Polarized Light Scattering for Rapid Observation of Bacterial Size Changes

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ABSTRACT The effect of changing growth conditions on the diameter of rod-shaped bacteria was studied in vivo with the use of polarized light scattering. The value of a ratio of scattering matrix elements was measured as a function of scattering angle at various times after nutritional "upshift" for two strains of *Escherichia coli* cells. The peak locations of the scattering function were calibrated against the diameter for rod-shaped bacteria. The peaks moved toward smaller angles as a function of time after upshift, indicating that the diameter was increasing. Under special conditions, substantial peak shifts occurred within a few minutes of growth condition change, indicating a rapid onset of growth in diameter. The rate of increase of the diameters after upshift was obtained from the angular shift of peak location. This rate was ~14 nm/min for *E. coli* K12 and ~9 nm/min for *E. coli* B/r at 37°C. The rate of diameter increase is smaller at lower temperatures. Experiments with *Bacillus megaterium* showed that any diameter change after nutritional upshift at 37°C is limited to at most a very small increase, at least for the strain and medium tested.

INTRODUCTION

Light scattering has been used for many years to obtain structural and density information about biomolecules and microorganisms (Bohren and Huffman, 1983; Koch, 1968; Salzman et al., 1990; Wyatt, 1968, 1969, 1970, 1973, 1993; Wyatt and Phillips, 1972). Detailed information about the scattering microorganism can be obtained by utilizing incident polarized light and determining the polarization state of the scattered light. Mathematically, polarized light scattering can be described by the following matrix equation:

$$\begin{bmatrix} I_s \\ Q_s \\ U_s \\ V_s \end{bmatrix} = \begin{bmatrix} s_{11} & s_{12} & s_{13} & s_{14} \\ s_{21} & s_{22} & s_{23} & s_{24} \\ s_{31} & s_{32} & s_{33} & s_{34} \\ s_{41} & s_{42} & s_{43} & s_{44} \end{bmatrix} \begin{bmatrix} I_0 \\ Q_0 \\ U_0 \\ V_0 \end{bmatrix} \quad (1)$$

where the four-component vector on the left-hand side describes the polarization state of the scattered light, and the vector on the right-hand side the polarization state of the incident beam. These four-component vectors are commonly known as Stokes vectors. The matrix, $\mathbf{S}_{ij}$, on the right-hand side of the equation transforms the incident to the scattered Stokes vector and is called the "scattering" or "Mueller" matrix. A detailed description of both the mathematics of the Mueller matrix and Stokes vectors and the instrumentation necessary to measure the matrix elements is given by Bohren and Huffman (1983) and Bronk et al. (1991).

Polarized light scattering measurements can be used to obtain each of the matrix elements of Eq. 1 as a function of the scattering angle for a given ensemble of scatterers, such as a suspension of bacteria contained in a cuvette, as indicated in the simplified sketch of the apparatus in Fig. 1. The particular ratio of the two matrix elements, S_{34}/S_{11}, measured as a function of scattering angle has been used for a number of years in attempts to rapidly identify microorganisms (Bickel and Stafford, 1981; Hunt and Huffman, 1973). It was found that measurements of the Mueller matrix elements plotted as a function of scattering angle give quite reproducible graphs, but change for a given species of bacteria as a function of growth conditions (Van De Merwe et al., 1989).

In subsequent research, using mostly rod-shaped bacteria, the relationship between the bacterial size and the different features in the graph of the Mueller matrix element ratio S_{34}/S_{11} versus angle was studied. The average cell length, which is easily measured microscopically, seemed to have a negligible effect on this graph under the experimental conditions used. On the other hand, using microscopic measurements of the bacterial cell diameter, it was found that the peak locations in the S_{34}/S_{11}-versus-angle graph seemed to be strongly correlated with the average cell diameter (Bronk et al. 1991, 1992; Van De Merwe and Bronk, 1989). The cell diameters, because their dimensions are close to the resolution of the optical microscope, are not as easily and accurately measured as the cell lengths. Although electron microscopy yields a much higher resolution, the reliability of electron microscopic measurements is limited because the cells shrink during the preparation for that technique. Either way, microscopic measurements of bacterial dimensions are time consuming, with limited accuracy depending on the number of cells measured. The correlation between the average cell diameter and the peak locations in the

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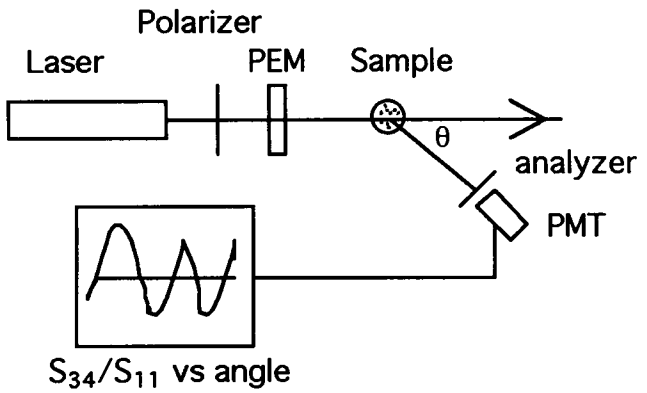


FIGURE 1 Simplified diagram of scattering apparatus. The laser light is linearly polarized before traversing a photoelastic modulator (PEM). The light is scattered from the microorganisms and is observed at an angle θ through a polarizer (analyzer) by a photomultiplier tube (PMT).

S_{34}/S_{11}-versus-angle graphs gives rise to a method for rapidly measuring diameters of rod-shaped bacteria in vivo. Theoretical computations have confirmed this experimental observed correlation (Bronk et al., 1995).

In a previous study (Stull, 1972) it was shown that light scattering from single levitated bacterial cells (*Staphylococcus epidermidis*) could be used to obtain a diameter by comparing the bacterial scattering to that of an equivalent sphere. However, the measurements were only suitable for obtaining diameters of cells that are close in shape to a sphere. Furthermore, they were performed on dried cells levitated in air, rather than cells suspended in a growth medium, so that the dynamics of bacterial size changes could not be followed. A study by Wyatt (1970) of total scattering versus angle (i.e., equivalent to measuring matrix element S_{11}) was used to obtain an average diameter and cell-wall thickness for spherical bacteria (*Staphylococcus aureus*) in water. Wyatt (1973) studied the scattering amplitude versus angle arising from a number of different bacterial scatterers. We believe that using polarized light for the scattering extends the capabilities for measuring dimensions of rod-shaped bacteria.

Many investigators (Brunschede et al., 1977; Cooper, 1991; Donachie et al., 1976; Grover et al., 1980, 1987; Kubitschek, 1990; Meyer et al., 1979; Pierucci, 1978; Trueba and Woldringh, 1980; Woldringh et al., 1977, 1980; Zaritsky et al., 1982, 1993; Zaritsky and Helmstetter, 1992) have studied bacterial cell growth and division as a function of different conditions, including "nutritional shift-up" experiments to elucidate the growth process. In the present paper, we report on our studies to monitor rapid changes in bacterial cell diameters, which were induced by rapid growth medium and growth condition changes. In a previous study (Bronk et al., 1992), we reported our microscopic measurements of diameters of two different strains of *Escherichia coli* bacteria. The cells in stationary phase have a measurably smaller diameter than cells in log phase. Stationary phase cells are cells grown over an extended period of time until a maximum cell density is reached and the cells stop dividing. A suspension of log-phase cells contains cells at relatively low density, with the number of cells growing exponentially. Zaritsky et al. (1982) found that the adjustment of the cell diameter during a nutritional shift is slow under the particular experimental conditions they utilized. However, we searched for and found conditions under which the adjustment of the diameters appeared to be rather rapid. We found that shifting some bacteria from early stationary phase conditions in minimum medium (nutritionally poor) to uncrowded conditions in fresh, nutritionally richer medium leads to a rapid change in size.

We describe experiments in which we monitor a measure of the average cell diameter for bacteria that were grown in a minimum medium to early stationary phase, and then transferred to a richer medium at low density. Measurements were taken immediately before the change in growth conditions, after dilution, and periodically during approximately one doubling time after the change. The experiments reported here indicate that the average diameter of *E. coli* bacteria responds very rapidly under these conditions at 37°C. The response is still rapid, but slower in rate when the cells are grown at a lower temperature.

MATERIALS AND METHODS

Experimental setup

The instrumentation has been described in detail in a number of earlier publications (Bickel et al., 1976; Bohren and Huffman, 1983; Bronk et al., 1991; Hunt and Huffman, 1973). In the following, we limit ourselves to a brief overview of the scattering apparatus, a simplified diagram of which is shown in Fig. 1.

Light from a 670-nm diode laser (Melles-Griot, model 56DLB112/P, 7 mW) is passed through a linear polarizer and subsequently through a photoelastic modulator (PME-8; HINDS International, Portland, OR), which causes the polarization of the emerging laser beam to alternate between left and right circular at a frequency of 50 kHz. The modulated laser beam, which is ~1 mm in diameter, passes through a suspension of the cells to be studied. The light scattered by the cells is passed through a linear polarizer (like an "analyzer") and then detected by a photomultiplier tube (PMT) (Hamamatsu R636), which is rotated around the sample at an angular velocity of ~1.0°/s. The 50-kHz component of the PMT signal, with the use of a lock-in amplifier (5208 two-phase lock-in amplifier-E G and G; Princeton Applied Research Corp.), measures the S_{34}/S_{11} ratio of elements of the matrix in Eq. 1.

The experimental setup is automated, with a computer recording the signal, which is averaged over 1–3 s per degree. The PMT was scanned through angles from near forward (20°) to near backward (150°) for some experiments. For other experiments, we scanned only over angles in a small range around a particular value at which the resulting graph showed a maximum, to follow rapid changes.

Biological

Two strains of *E. coli* bacteria were used for the experiments reported here: *E. coli* K 12 (ATCC 49439) and *E. coli* B/r (ATCC 12407). We also utilized *Bacillus megaterium* (ATCC 13632), which is listed as a nonsporulating strain in the American Type Culture Collection catalog. The two different media used were (per liter): M1t, consisting of 2.0 g NH_4Cl, 6.0 g Na_2HPO_4 (anhydrous), 3.0 g KH_2PO_4, 3.0 g NaCl, 0.05 g tryptophan, 0.25 g $MgSO_4 \cdot 7H_2O$, and 2.0 g glucose (Mg salt and glucose solutions

sterilized separately), with a pH brought to 7.0 after autoclaving; and LB (ATCC medium 1065), consisting of 10.0 g NaCl, 10.0 g tryptone, and 5.0 g yeast extract (Difco 0127) with a measured pH of ~6.9. The buffers used were phosphate-buffered saline (PBS) at pH ~7.4 (Sigma, St. Louis, MO), or unbuffered sterile saline (SS), consisting of 0.9% NaCl, pH ~5.7–6.7.

The suspension of bacteria was contained in a cylindrical quartz cuvette (19 mm inner diameter, 22 mm outer diameter, and 70 mm high, with the suspension ~2.5 cm deep), which was immersed in water in a glass dish (~7 cm diameter). We maintained a steady-state temperature of 37°C by heating the water in the dish with a DC current through a resistor (power ~8 W).

Three different protocols were followed in preparing the bacterial cells for the upshift experiments. Protocol 2 is a slight modification of protocol 1. Protocol 3 was used with the particular strains indicated in the Results section, because it seemed to give a more pronounced upshift effect for these cells. The protocols were chosen after preliminary experimentation to give large changes within a single doubling time, so that cell doubling would not require dilutions during the course of these scattering experiments.

Protocol 1

1. *E. coli* B/r is grown overnight in M1t medium (18–20 h) at 37°C in a flask in a shaker bath with good aeration.
2. The cells are spun down and resuspended in saline at an optical density at 600 nm of ~0.1.
3. After an initial scattering curve is obtained, an equal volume of prewarmed, double-concentration (2×) LB is added to the cells in the cuvette in the scattering apparatus. This was time "zero" for the scattering experiment.

Steps 2 and 3 were carried out either at room temperature (~24 C) or at 37°C, as indicated in the particular experiment, while the growth of the cells continued in the cuvette. The cell suspension was stirred regularly.

Protocol 2

Same as protocol 1, but using PBS for step 2.

Protocol 3

1. Overnight growth in LB (18 to 20 h) at 37°C.
2. Dilution into fresh LB and growth for 5 or 6 h at 37°C to late log phase.
3. Spin-down and resuspension in PBS at OD ~0.1.
4. Step 3 from Protocol 1.

We carried out several control experiments to show that moving the bacterial cells from medium to PBS or SS (protocols 1 and 2, step 2) had a negligible effect on the location of the peaks relative to the changes reported after upshift (i.e., shift of the peaks was less than a degree). We also performed a similar control experiment in which stationary phase bacteria were diluted into spent medium obtained by removing the bacteria from medium after an overnight growth. There was the same lack of a shift of the peaks in this case.

RESULTS

Scattering curves of S_{34}/S_{11} as a function of scattering angle are presented in Fig. 2 for an upshift in nutrition for *E. coli* B/r, using protocol 1. This timed sequence of measurements allows one to follow the dynamics of the diameter change, because the locations of the peaks in the graph give a measure of the average bacterial diameter (Bronk et al., 1992). The initial average diameter for these bacteria after overnight growth in the present conditions is ~0.60 μm, as measured by optical microscopy (Bronk et al., 1992). A

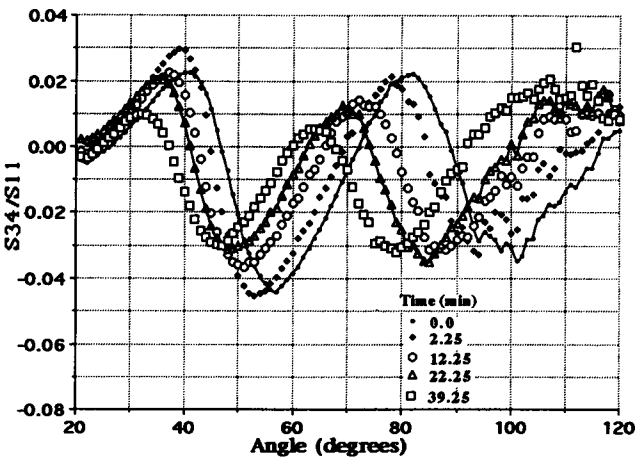


FIGURE 2 Scattering curves for S_{34}/S_{11} versus angle for *E. coli* B/r after upshift at 37°C in nutrition to rich broth after overnight growth in minimum medium (protocol 1). Times indicate time after upshift.

displacement of a particular peak toward the forward direction (*to the left in the graph*) indicates that the diameter has become larger with the addition of fresh medium. This has been confirmed for randomly oriented rod-shaped and spheroidal cells, both theoretically and experimentally, in a range of parameters (length, diameter, optical constants) that includes those of this paper (Bronk et al., 1992, 1995; Van De Merwe et al., 1989; Van De Merwe and Bronk, 1989). The times indicated are the number of minutes of growth after the upshift of medium with about 1/2 min uncertainty.

The decrease in peak heights with time may be attributed to two causes. A broadening of the size distribution probably occurs during the interval after upshift, and some multiple scattering may occur at later times, because of an increase in optical density of the bacterial suspension. It is hoped that these studies will be extended in later experiments to the changes that occur more gradually during normal batch culture procedures. Preliminary data show that for *E. coli*, the more gradual changes during the period 60–100 min are easily followed as the cell diameters stop increasing and slowly decrease toward their stationary phase values.

In Fig. 3, a graph is plotted with points derived from the scattering graphs for two experiments similar to that used for Fig. 2, except that protocol 2 was used (PBS instead of saline). The plotted points in Fig. 3 represent the angle of the second maximum (near 83° for the particular cells used) at various times after the upshift. To correlate the location of this peak with a diameter size, we utilized our data, reported in figure 5 of a previous paper (Bronk et al., 1992). We performed additional size calibration runs, using stationary-phase cells of the three types used in this project. The peak locations were then calibrated against the diameter size as previously reported (Bronk et al., 1992). We assumed that, to a good approximation, the departure from an ideal log-phase distribution of the diameters and lengths would have

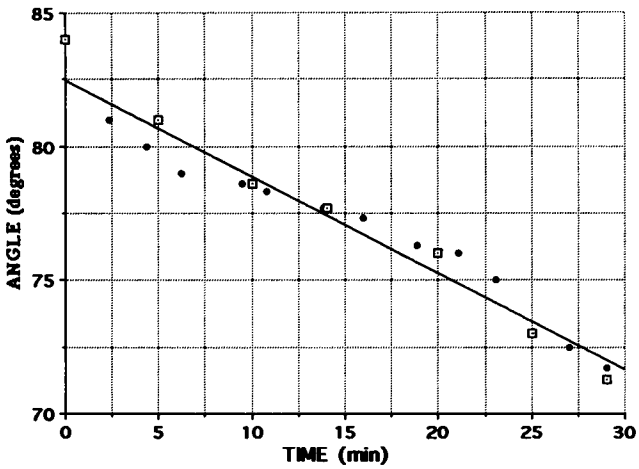


FIGURE 3 Angular location versus time for the second maximum of the curves for an experiment similar to that of Fig. 2, but using a buffer (PBS) instead of saline (protocol 2).

a negligible effect on peak locations for randomly oriented cells.

The results of several experiments, as described above, are summarized in Table 1. As an approximation, the rate of change of the diameter in degrees per minute was obtained from a linear fit to the data as plotted in Fig. 3. This value was converted to nanometers by using a conversion factor obtained from the slope of the calibration graph. The value of the conversion factor we used was adjusted for the slightly longer laser wavelength used in the present experiments by multiplying by the ratio of the wavelengths (i.e., 633/670). The corrected conversion factor, C, for the second maximum is 21.0 ± 2.0 nm/degree and 11.6 ± 1 nm/degree for the third maximum. For *E. coli* B/r with protocol 1, the average angular rate of change was 0.43°/min, which, when multiplied by C, gives 9.0 nm/min as the average rate of diameter increase, and for protocol 2, the average rate of change was very close at 9.2 nm/min.

Similar experiments were performed with protocol 1 at room temperature (~24 C). As one would expect, the average rate of change of the diameters slows with a lowering of growth temperature. The rate of diameter increase at room temperature was 4.2 nm/min for *E. coli* B/r. Furthermore, experiments were performed with the K12 strain of *E. coli*, with the protocol changed slightly to allow the bacteria to resume growth into and through log phase and into late log phase (protocol 3), at which time a nutritional upshift took place. Two temperatures were again used, in this case 37°C and 30°C, for growth before and after the upshift. A timed series of scattering curves taken after the nutritional upshift at the lower temperature is shown in Fig. 4. In this case, the second maximum for the S_{34}/S_{11} curve starts at ~71°, an angle closer to the forward direction. This is expected from previous measurements, which indicated that the K12 strain grown overnight in LB had a larger diameter (~1.0 μm) than the B/r cells grown overnight in M1t medium (~0.6 μm). The linear graphs obtained by plotting the angular location of the second maximum are shown in Fig. 5 for both temperatures. The respective rates of increase for the average bacterial diameters for *E. coli* K12 with protocol 3 were 13.6 nm/min and 8.4 nm/min for the 37°C and 30°C temperatures, respectively.

We also examined the effect of an upshift on the diameter of a different bacterial species, a nonsporulating strain of *Bacillus megaterium*. This bacterium has a much larger diameter (~1.4 μm) than *E. coli*, both for stationary phase and for log-phase growth. The scattering curve obtained from one such experiment is shown in Fig. 6. There is a very small but definite angular shift of the third peak. As indicated by this very small shift, the effect of the nutritional upshift on the diameter is at most very small. This was not totally unexpected, because our microscopic measurements (Bronk et al., 1992) indicated that log-phase and stationary-phase diameters of these cells are the same to within experimental error, when the cells were grown in the medium used for the present experiments. This is also true for *Bacillus subtilis*, although the average length for both species of bacilli is substantially greater in log phase than in stationary phase.

TABLE 1 Summary of rate of diameter growth for several bacterial strains

Bacteria protocol, temperature	Second peak (degrees) initial angle	Angular rate ± SD (degrees/min)	Rate of increase of diameter ± SD (nm/min)	No. of experiments
E. coli B/r protocol 1, 37°C	82.8 ± 3.8	0.43 ± 0.08	9.0 ± 1.9	6
E. coli B/r protocol 1, 24°C	83.0 ± 1.5	0.20 ± 0.07	4.2 ± 1.5	3
E. coli B/r protocol 2, 37°C	83.0 ± 1.7	0.44 ± 0.06	9.2 ± 1.5	3
E. coli K12 protocol 3, 37°C	71.0 ± 1.5	0.65 ± 0.04	13.6 ± 1.5	4
E. coli K12 protocol 3, 30°C	70.8 ± 1.3	0.40 ± 0.014	8.4 ± 0.8	3
	Third peak (degrees)	Total change (degrees) in 15 or 30 min	Total increase (nm)	
B. megaterium nonsporulating, protocol 3, 37°C	60 or 61	1.2	13.1 ± 3.0	2

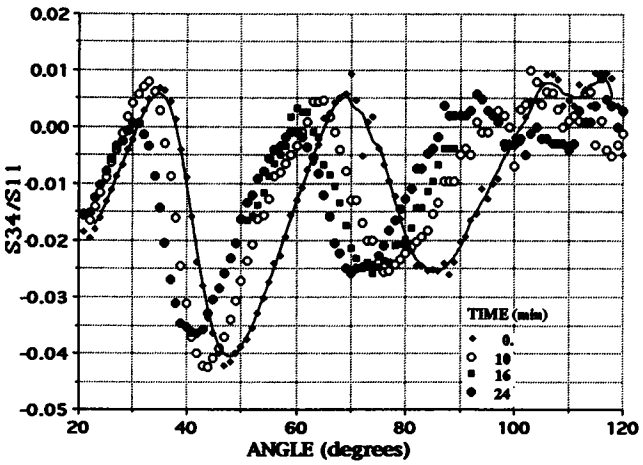
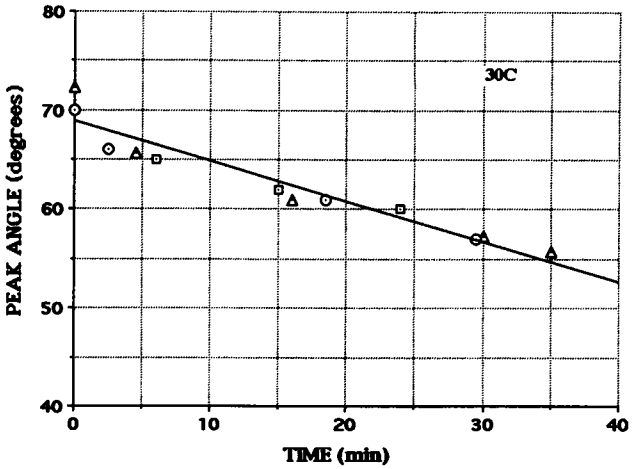


FIGURE 4 Graphs of S_{34}/S_{11} versus angle for *E. coli* K12 at various times after nutritional upshift from late log phase at 30°C (protocol 3).



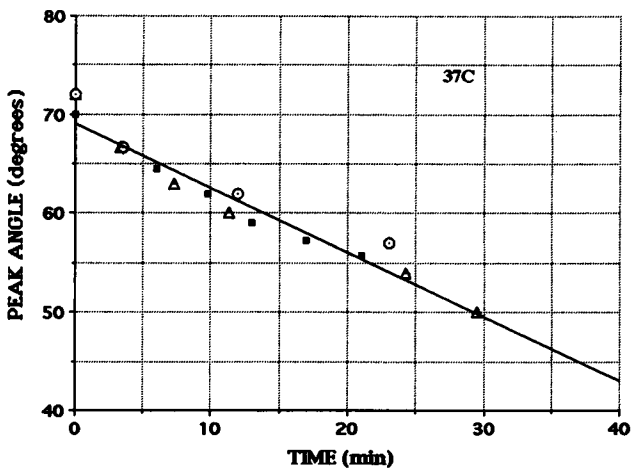


FIGURE 5 Graphs of angular location versus time for second maximum from scattering graphs for experiments with *E. coli* K12 like that of Fig. 4. (*a*) 30°C. (*b*) 37°C.

The results of the various experiments are summarized in Table 1. The estimated errors are obtained from standard deviations over the several experiments for the angular rate of change. These are combined with the indicated standard deviation in the conversion factor, using standard rules for error propagation to obtain a standard deviation in the rate of diameter increase.

DISCUSSION

The results demonstrate that polarized light scattering can be used to monitor rapid changes in average bacterial cell diameters in a suspension of a growing culture. The technique is sensitive and reproducible. In the current setup, the usable OD of the sample is limited to the range between ~0.02 and 0.15 (limits due to signal-to-noise and multiple scattering effects). Using more intense laser light and a larger beam diameter could extend the useful range of OD's to lower values.

The results show that the rate of change of a bacterial diameter depends on the type of cell, the temperature, and the nutritional conditions before and after the upshift (graphs not shown).

From previous experiments and calculations (Bronk et al., 1992, 1995) it became clear that changes of cell length hardly affect the location of the peaks in the scattering graphs used for randomly oriented rod-shaped bacteria. This was concluded following our observations that resulting changes in the peak location in the graphs for a particular bacteria, from different laser wavelengths, were similar to the changes due to different cell sizes (Van De Merwe et al., 1989). (Note that a longer wavelength is equivalent to smaller scatterers, for elastic scattering.) Further experiments with several different species of cells showed that the peak shift was linearly correlated with microscopically measured diameters for rod-shaped bacteria, but not at all correlated with different lengths for these cells suspended in random orientation (Bronk et al., 1992). Calculations were then made by using the coupled-dipole theoretical model for electromagnetic scattering from micron-sized objects (Bronk et al., 1995). Calculations made with this model were verified to give identical results for the Mueller matrix elements for spheres, where the scattering is calculated exactly by using the equations of Mie theory. In this case the same result was obtained for randomly oriented rod-shaped bacteria. (For oriented cells, the peak location is affected by cell length, but this effect washes out when averaged over random orientations.) Thus we verified, both experimentally and theoretically, that angular peak location in the S_{34}/S_{11} graph can be converted to the cell diameter for rod-shaped bacteria under the conditions used in the present experiments.

At 37°C, the cell diameters of the *E. coli* bacteria can rapidly increase its diameter with a shift to a richer or fresher medium. At a lower temperature, the diameters also increase, but at a much lower rate. The diameters of the bacteria are hardly affected by a shift of the cells from medium to saline or PBS, as indicated in control experi-

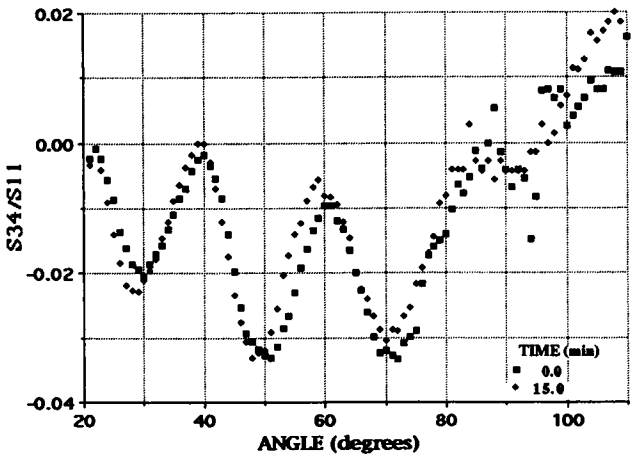


FIGURE 6 Scattering graph of S_{34}/S_{11} at two times after nutritional upshift for *B. megaterium* cells (protocol 3).

ments, which generally show negligible shift in the scattering curves.

The *B. megaterium* bacteria, the diameters of which are much larger, changed their diameter by a very small amount when tested under the same conditions. We note that in 30 min after upshift at 37°C, *E. coli* K12 had undergone an increase in diameter ~20 times that of the much larger bacillus. The relatively negligible change of diameter for *B. megaterium* cells during growth cannot be fully assessed at this time, except to say that there appears to be no significant role for change in diameter for these cells under the present conditions as there evidently is for *E. coli* cells.

An additional, possibly interesting observation is that the rate of change of the diameter after upshift differs by roughly the same factor, ~1.5, as their diameters differ at the beginning of the upshift for the two strains of *E. coli* cells (at 37°C and using somewhat different protocols).

The present experiments were performed with bacteria shifted from stationary phase or late log phase to an enriched medium under conditions in which rapid growth was possible. For the particular conditions we used, these experiments indicate that for some bacteria, the cell diameter can respond within minutes to changing growth conditions, although the change may be very small or absent for other bacteria.

The present technique seems like an ideal method for investigating cell diameter changes during a cell cycle with the use of synchronized cultures. These measurements could provide a sensitive measurement of the change in a cell's diameter in the course of a single cycle. Future studies could also be done with scattering from aligned bacteria. Preliminary modeling studies (Bronk and Druger, unpublished observations) have shown that in this case, the graph of S_{34}/S_{11} is sensitive to cell length. Measuring in vivo size changes for a single bacterial cell appears to be a technical possibility that could lead to interesting results, but which is beyond our present capability. The fact that this technique is currently used for statistical averages over several thousand bacteria is a limitation. However, such a statistical average also confers an advantage in that it is intrinsically much faster in yielding average changes than any expected direct microscopic measurement.

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