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Structural changes in the cells of some bacteria during population growth: An FTIR-ATR study

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Structural changes occurring in the cells of several bacteria during their growth curves have been investigated by Fourier transform infrared (FTIR) spectroscopy using the sampling technique of attenuated total reflectance (ATR). Spectra reflect all of the components of the cells including the cell walls, cell membranes, internal structures and the cytoplasm. The bacteria studied were *Bacillus stearothermophilus*, *Halobacterium salinarium*, *Halococcus morrhuae* and *Acetobacter aceti*. All species showed significant spectral changes during their growth curves, indicating structural changes in the cells during increases in cell numbers. The major change for *B. stearothermophilus* was in the lipid content which was at a maximum during the exponential phase of the growth curve. For the halophiles *H. salinarium* and *H. morrhuae* the major change was that the concentration of sulfate ion in the cells varied during the growth curve and was at a maximum during the mid-part of the exponential phase of the growth curve. *A. aceti* cells showed increasing polysaccharide content during the growth curve as well as maximum lipid content during the exponential phase of growth.

Index Headings: **infrared spectroscopy; FTIR; attenuated total reflectance; bacteria; growth curve; cellular structure**

INTRODUCTION

There have been many reports on the use of vibrational spectroscopy for the study of bacteria. The main aim of most of these studies was to use the techniques of infrared or Raman spectroscopy for the rapid identification of microbial species.[1-4] This work has been the subject of recent reviews.[5,6] A spectroscopic approach appears to have great potential for studying bacterial populations because of its speed, simplicity and the high information content of vibrational spectra. While both major techniques of vibrational spectroscopy, infrared and Raman, have been used for microbial studies, infrared spectroscopy has found far wider use. Infrared spectroscopy is more widely available, is easier to use than Raman spectroscopy, and does not suffer from interference by fluorescence. The major drawback of infrared spectroscopy is that microbial suspensions must be dried before spectra are collected because of the very strong absorption of the water molecule.

Microbial growth is characterised by an initial period of slow growth (the lag phase) followed by an exponential growth phase in which the number of cells doubles every unit of time, known as the generation time. Generation times for bacteria vary widely and range from as little as 10 min to as long as several days. It is known that the molecular structure of some cell components can change slightly during growth, and that this parameter of "cell maturity" can be a confounding variable when using spectroscopic techniques coupled with chemometrics to distinguish different cells, e.g. cancer cells from normal cells.[7,8] However, the molecular structural changes occurring in bacterial cells during growth have not been well studied. Zeroual *et al*[9] used Fourier transform infrared (FTIR) spectroscopy to investigate structural changes to *Bradyrhizobium japonicum* grown under different incubation conditions, including in liquid or on solid media. They found that significant spectral differences occurred when the bacteria were transferred between solid and liquid media and they concluded that such changes were due to changes in the bacterial wall components which are different in the solid and liquid media.

Generally, infrared spectra of bacteria are collected in transmission mode by placing a small amount of bacterial suspension on a suitable IR transparent window, such as zinc selenide, followed by drying. IR photoacoustic spectrosocopy[3] and reflection-absorption methods[4] have also been applied to bacterial samples. This paper describes an alternative approach for the measurement of IR spectra of bacterial suspensions which uses the technique of attenuated total reflectance (ATR). ATR is a well-known technique for measuring IR spectra[10,11] but has found relatively little use in bacterial studies[12-17] The basis of the method is that IR light is directed into a crystal material at such an angle that it is totally internally reflected at each reflection it makes inside the crystal. The light exits the crystal and is directed to the detector. At each reflection a part of the light, called the evanescent wave, passes beyond the crystal interface and interacts with a sample placed in close contact with the crystal. In this way an IR spectrum may be collected. ATR accessories for IR spectrometers have been available for many years either with the crystal held vertically, or more recently, horizontally. Accessories with a vertical crystal are not suitable for dry microbiological samples, as good contact must be made between the sample and the crystal surface. Typical horizontal ATR accessories have had rather large crystals which may measure as much as 1 x 4 cm and therefore require a substantial amount of sample. However, recently ATR accessories have become available, for use with FTIR spectrometers, which have crystal surfaces which are circular and much smaller with a diameter of the order of a few millimetres. Hence, high quality spectra can be obtained simply and quickly from small amounts of bacterial suspensions.

In this paper we present FTIR-ATR spectra taken during the growth curves of four bacteria and discuss how the spectra indicate structural changes occurring in the cell material

during different phases of the growth curves of these bacteria. The particular bacteria were chosen because they were readily available to us, and because they are safe to use in a normal laboratory environment.

MATERIALS AND METHODS

Bacterial cultures

Acetobacter aceti sp. aceti was cultured in Mannitol broth prepared from the following components: yeast extract, 5.0 g; bacteriological peptone, 3.0 g; mannitol, 25.0 g and distilled water, 1.0 L. The final pH of the Mannitol broth was adjusted to 6.0 prior to autoclaving at 121 °C for 15 min. This organism is characterised by slow growth (5 days) at a temperature of 28 °C.

Bacillus stearothermophilus is characterised by rapid growth (24 hrs) at a temperature of 55 °C in Tryptone Soy Broth (TSB) (Oxoid). The final pH of the TSB was adjusted to 7.2 prior to autoclaving at 121 °C for 15 min.

Halobacterium salinarium and *Halococcus morrhuae* are characterised by slow growth (7 days) at a temperature of 30 °C in Halophile broth containing three components as follows: A – Oxoid peptone, 10.0 g; trisodium citrate 2.0 g; KCl, 3.0 g; in distilled water, 200.0 mL: B – NaCl, 200.0 g in distilled water, 600.0 mL: C – $MgSO_4.7H_2O$, 20.0 g in distilled water, 200.0 mL. Adjustment of the pH to 7.2 was performed and each component autoclaved separately at 121 °C for 15 min. After autoclaving, all of the components of the media were aseptically combined.

Growth Media Removal

Great care was employed to ensure sample spectra obtained were free from growth media effects. Therefore a rinsing procedure was used as follows:
The sample culture was placed in a sterile 1.5 mL Eppendorf tube and microfuged at 3500 rpm for 10 min to pellet the bacterial cells. The supernatant was discarded and a volume of rinse solution equal to the original sample volume was added and mixed. The sample was microfuged a second time for 10 min and the supernatant again discarded. The rinse solution used was 0.7 % NaCl solution for *A. aceti* and *B. stearothermophilus*, and 3.0 M NaCl solution for *H. salinarium* and *H. morrhuae.*

Growth curve study

0.1 mL stationary phase culture of each bacterium was added to 50.0 mL standard growth medium in stoppered sterile 250 mL side-arm flasks. Flasks were placed into a shaking incubator set at 100 rpm and temperatures of 55 °C for *B. stearothermophilus,* 28 °C for *A. aceti*, and 30 °C *H. salinarium* and *H. morrhuae*. Optical Density (OD) readings at 540 nm were taken at hourly intervals for 24 hr for *B. stearothermophilus*, and at 6 hr intervals for *A. aceti*, *H. salinarium* and *H. morrhuae* using a bench spectrophotometer. 0.2 mL samples of bacterial culture were taken at corresponding intervals and rinsed in accordance with the rinsing procedure above. 0.1 μL samples were air-dried onto the ATR objective for 50 min before spectral collection.

FTIR-ATR spectroscopy

Infrared spectra were obtained using a Nicolet 870 Nexus Fourier Transform infrared spectrometer equipped with a DTGS TEC detector and an ATR objective (Nicolet Instrument Corp., Madison, WI). An Optical Path Difference (OPD) velocity of 0.6329 cm s^{-1}. The aperture was set to 100, and a gain of 8 was used. The ATR accessory utilised was a Smart Endurance single reflection ATR accessory equipped with a composite diamond IRE with

0.75 mm sampling surface and a ZnSe focusing element. 0.2 mL bacterial samples were rinsed using the rinsing procedure outlined above (0.2 mL rinse solution was added) and a 1.0 μL sample was then air-dried onto the diamond IRE for 50 min to remove excess water before spectral collection. Spectra were collected in the spectral range 4000-525 cm^{-1} with 128 scans and 4 cm^{-1} resolution, and are corrected for the wavelength dependence of the ATR experiment. Sample spectra were collected in single beam mode then the IRE was cleaned immediately and a background spectrum collected. This was to minimise CO_2 and water vapour effects. Data manipulation when required was done using the GRAMS32 computer software package (Galactic industries, Salem, NH). All spectra were normalised to the amide I band around 1650 cm^{-1} and are displayed without absorbance values. However, typically the absorbance value of the amide 1 band in the raw spectra was about 0.3 absorbance units. In many figures spectra are displaced for clarity.

RESULTS AND DISCUSSION

ATR spectroscopy

The penetration depth of the IR radiation into the sample in an ATR experiment depends directly on the wavelength, but it is also affected by other parameters of the system as given in the following equation, which is due to Harrick.[10] Penetration depth is defined as the depth at which the intensity of the evanescent wave has dropped to 1/e of its value at the surface.

$$\text{Penetration Depth} = \frac{\lambda}{2\pi \left(n_p^2 \sin^2\theta - n_s^2\right)^{1/2}}$$

where λ = wavelength
θ = angle of internal reflection
n_p = refractive index of crystal
n_s = refractive index of sample

For a diamond IRE (RI = 2.4) with incident angle of 50° and assuming an RI of approximately 1.5 for bacterial cell material, the depth of penetration is calculated to be about $\lambda/5$, i.e. about 0.5 μm at 4000 cm^{-1}, and about 2 μm at 1000 cm^{-1}. Given that the bacteria under study have elongated shapes with a diameter of 1-2 μm and are likely to collapse somewhat when air-dried onto the surface of the IRE, then it is likely that the ATR measurement is seeing at least one layer of whole cells, and possibly several layers in parts of the spectrum. Hence, the ATR spectrum of individual bacteria reflects the sum of the molecular structures of the whole organism, including cell wall and membranes, the internal structures and the cytoplasm.

Fig. 1. shows the FTIR-ATR spectra of the four bacteria. The spectra are of high quality (good signal-to-noise ratio) and are typical of spectra previously reported for bacteria.[6] The most prominent features are an intense broad peak around 3400 cm^{-1}, assigned to O-H stretch and residual water stretching modes, a group of overlapped bands around 2900 cm^{-1}, assigned to C-H stretching modes, the amide I band near 1650 cm^{-1}, and the amide II band near 1550 cm^{-1}. Careful examination of all of the spectra shows that, while broadly similar, there are significant differences between the spectra particularly in the spectral region 1500–500 cm^{-1}. However, as will be noted later in this paper, these spectra are merely ‘snapshots’, each taken at a particular point in the growth curve of that organism, and it is not possible to associate an exact spectrum with a particular organism.

Growth curve studies: B. stearothermophilus

B. stearothermophilus is a relatively quick growing organism. Its growth curve, as measured by the optical density at 540 nm, is shown in Fig. 2. Samples were taken from the culture at various times during the lag phase, the exponential phase, and the stationary phase and FTIR-ATR spectra were collected. Examples of these spectra are shown in Fig. 3. Examination of the spectra shows that there are significant changes in the spectra, notably in the C-H stretching region around 2900 cm^{-1}, and in the amide I band near 1650 cm^{-1}. In the C-H stretching region the spectra show that two bands at 2918 cm^{-1} and 2850 cm^{-1} increase in intensity during the exponential growth phase but are less intense during the lag and stationary phases. This is shown more clearly in Fig. 4 which shows the difference spectrum obtained when the spectrum of the 13.5 h sample is subtracted from that of the 8 h sample. In the difference spectrum the bands at 2918 and 2850 cm^{-1}, assigned to antisymmetric and symmetric methylene ($-CH_2-$) stretching modes of long chain lipid molecules,[6] respectively, occur as positive peaks indicating increased intensity at 8 h compared with that at 13.5 h. There is also a weaker positive peak near 1738 cm^{-1} which can be assigned to the ester carbonyl group of the lipid molecules. The spectra therefore indicate that the lipid content of the cell material increases significantly during the exponential part of the growth curve. A plot of the integrated area under the 2850 cm^{-1} band against the time after inoculation is shown in Fig. 5. It shows clearly that the increase in lipid content happens during the mid-part of the exponential phase.

There is also a change in the position of the amide I band during the growth curve of *B. stearothermophilus* (Fig. 6). At 3 h and 8 h (spectra A and B, respectively, in the Figure) during exponential growth the peak position of the amide I is near 1650 cm^{-1}, but during the stationary phase of growth the position shifts to near 1640 cm^{-1}. The position of the amide 1 band gives an indication of the average secondary structure of the proteins and this shift could indicate that there is an increase in α-helical compared with β-sheet protein structures during the exponential phase when the cells are rapidly dividing.[18] The smaller shift in the amide II band from about 1545 to 1540 cm^{-1} also supports increased α-helical character during the exponential phase. However, the system is very complex as the spectrum reflects all of the cell material, so it is difficult at this stage to understand the significance of these variations.

Growth curve studies: H. salinarium and H. morruhae

Examination of the growth curve for the halophile *H. salinarium* (Fig. 7) shows this organism exhibits much slower growth than the thermophile *B. stearothermophilus* (Fig. 2). *H. salinarium* takes around 120 h after inoculation to reach the stationary phase compared with around 10 h for *B. stearothermophilus*. Samples were taken from the culture of *H. salinarium* at various intervals between 48 h and 128 h, FTIR-ATR spectra were collected and these are displayed in Fig. 8. These spectra show little variation in the C-H stretching bands around 2900 cm^{-1} indicating that the lipid content does not vary during the growth curve. Nor is there significant variation in the position and relative intensities of the amide I and amide II bands, showing consistency of protein structure during the growth curve. However, there are large differences in the intensity of three bands in the low wavenumber part of the spectra at 1108, 995 and 618 cm^{-1}. These bands can all be assigned to sulfate ion, for which Nakamoto[19] gives three bands in this spectral region at 1105, 983 and 611 cm^{-1}. The small difference in band positions may be due to the fact that Nakamoto's positions are for the solid state, whereas in the air-dried microorganism the sulfate ion may be wholly or partly in aqueous solution. The variation in intensity of one of the sulfate bands, 618 cm^{-1}, with time, together with the growth curve for *H. salinarium* is shown in Fig. 9. As with *B.*

stearothermophilus, the largest change is in the mid-part of the exponential phase. It is interesting to note that the maximum sulfate content of the cells corresponds exactly with a point in the growth curve where there is a slight increase in optical density. This could mean that there actually is a slight increase in growth rate at this point, or that cells with an increased content of sulfate ion have a slightly higher optical density.

It is known that *H. salinarium* incorporates sulfate into the cell wall.[20] If the sulfate seen in the spectra was due only to this, then it would be expected to accumulate during the growth curve. As this does not happen, it can be concluded that the sulfate ion is being metabolised, most likely being reduced to sulfide ion. Hence sulfate ion appears to concentrate in the cells during the most active part of the growth curve when cells are undergoing a high rate of mitosis. Although *H. salinarium* is best known for its halophile properties, it is also known to be sulfate reducing.[20]

The spectroscopic results for *H. morruhae* (data not shown), another halophile, exactly parallel those of *H. salinarium*, showing increased concentration of sulfate ion during the mid-part of the exponential phase of the growth curve.

Growth curve studies: A. aceti

A. aceti is a relatively slow growing organism taking around 100 h to reach the stationary phase. The optical density growth curve is shown in Fig. 9. This curve looks very different from the equivalent curves for *B. stearothermophilus* and *H. salinarium* mainly because *A. aceti* has a tendency to clump, consequently optical density measurements are not as meaningful. However, the growth curve does give some idea of the position of the exponential phase.

FTIR-ATR spectra of *A. aceti* taken at various times during the growth curve are shown in Fig. 10. Careful analysis of these spectra reveals that there are considerable differences between spectra from different parts of the growth curve. Firstly, the C-H stretching region around 2900 cm^{-1} shows a modest increase in bands at 2918 and 2850 cm^{-1}. As was previously discussed for *B. stearothermophilus*, these bands are assigned to antisymmetric and symmetric methylene stretching modes, respectively, and can be related to the amount of lipid in the sample. The results therefore show a small increase in the amount of lipid present in the cells during the exponential phase.

Secondly, there is a large increase in a broad band centred at 1050 cm^{-1} during the growth curve. Bands in this spectral region can be assigned to the C-O stretching mode of polysaccharides present within the cell walls and membranes,[21,22] or to the presence of smaller carbohydrates or related compounds.[14] Fig. 9. also shows a plot of the variation of the integrated area of the band at 1050 cm^{-1} against time from inoculation. This plot indicates that as the growth curve continues, the amount of carbohydrate in the cells increases until it plateaus during the stationary phase. In fact, the increase in the area of the carbohydrate band at 1050 cm^{-1} closely follows the growth curve. The main difference is that in the stationary phase the optical density drops showing fewer live cells, while the area of the 1050 cm^{-1} band remains constant showing that the remaining cells have consistent structure.

The behaviour of *A. aceti* is quite different from the behaviour of the other bacteria studied which showed changes which were at their maximum during the mid-section of the exponential phase but were insignificant by the time the stationary phase was reached. Changes in carbohydrate content of cells during the growth curve have been reported previously[14] for microcolonies of *Candida albicans*. In that case the authors noted that the highest carbohydrate content was to be found at the edge of the colony in the most metabolically active cells and they speculated that this was due to increased uptake of glucose from the medium by the active cells.

For *A. aceti* there appear to be permanent changes to the polysaccharide content of the cells as the growth curve continues. Hence these changes are probably not related simply to cellular changes occurring before cell mitosis, which is the most likely explanation for structural changes which occur only during the exponential phase of the growth curve.

CONCLUSIONS

FTIR spectroscopy utilizing the ATR sampling technique has been shown to be a useful technique for obtaining information about the structural changes in the cell material of bacteria during their growth curve. The method is simple and excellent noise-free spectra are obtained. The information contained in the spectrum is calculated to originate from the whole air-dried cell and therefore includes the structural features of the cell walls and membranes, internal structures and the cytoplasm.

All bacteria studied showed significant spectral differences during their growth curves. Typically, the largest variations occurred during the mid-part of the exponential phase of growth. The thermophile, *B. stearothermophilus*, showed increased intensity of methylene stretching bands during the exponential phase indicating increased lipid concentration in the cells during this part of the growth curve. The spectra also showed variations in the amide I band which indicates either different proteins, or different protein conformations, during the exponential phase. The halophiles, *H. salinarium* and *H. morrhuae*, showed large spectral changes indicating that the concentration of sulfate ion was varying widely during the growth curve and was at a maximum in the mid-part of the exponential phase before falling to much lower levels in the stationary phase. The acid tolerant species, *A. aceti*, showed changes in lipid content during the exponential phase in a similar manner to *B. stearothermophilus.* The spectra of *A. aceti* also showed that the amount of polysaccharide in the cells was increasing during the growth curve. However, the changes followed a very different pattern from those previously mentioned because the increase was maintained by cells in the stationary phase and was not restricted to the exponential phase of the growth curve. Structural changes that occur in the exponential phase are likely to be related to active cell division. The continuously increasing concentration of polysaccharide during the growth curve cannot be explained in the same way.

An outcome of this study is that each species behaved quite differently, except for the two species of halophiles. Given that only four species were studied, a great deal more work is required to understand the variation of spectra and structure during the growth curves of a much wider range of bacteria.

Clearly, the results of this study have ramifications for the use of infrared spectroscopy for the identification of bacterial species and strains. It highlights the difficulties of correlating a single IR spectrum with a particular bacterial species, and shows that great care must be taken to standardize bacterial growth conditions to allow for variation during the growth cycle. It may be that a range of spectra should be taken into consideration for each bacterial species, in which case spectral databases for bacterial identification may be much larger than previously thought.

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Figure Captions

1. FTIR-ATR spectra of the four bacteria used in this study: A) *H. salinarium*; B) *H. morrhuae*; C) *B. stearothermophilus* and D) *A. aceti*.

2. Growth curve of *B. stearothermophilus* grown at 55 °C

3. FTIR-ATR spectra of samples of *B. stearothermophilus* grown at 55 °C taken at various times during growth: A) 3.0 h; B) 6.5 h; C) 8 h; D) 11.0 h; and E) 13.5 h.

4. Spectral subtraction of *B. stearothermophilus* spectra showing increase in lipid content during exponential growth phase: A) 13.5 h; B) 8.0 h; and C) Difference spectrum B) minus A).

5. Plot of intensity of CH_2 symmetric stretching band at 2850 cm^{-1} against time from inoculation (–•–). Also shown on the same timescale for comparison is the *B. stearothermophilus* growth curve as measured by the optical density at 540 nm (♦).

6. Expansion of the spectral region 1800-1300 cm^{-1} for spectra of *B. stearothermophilus* samples to show changes in the amide I (around 1650 cm^{-1}) and amide II (around 1545 cm^{-1}) bands: A) 3.0 h; B) 8.0 h; C) 11.0 h; and D) 13.5 h.

7. Variation of intensity of sulfate band at 618cm^{-1} during the growth curve *of H. salinarium* (–•–), together with the growth curve as measured by optical density at 540nm(♦).

8. FTIR-ATR spectra of *H. salinarium* taken at various times during the growth curve: A) 60.0 h; B) 90.0 h; C) 96.0 h; D) 102.0 h; E) 12.02 h; and F) 128.0 h..

9. Variation of intensity of band at 1050 cm^{-1} during the growth curve of *A.aceti* (•), together with the growth curve as measured by optical density at 540nm (♦).

10. FTIR-ATR spectra of *A. aceti* taken at various times during the growth curve: A) 74.0 h; B) 75.0 h; C) 76.0 h; D) 93.0 h; E) 94.0 h; F) 99.0 h; G) 100.0 h; H) 101.0 h; and I) 120.0 h.