The Infrared Spectra of Some Acetic Acid Bacteria

By A. W. SCOPES

Brewing Industry Research Foundation, Nutfield, Redhill, Surrey

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SUMMARY

The infrared spectra of 22 strains of the genus *Acetobacter* were significantly different from those of 9 strains of the genus *Acetomonas*, with one exception. Within each genus, however, the spectra formed smooth graduated series which showed no sudden differences which could be correlated with species boundaries, and exhibited no features which could be traced to the presence or absence of particular biochemical properties. These results support the division of the acetic acid bacteria into two genera *Acetobacter* and *Acetomonas* and render unlikely the existence of distinct species within the genera.

INTRODUCTION

It is generally agreed that, on the basis of their morphology and biochemical activities, the acetic acid bacteria can be divided into two distinct groups. Asai (1934, 1935), who first proposed such a duality, suggested that strains which he described as 'scarcely able to oxidise ethanol but which nevertheless produce considerable amounts of gluconic acid', should be separated from the genus *Acetobacter* into a new genus called *Gluconobacter*. Frateur (1950) on the other hand divided the acetic acid bacteria into four groups but kept them all within the single genus *Acetobacter*, the differences between the groups being based mainly on the oxidative metabolism of the organisms. Strains in the peroxydans, oxydans and mesoxydans groups oxidize lactate to carbonate, and ethanol through acetic acid to carbon dioxide, while the suboxydans group consists of bacteria which produce no carbonate from lactate and which oxidize ethanol no further than acetic acid. With regard to morphological criteria, motile strains in all groups were thought to possess polar flagella (*Bergey's Manual*, 1948) until Leifson (1954) showed that only bacteria in Frateur's suboxydans group were polarly flagellate and that motile strains in the other groups had peritrichous flagella. This led him to suggest a phylogenetic difference between the two types and to give generic status to the suboxydans group under the name *Acetomonas*. Asai & Shoda (1958) confirmed the existence of the two types of flagellation but proposed the use of Asai's original name *Gluconobacter* for the new genus. Shimwell (1958) and Shimwell & Carr (1959) extended Leifson's findings, the first mentioned author (1958) re-defining *Acetomonas*. Although this distinction between the genera appears to be well founded, Shimwell (1956, 1957) reported that most of the biochemical properties used in the classification of the component species are labile; he concluded (1959*a*) that the variability is so widespread that *Acetobacter* strains are virtually unclassifiable within the genus. Similarly Shimwell & Carr (1959) suggested that the differences between strains of *Acetomonas* did not merit varietal status. However, Frateur (1950) remarked that the bio-

chemical properties of his strains had not varied for 20 years, while DeLey (1961) reported that he had not encountered the exceptionally high mutation rates reported by Shimwell. DeLey (1961) surveyed the carbohydrate metabolism of organisms representative of the whole range of the acetic acid bacteria, and concluded that within both *Acetobacter* and *Gluconobacter*, which term was used in preference to *Acetomonas*, there is probably a continuous range of strains. Immediate neighbours in the series differ only in the possession of one or at most two enzymes and hence exhibit very similar biochemical properties. Two strains some distance apart will have different enzymic make-ups and will consequently differ in their metabolism.

The biochemical properties of a cell are an expression of its overall chemical composition, hence a classification of unicellular organisms based on the latter might be expected to lead to groupings very similar to those encountered in biochemical classifications. One method of investigating the molecular composition is by means of infrared spectrophotometry. The infrared spectrum of a simple or moderately complicated molecule is unique, and under appropriate conditions can be used for qualitative and quantitative analysis. In more complex molecules, however, the absorption bands become more diffuse and their spectra are no longer a means of absolute identification, although molecules of similar composition have similar spectra and the intensity of the absorption bands still indicates the amount of the substance present. The spectrum of a bacterial cell should thus reflect its total molecular composition and under suitable circumstances might be an aid to its classification.

Stevenson & Bolduan (1952) were the first to use infrared spectrophotometry for the identification of bacteria and Riddle *et al.* (1956) and Kenner, Riddle, Rockwood & Bordner (1958) extended the work and devised a method for obtaining reproducible spectra and for their comparison. A statistical analysis of the differences between bacterial spectra (Greenstreet & Norris, 1957) suggested that it was possible to distinguish between different genera and species, and in some cases between strains within a given species. Goulden & Sharpe (1958) examined the spectra of strains of lactobacilli and found that the spectra fell into five distinct classes. The boundaries of the spectral groups corresponded to certain physiological and serological divisions commonly accepted in the genus. Accordingly, the infrared spectra of representative strains of acetic acid bacteria have been examined to see whether this method could give further information on the interrelationships of these organisms.

METHODS

Nomenclature. In the following sections, strains in Frateur's suboxydans group are referred to as *Acetomonas* (abbreviated to *As.*). Frateur's specific names are used for both *Acetomonas* and *Acetobacter* merely to indicate the biochemical properties of the strains. The validity of the species concept in the acetic acid bacteria is discussed below.

Organisms and media. The strains used, their origins and properties are shown in Table 1. Cultures were maintained on slopes of the acetobacter medium of Haynes, Wickerham & Hesseltine (1955) and subcultured every 6-8 weeks. On isolation or on arrival strains were identified or checked by the methods of Frateur (1950), being grown for this purpose on Williamson's medium A (1959). The liquid medium

Table 1. The strains of acetic acid bacteria used, their source and properties

Strain no.	Type	Source	Catalase	Acid from glucose	Lactate to carbonate	Ethanol to acetic acid	Acetate to carbonate	Dihydroxy-acetone from glycerol	Cellulose production	Pigment	Growth in Hoyer's medium
8618	<i>Acetobacter peroxydans</i>	NCIB*	+	—	+	+	+	+	+	—	+
4938	<i>A. ascendens</i>	NCIB	+	—	+	+	+	+	+	—	+
38	<i>A. ascendens</i>	Brewer's yeast	+	—	+	+	+	+	+	—	+
131	<i>A. estunense</i>	Dr J. G. Carr†	+	—	+	+	+	+	+	—	+
53	Nov. spec.?	Brewer's yeast	+	—	+	+	+	+	+	—	+
8620	<i>A. lovainense</i>	NCIB	+	+	+	+	+	+	+	—	+
8163	<i>A. rancens</i>	NCIB	+	+	+	+	+	+	+	—	+
2/1	<i>A. rancens</i>	Dr J. G. Carr	+	+	+	+	+	+	+	—	+
4	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
13	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
14	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
25	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
39	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
43	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
52	<i>A. rancens</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
6249	<i>A. turbidans</i>	NCIB	+	+	+	+	+	+	+	—	+
8622	<i>A. mesoxydans</i>	NCIB	+	+	+	+	+	+	+	—	+
8747	<i>A. mesoxydans</i>	NCIB	+	+	+	+	+	+	+	—	+
B 4	<i>A. mesoxydans</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
142	<i>A. aceti</i>	Dr J. G. Carr	+	+	+	+	+	+	+	—	+
8554	<i>A. aceti</i>	NCIB	+	+	+	+	+	+	+	—	+
B 3	<i>A. aceti</i>	Brewer's yeast	+	+	+	+	+	+	+	—	+
8086	<i>Acetomonas melanogena</i>	NCIB	+	+	—	+	+	+	+	+	+
8084	<i>As. melanogena</i>	NCIB	+	+	—	+	+	+	+	+	+
1	<i>As. suboxydans</i>	Brewer's yeast	+	+	—	+	+	+	+	+	+
9	<i>As. suboxydans</i>	Brewer's yeast	+	+	—	+	+	+	+	+	+
10	<i>As. suboxydans</i>	Brewer's yeast	+	+	—	+	+	+	+	+	+
27	<i>As. suboxydans</i>	Brewer's yeast	+	+	—	+	+	+	+	+	+
50	<i>As. suboxydans</i>	Brewer's yeast	+	+	—	+	+	+	+	+	+
22	<i>As. suboxydans</i>	Brewer's yeast	+	+	—	+	+	+	+	+	+
4943	<i>As. capsulata</i>	NCIB	+	+	—	+	+	+	+	+	+

* NCIB—National Collection of Industrial Bacteria, D.S.I.R. Torry Research Station, Aberdeen, Scotland.

† Dr J. G. Carr, University of Bristol, Department of Agriculture and Horticulture, Research Station, Long Ashton, Bristol.

used for growing bacteria to obtain their spectrum was a liver-extract medium of the following composition (v/v). Solution A, 70 %; liver extract (Haynes *et al.* 1955), 10 %; citrate + phosphate buffer (0.5 M, pH 4.0) 20 %. Solution A consisted of (w/v) yeast extract (Difco) 0.7 %, casein hydrolysate (Difco) 1.45 %, glucose 0.7 %. Cultures on solid media were grown at 30°, whereas the liquid cultures were grown at 25° on a reciprocating shaker.

Procedure for obtaining infrared spectra

Bacteria from a stock slope were streaked on medium A and incubated at 30° for 2 or 3 days. A colony was picked off and transferred to 10 ml. of liver-extract medium, while at the same time the biochemical properties of the culture were checked. When there was visible growth in the liquid medium (usually after 24–48 hr.), from 1 to 5 ml. were inoculated into 100 ml. of fresh medium, the size of inoculum depending on the amount and rate of growth in the initial culture. This second subculture was incubated for about 24 hr. and the bacteria harvested. They were washed twice with 0.85 % (w/v) sodium chloride solution, once with distilled water and then suspended in about 1 ml. of distilled water. A few drops of this suspension were transferred to a silver chloride plate and dried to a film under an infrared heater. The plate was slightly tilted while it dried so that the thickness of the film varied along its length. The infrared spectrum was then run from 650 to 2000 cm^{-1} by using a Perkin Elmer 137 Infracord spectrophotometer. An untreated silver chloride plate with the same transmission as the sample plate was placed in the reference beam and the position of the sample plate adjusted so that the depth of the absorption band at 1640 cm^{-1} was > 70 % of a full-scale deflexion and the transmission at 1175 cm^{-1} was 50 %.

Comparison of spectra. In spite of these adjustments of sample film thickness the intensity of the absorption bands differed from sample to sample. To compare the spectra it was therefore necessary to cut the trace into sections and to superimpose the lines in each section by an appropriate shift of the transmittance scale (Riddle *et al.* 1956).

RESULTS

Spectra of some strains of *Acetobacter* and *Acetomonas* are shown in Figs. 1 and 2. The traces are very similar, but differences can be seen on the low frequency side of the band at 1080 cm^{-1} , in the shape of this band and the other main band at 1240 cm^{-1} and in the relative intensities of the weak bands and troughs in the region 750–950 cm^{-1} . These features are discussed below. The reproducibility of a spectrum was good; thus when spectra obtained on different occasions from the same strain were compared, the traces differed by less than 1 % over the whole wavelength range.

Comparisons between Acetobacter and Acetomonas strains

Significant differences between the spectra of strains of *Acetomonas* and *Acetobacter* were found only in the region 900–1080 cm^{-1} . In *Acetomonas*, the bands at 910 and 970 cm^{-1} were less well resolved. The absorption on the low frequency side of the 1080 cm^{-1} band was also stronger so that all these bands ran together giving a more or less constant increase in absorption from 900 cm^{-1} usually interrupted by shoulders in the region of 910 and 970 cm^{-1} (Fig. 2c). Spectra of *Acetobacter*, on the

other hand, generally showed a definite band at 910 cm^{-1} with the absorption on the high frequency side usually equal to or less than that on the lower side, and a definite narrow band at 970 cm^{-1} with a shallow trough before the sharp rise of the 1080 cm^{-1} band (Fig. 1a).

Fig. 1. Spectra of various *Acetobacter* strains: (a) 8618, *A. peroxydans*; (b) 38, *A. ascendens*; (c) 13, *A. rancens*; (d) 8747, *A. mesoxydans*; (e) B3, *A. aceti*.

Spectra of Acetomonas strains

Nine strains of *Acetomonas* were examined, five of *As. suboxydans*, one non-ketogenic variant of *As. suboxydans*, two strains of *As. melanogena* and one of *As. capsulata*. The spectra of all the *As. suboxydans* strains exhibited the typical generic features mentioned above and differed only in the extent of the shoulders at 910 and 970 cm^{-1} . With respect to this difference, the six traces showed a smooth graduation from strain 27 (Fig. 2b) which had no appreciable shoulder at the higher frequency and a broad weak one at the lower, to strain 50 (Fig. 2d) which showed merely a change of slope at 910 cm^{-1} and a broad flat shoulder at 970 cm^{-1} . The spectrum of the non-ketogenic variant fell near the middle of the series, differing from the other strains only in a slight intensification of the bands at 810 and 870 cm^{-1} . It may be noted that in the spectra of *Acetobacter* strains loss of ketogenic

ability was associated with a diminution of the intensity of these same bands, the opposite of the present case.

The spectrum of the strain of *Acetomonas capsulata* (Fig. 2e) differed markedly from normal. From 900 to 1030 cm^{-1} the trace was typical of an *Acetomonas* but at higher frequencies the general level of absorption was lower than usual and com-

Fig. 2. Spectra of various *Acetomonas* strains: (a) 8086, *A. melanogena*; (b) 27, *A. suboxydans*; (c) 9, *A. suboxydans*; (d) 50, *A. suboxydans*; (e) 4943, *A. capsulata*.

pletely altered the shape of the curve. The most noticeable differences were the weakness of the band at 1240 cm^{-1} and the height of the trough at 1180 cm^{-1} . Since the enzymic constitution of this strain is not very different from other *Acetomonas* strains (DeLey, 1961) the spectral difference is probably due to the capsular material which this organism produces.

The spectra of the two strains of *Acetomonas melanogena* investigated (Fig. 2a) were both exceptional in that they showed no *Acetomonas* characteristics, the traces being indistinguishable from those of some *Acetobacter*. There is, however, no doubt that biochemically, these two strains are varieties of *Acetomonas* since they cannot be made to over-oxidize ethanol or to produce calcium carbonate when grown on calcium lactate + yeast-extract agar. This anomaly is discussed below.

Spectra of Acetobacter strains

As the biochemical versatility of the strains increases in the series from *Acetobacter peroxydans* through the oxydans group to *A. mesoxydans* and *A. aceti* there are gradual changes in the spectra. The most obvious when the traces are superimposed is a slight overall increase in absorption. The increase is most noticeable in the bands at 1380 and 1450 cm^{-1} , the trough in the region 1330 cm^{-1} and the shoulder or weak band at 970 cm^{-1} . The two main bands at 1080 and 1240 cm^{-1} become slightly stronger and more diffuse while the concavity of the long wavelength side

Fig. 3. The range of spectra from *Acetobacter rancens* strains: (a) 38, *A. ascendens*; (b) 4, *A. rancens*; (c) 25, *A. rancens*; (d) 52, *A. rancens*; (e) 43, *A. rancens*; (f) 8747, *A. mesoxydans*.

of the 1080 cm^{-1} band grows more pronounced. The absorption from 800 to 950 cm^{-1} increases a little and the weak bands and troughs in this region tend to become stronger (Fig. 1a-e). There are no abrupt changes in the spectra and no features which can be correlated with definite biochemical properties. For instance, the four strains examined which are not able to produce acid from glucose, *A. peroxydans*, two strains of *A. ascendens* and strain 53, a Hoyer positive variant of *A. ascendens*,

all show a weak absorption minimum in the low wavelength range at 835 cm^{-1} (Fig. 1*a, b*). In *A. rancens*, which has the property of acid production from glucose, the minimum tends to move to the trough at 935 cm^{-1} (Fig. 1*c*). However *A. rancens*, strain 4, which produced acid more slowly than the other strains and may therefore be regarded as falling between *A. rancens* proper and the strains which fail to produce acid, showed a level trace (Fig. 3*b*). Hence as the property of acid production is acquired there is a gradual change in the shape of the spectrum. An absorption minimum at 835 cm^{-1} is also found in the spectra of strains of the mesoxydans group (Fig. 1*d*); since these organisms readily acidify glucose, the minimum at 835 cm^{-1} cannot be associated with the ability to metabolize glucose to gluconic acid.

Acetobacter mesoxydans and *A. rancens* differ biochemically in that the former is able to produce dihydroxyacetone from glycerol. In the spectra of *A. mesoxydans* strains the low frequency absorption minimum has returned to 835 cm^{-1} , not now as the lowest point in a fairly flat trace but as a distinct trough (Fig. 3*f*). One of the strains of *A. rancens* examined (no. 43), produced a similar curve (Fig. 3*e*). Other strains of *A. rancens* show two minima at 835 and 935 cm^{-1} (Fig. 3*d*), and others only one minimum at 935 cm^{-1} (Fig. 3*c*). As mentioned in the preceding paragraph strain 4 has a flat trace in this region. We thus see that the spectra of strains all classified biochemically as *A. rancens* form a smoothly graduated series from spectra almost indistinguishable from those of *A. ascendens* through traces typically *A. rancens* to those closely resembling *A. mesoxydans*. This behaviour is illustrated in Fig. 3.

The lack of correlation between biochemical behaviour and spectral features is also seen on considering the three pairs of strains: *Acetobacter lovaniense* and *A. rancens*; strain 53 and *A. ascendens*; *A. aceti* and *A. mesoxydans*. The first named in each case differs biochemically from its partner only in its ability to grow in Hoyer's medium (Table 1). Examination of the spectra reveals no consistent difference between the traces of the pairs and the Hoyer-positive strains have no particular features which can be associated with their ability to grow in this medium. Similarly, the gain of ketogenic power in the mesoxydans group brings about no change in the spectrum which can be associated solely with ketogenesis. Indeed such change as is observed is exactly opposite to that brought about in the spectra of strains of *Acetomonas* by the same biochemical property.

In summary, spectral features change gradually and on the whole parallel the biochemical changes. The spectra of a group of strains having the same biochemical properties form a complete range linking the spectra of their neighbours in the biochemical classification.

DISCUSSION

Greenstreet & Norris (1957) and Kenner *et al.* (1958) showed that to obtain reproducible bacterial spectra, instrumental conditions, preparation of the specimen and growth of the culture must be carefully controlled. The chemical composition of bacteria from a batch culture varies with the age of the culture, and also depends on the nature of the medium in which they are grown (Herbert, 1961). On solid media, even after a short incubation, cells of very different composition are found, since the environment of a cell differs according to its position in the colony. Cells on the

edge of a colony have access to the full range of nutrients in the medium while the older cells at the centre have less nutrient available and also experience a higher concentration of waste products. In a shaken liquid culture, on the other hand, all the cells have the same environment and in a sample taken during exponential growth the range in composition of individual organisms should be minimal. It was for this reason that liquid cultures were used in the present study and the cells harvested during exponential growth.

DeLey (1961) showed that of all the acetic acid bacteria the mesoxydans group contain the greatest variety of enzymes and that as one progresses through the oxydans group to the peroxydans in one direction and to *Acetomonas* in the other, the number of enzymes becomes less. In addition, Shimwell & Carr (1960) have pointed out that *Acetomonas* strains lack the enzymes of the citric acid cycle. The infrared absorption parallels these changes, increasing from peroxydans to mesoxydans and then decreasing with the change to *Acetomonas*.

The species concept in the acetic acid bacteria

There are two proposals in the literature for abolishing the division of *Acetobacter* and *Acetomonas* into species. One suggestion (Shimwell, 1959*a*) is based on the extreme lability of the biochemical properties used as the basis for classification. Other investigators, however, have found the biochemical reactions of certain strains to be constant with time and have consequently been able to apply Frateur's classification. In the present work a constant watch was kept on the cultures for any aberrant colony forms and for loss or gain of biochemical properties. No instances of the latter were observed while on the very few occasions when an atypical colonial form appeared in a culture, it was found when isolated and grown up to have the same biochemical properties as its parent. The discrepancy between the findings of Shimwell and of other investigators is probably explained by the different conditions used. In spite of the insistence of the former author on the variability of the acetic acid bacteria, under suitable standardized conditions the properties of the strains comprising these species remain constant and a classification is therefore possible.

DeLey (1961) suggested that the concept of species in the acetic acid bacteria should be abandoned because he found a series of gradual changes in the enzymic make-up and biochemical characteristics of the strains, with no sudden breaks corresponding to species boundaries. In the infrared spectra also this smooth graduation is observed and there are no indications of any discontinuities which can be associated with divisions within the genera. The present work supports DeLey's contention that the species boundaries are artificial and his suggestion that strains of *Acetobacter* and *Acetomonas* should be regarded as variations within two biotypes (Winogradsky, 1952), the specific names being used merely to indicate their particular biochemical properties.

It is of interest to recall that Asai & Shoda (1958) reported, and DeLey (1961) confirmed, some strains commonly regarded as *Acetomonas* which nevertheless oxidized acetate. One of these, a strain of *Acetomonas melanogena* which Asai & Shoda stated had polar flagella was later shown by Shimwell & Carr (1959) to be peritrichously flagellate and so to be, in fact, an *Acetobacter*. On the other hand other strains of *Acetomonas melanogena* fail to oxidize acetate. The strains of this organism

examined in the present work were of this type, and one of them which was motile was found to have polar flagella, and must therefore be regarded as a true *Acetomonas* in spite of giving an *Acetobacter* type spectrum. It would thus seem that bacteria previously classified as *Acetomonas melanogena* are now to be found on both sides of the generic boundary. The position of other acetate-oxidizing *Acetomonas* strains still awaits clarification. Examination of the type of flagella of motile strains would give further information on the position of this boundary. However, it has been found difficult to obtain unequivocal results on flagellation by the use of staining methods (Shimwell, 1959*b*) and the problem must await a comprehensive study by electron microscopy.

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