lindneri*, *Zymomonas mobile*) and *Achromobacter anaerobium* Shimwell (synonym: *Saccharomonas anaerobia*). It is proposed that the generic name *Zymomonas*, Kluver & van Niel (1936) be adopted for the organisms isolated by Lindner (1928) and Shimwell (1937) and the organism described in this paper. The distinctive character which places these three organisms together in a separate genus in the tribe Pseudomonadeae is their ability to ferment glucose to give almost a theoretical yield of ethanol.

Sweet low-acid ciders and perries (less than 0.5 g. malic acid/100 ml.) are subject to a disorder called cider sickness. In the early stages of the disorder a thin haze develops, then copious gas production follows as sugar is fermented to ethanol and carbon dioxide. A full fruity aroma develops due to the liberation of acetaldehyde. Later the aroma and flavour become very harsh and a dense precipitate of an aldehyde-tannin complex forms. Cider so spoiled never regains its former quality, although after storage for 6 months it may be used in blending. The disorder was recognized in England in 1903 (Lloyd), and although Barker & Hillier (1912) isolated an organism from sick cider (which they called the cider-sickness bacillus) their description of it is incomplete by modern standards and they made no attempt to classify it. It seems probable, however, that their isolate and those made by Millis (1951) are identical. Grove (1914, 1916) and Barker (1948) described various aspects of the disorder and practical measures to combat it. In France a similar problem exists. Warcollier (1928) recorded the condition under a number of names, *la tourne* and *la pousse*. Unfortunately these names were given to disorders caused by lactobacilli and the confusion has been perpetuated in Charley's translation (1949). However, the name *framboisé*, now apparently in current use in France, was applied to the disorder by Warcollier (1939), who isolated a mobile anaerobic cocco-bacillus from ciders suffering from this disorder. But later Guittonneau, Mocquot & Tavernier (1940) attributed the development of *framboisé* in cider to the symbiotic growth of yeast and acetic acid bacteria. They claimed that in *framboisé* cider a delicate balance between yeast and

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acetic acid bacteria existed which allowed the oxidation of ethanol by the bacteria to proceed as far as acetaldehyde. This occurred only when the balance was correct and the concentration of oxygen was limiting. They claimed that the accumulated acetaldehyde was responsible for the fruity aroma and the copious precipitate associated with this disorder. Auclair (1955) and Barret (1955) also studied *framboisé* cider. Their results indicated that this disorder, whose outward characters appeared identical with those of cider sickness, may be caused by an organism similar to, if not identical with, the cider-sickness bacillus.

The present study describes the characters of the cider-sickness bacillus and shows its relationship to the organisms *Termobacterium mobile* Lindner (1928) (*Pseudomonas lindneri*; *Zymomonas mobile*) and *Achromobacter anaerobium* Shimwell (1937) (*Saccharomonas anaerobia*).

METHODS

Organisms. Two strains, 1 and 2, of the cider-sickness bacillus were isolated from ciders and perries examined at the Research Station, Long Ashton, Bristol. *Achromobacter anaerobium* NCIB 8227 (*Saccharomonas anaerobia*) was obtained from Mr H. J. Bunker, London. Three strains of *Termobacterium mobile* (*Pseudomonas lindneri*, *Zymomonas mobile*) were obtained from the late Prof. A. J. Kluyver, Delft. Strain L192 was the original received from Lindner 25 years ago and strains 2 and 3 were isolated in Java.

Media

Isolation medium. Apple juice was treated overnight with 1 g. Pectozyme (Messrs Norman, Evans and Rais Ltd., Dudley Road, Manchester) per 100 ml., filtered and 1% Difco yeast extract added. The pH value was adjusted to 4.5 and the medium filtered again. Agar to 3% (w/v) was added to make a solid medium. The medium was autoclaved at 7 lb./sq.in. for 15 min. On storage for c. 4 weeks, the liquid medium develops a fine dark precipitate which makes it unsatisfactory for some purposes, although growth is in no way inhibited.

Stock culture medium. Cultures were maintained as stabs in screw-cap bottles containing apple juice diluted 1 part+3 of water, +1% (w/v) Difco yeast extract, solidified with 10% (w/v) gelatin at pH 5.5. They remained viable in this medium for at least 17 weeks.

Carbohydrate fermentation tests. Seitz-filtered carbohydrate solutions were added to sterile 1% Difco yeast extract at pH 5.5, to give a final concentration of 1% carbohydrate. Bromocresol green was used as indicator.

Indole production. 1% Difco yeast extract containing 2 mg. DL-tryptophan/ml. at pH 5.5 was used. The indole reagent was made as recommended by Mackie & McCartney (1946). The test was performed after incubation for 10 days.

Nitrate reduction. 1% Difco yeast extract, 0.4% NaNO₃, 1% glucose, pH 5.5; α -naphthylamine and sulphanic acid solutions were used for the detection of nitrite (Topley & Wilson, 1946). The test was performed after incubation for 10 days.

Ammonia production. 1% Difco yeast extract + 1% glucose (adjusted to pH 5.5). After incubation for 10 days, Nessler's reagent was used to test for the presence of ammonia.

Hydrogen sulphide production. The same medium was used as that for detecting ammonia production. Lead acetate paper suspended from the plug was used to detect hydrogen sulphide.

Methyl red and Voges-Proskauer tests. The medium contained 0.5% glucose + 0.5% K_2HPO_4 + 0.5% Difco yeast extract + 0.5% Difco peptone; the pH value was adjusted to 6.5. O'Meara's modification (Mackie & McCartney, 1946) was used to detect the presence of acetylmethylcarbinol after incubation for 10 days. Methyl red solution was made as recommended in Mackie & McCartney (1946); the test being performed after incubation for 10 days.

Temperature. All cultures were incubated at 25° unless otherwise stated.

Isolation procedure. The isolation of spoilage bacteria from cider in the presence of large amounts of yeast presents considerable difficulty. By adjusting the pH value of the apple juice medium to 4.5, and using an anaerobic atmosphere, bacteria whose true habitat is not cider are discouraged, but these conditions allow both yeasts and cider bacteria to grow.

Attempts to separate yeast and bacteria by differential centrifugation or filtration were unsuccessful. Better results were obtained by adding to the isolation medium substances which inhibited the growth of yeasts but not of bacteria. Green & Gray (1950) found Actidione (The Upjohn Co., Kalamazoo, Michigan, U.S.A.) helpful in the isolation of bacteria from brewery materials, and at 10 $\mu\text{g./ml.}$ in apple juice medium this compound suppressed the growth of most cider yeasts whilst allowing lactobacilli, acetic acid bacteria and the cider-sickness bacillus to grow. Malachite green in apple juice medium (250 $\mu\text{g./ml.}$) retarded the growth of yeasts and inhibited lactobacilli completely. Since it had little effect on the cider-sickness bacillus or on acetic acid bacteria, it was used for their isolation. Actidione was used, however, for all routine investigations since it allowed all the common cider-spoilage bacteria to grow. Phillips & Hanel (1950) claimed that moulds were inhibited by Actidione, but this did not apply under the conditions of these experiments. The procedure finally adopted for the isolation of bacteria in cider was to centrifuge a 10 ml. sample of cider, plate the deposit on apple juice medium containing 10 $\mu\text{g. Actidione/ml.}$, and incubate the plate anaerobically for 4-5 days.

RESULTS

The sickness organism, whose properties are described in detail later, was isolated from the thirty naturally sweet sick ciders or perries examined. When inoculated into pasteurized cider or apple juice these isolates all produced a vigorous ethanolic fermentation with an aroma and flavour typical of the original disorder, as judged by a panel of experienced cider-tasters. Two strains were found to differ only in morphological appearance; these have been called strains 1 and 2.

Morphology. Strain 1 showed short rods mainly occurring as diplobacilli,

3.5–4.0 μ . long and 1.5 μ . wide, but also singly 2.0 μ . long and 1.5 μ . wide. Gram-negative, staining evenly. No spores or capsules detected. When grown in unfavourable conditions or when old, the organisms became pleomorphic. Actively motile in young cultures in glucose yeast-extract medium, but frequently non-motile in apple juice and cider.

Strain 2 was like strain 1 in shape, size and motility of the individual organisms. In addition, this strain formed rosettes or clumps of organisms which, in young cultures, swarmed over each other; whole clumps moved about in a hanging-drop preparation.

Cultural characters. With strain 1 on apple-juice agar medium under anaerobic incubation, the colonies grew to 0.5 mm. diam. in 2 days. At 7–8 days the colonies had reached maximum size, 3–5 mm. diam. The colonies were circular, regular, entire edged, low convex, butyrous, opaque and of pale buff after 5 days. A sickly sweet aroma, quite characteristic, was produced and persisted for about a week. In apple-juice liquid medium during the first 2–3 days there was dense and even turbidity, but as fermentation ceased, the supernatant fluid cleared and a creamy compact deposit formed which darkened with age as a tannin-aldehyde complex settled out.

With strain 2, under the same conditions, the organism produced colonies which were of high-convex, dewdrop appearance, and which were less opaque than strain 1. At 8–10 days, the colonies showed denser patches in a watery buff-coloured background; at the same time they flattened and spread so that colonies which were discrete at 4 days gradually merged to produce a solid streak of growth. In apple-juice liquid medium, the clumps of organisms seen microscopically caused a dense granular turbidity when fermentation was active. On standing a week, the supernatant fluid became clear, with a flocculent deposit which was cream at first but later darkened.

Biochemical and physiological characters. The results of tests on the cider-sickness bacillus, *Termobacterium mobile* and *Achromobacter anaerobium*, are shown in Table 1.

Glucose fermentation. Both strains of the cider-sickness bacillus gave almost theoretical conversion of glucose to ethanol (see Table 2). The mechanism of this fermentation has not been studied for the cider-sickness bacillus, but Gibbs & DeMoss (1951, 1954) and DeMoss (1953) established that whilst the products of glucose fermentation by *Termobacterium mobile* (*Pseudomonas lindneri*) are similar to those of a yeast fermentation, the mechanism is different. They found that carbon dioxide was formed from C₁ and C₄ of glucose, whilst the methyl group of ethanol was derived from C₃ and C₆ of glucose. Gunsalus, Horecker & Wood (1955) cited unpublished data of Roa & Gunsalus that *T. mobile* does, however, possess a yeast-like carboxylase.

Sulphur dioxide tolerance. Since sulphur dioxide is a permissible preservative in English cider it was of practical importance to test the tolerance of the organism to it. Sulphur dioxide was added to apple juice medium at 100, 200, 300, 400, 500, 750, 1000 and 2000 μ g./ml. Growth was delayed 8 days at 500 μ g./ml.; concentrations above 750 μ g./ml. inhibited growth completely. There was no inhibition, however, at the legally permissible concentration of

Table 1. Comparison of *cider-sickness bacillus* with *Termobacterium mobile* and *Achromobacter anaerobium*

Cider-sickness bacillus	Growth in fluid medium		pH range	Optimum temperature (° C.)	Motility	Acetylmethyl-carbinol production	Methyl-red test	Nitrite production	Indole production	Catalase production	H ₂ S production	Gelatin liquefaction	Sorbitol	Dulcitol	Mannitol	Raffinose	Rhamnose	Arabinose	Xylose	Sucrose	Maltose	Fructose	Lactose	Galactose	Glucose
	(a) Strain 1	(b) Strain 2																							
(a) Strain 1		Dense even turbidity, later formed a compact deposit	3.5-7.9	30	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	G
(b) Strain 2		Dense granular turbidity, later formed a diffuse deposit	3.5-7.9	30	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	G	-	-	G
<i>Achromobacter anaerobium</i>		Dense slightly granular turbidity, later formed a semi-flocculent deposit	3.4-7.5	30*	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	G	-	-	G
<i>Termobacterium mobile</i>		Dense granular turbidity, later formed a flocculent deposit	0	30*	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-	-	G	-	-	-	G
(a) L.192		Dense even turbidity, later formed a compact deposit	0	30*	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	G	-	-	G
(b) Strain 2		Dense granular turbidity, later formed a flocculent deposit	0	30*	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	G	-	-	G
(c) Strain 3		Dense granular turbidity, later formed a diffuse flocculent deposit	0	30*	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	G	-	-	G

Note. G = gas production and growth; ± = growth sometimes occurred but no gas production; - = no growth or negative test; * = as quoted in literature; 0 = information not available.

200 $\mu\text{g.}/\text{ml.}$ It seems probable that aldehyde liberated by the sickness bacillus combines rapidly with the sulphur dioxide to render it inactive.

Tolerance to various pH values. Grove (1914) observed that low-acid sweet ciders were particularly susceptible to cider sickness; the present studies have shown that the pH value of a cider, rather than its titratable acidity, controls the growth of the causative organism. Samples of apple-juice medium were adjusted to different pH values and inoculated. From pH 2.5 to 3.4 no growth was observed, but at pH values of 3.5 and 3.6 growth sometimes occurred. From pH 3.7 to 8.0 growth was vigorous, although the onset of growth was delayed at either end of this range. Of twenty-three naturally sweet sick ciders, twenty-one were within the range of pH 3.7–4.1, while the remaining two ciders were on the tolerance threshold of pH 3.4–3.6.

Table 2. *Fermentation of glucose by the cider-sickness bacillus*

	Glucose (g./100 ml.)		Alcohol produced (g./100 ml.)	Ratio: alcohol glucose (g./g. %)
	Sterile medium	After fermentation		
Strain 1	4.58	0.004	2.22	48.5
		0.009	2.23	48.8
Strain 2	4.58	0.016	2.25	49.4
		0.016	2.25	49.4

DISCUSSION

In their sugar reactions and other properties, both strains of the cider-sickness bacillus were almost identical with *Achromobacter anaerobium*, and these organisms differed principally from *Termobacterium mobile* in their inability to ferment sucrose (see Table 1). All three organisms fermented glucose almost quantitatively to ethanol and carbon dioxide (see Table 2, Kluwyer & Hoppenbrouwers, 1931; Shimwell, 1937). When inoculated into pasteurized cider *A. anaerobium* and *T. mobile* grew and fermented the sugars vigorously. The resulting spoiled ciders were sampled in random order by an experienced panel of cider-tasters. They agreed unanimously that the flavours which these organisms induced in cider were similar although not identical with cider sickness. It seems, therefore, that all three organisms are closely related and should be placed in the same genus.

Lindner (1928) named his original isolate *Termobacterium mobile*; Kluwyer & Hoppenbrouwers (1931), who studied this organism in some detail and discussed its taxonomy, considered that it should be called *Pseudomonas lindneri* under the Lehmann-Neumann system. Kluwyer & van Niel (1936), in their paper on a natural system for the classification of bacteria, proposed a new genus *Zymomonas* in the tribe Pseudomonadeae and that Lindner's organism should be the type species (*Zymomonas mobile*). Shimwell (1937) isolated a similar organism from beer and placed it in the genus *Achromobacter*. *Bergey's*

Manual of Determinative Bacteriology (1948) adopted *Pseudomonas lindneri* as the name for Lindner's isolate, with *Termobacterium mobile* as a synonym, but this classification made no mention of the proposed new name *Zymomonas mobile*. Later Shimwell (1950), who was not satisfied with the generic names *Achromobacter* or *Pseudomonas*, proposed the creation of a new genus *Saccharomonas* in the tribe Pseudomonadeae to contain his isolate from beer and Lindner's organism. However, since Kluver & van Niel have precedence for the name *Zymomonas* for these same organisms, it is proposed to adopt their nomenclature.

The chief difference observed between *Zymomonas anaerobia* Shimwell, and the cider-sickness bacillus, was in the change which the two organisms produced in cider. Although this is important from a technical point of view, it is of minor significance in bacteriological classification. *Z. mobile* Lindner differs not only in its effect on cider, but also in its ability to ferment sucrose. It is considered, therefore, that the cider-sickness bacillus is different from *Z. mobile* but that it resembles *Z. anaerobia* so closely that it can be regarded as a variety of that species. Prof. B. T. P. Barker, in a private communication, has suggested the variety name should be *pomaceae*. It is proposed then that the cider-sickness bacillus be called *Z. anaerobia* var. *pomaceae*. A subculture of *Z. anaerobia* var. *pomaceae* strain 1 has been deposited at the National Collection of Industrial Bacteria, Teddington, Middlesex, England.

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