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Production of sound waves by bacterial cells and the response of bacterial cells to sound

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Bacterial cells enhance the proliferation of neighboring cells under stress conditions by emitting a physical signal. Continuous single sine sound waves produced by a speaker at frequencies of 6–10, 18–22, and 28–38 kHz promoted colony formation by *Bacillus carboniphilus* under non-permissive stress conditions of high KCl concentration and high temperature. Furthermore, sound waves emitted from cells of *Bacillus subtilis* at frequencies between 8 and 43 kHz with broad peaks at approximately 8.5, 19, 29, and 37 kHz were detected using a sensitive microphone system. The similarity between the frequency of the sound produced by *B. subtilis* and the frequencies that induced a response in *B. carboniphilus* and the previously observed growth-promoting effect of *B. subtilis* cells upon *B. carboniphilus* through iron barriers, suggest that the detected sound waves function as a growth-regulatory signal between cells.

Key Words——*Bacillus carboniphilus*; *Bacillus subtilis*; bacteria; growth regulation; signal response; sonic signal; sound; ultrasonic

Cell biologists often have difficulty culturing highly diluted animal or plant cells (Watson et al., 1993). Once the cells reach a certain density, their growth is markedly facilitated. Individual cells probably produce a signal or signals that stimulate the growth of other cells. However, the signal intensity is too weak in diluted cultures unless a layer of homologous or foreign cells, called a feeder layer or host or nurse cells, is added to the diluted culture (Muir et al., 1954). Even unicellular microorganisms cannot form colonies on an agar plate if inoculated under unfavorable growth conditions such as high salt concentrations or high temperatures (Matsuhashi et al., 1995). However, they do start to grow and form colonies under these conditions, which are ordinarily non-permissive, when they receive signals from large growths of neighboring homologous or heterologous cells (Matsuhashi et al., 1997b). Certain chemicals may play an important role in this cell-to-cell growth regulation (Kaiser and Losick, 1993; Swift et al., 1996). Recently, we proposed another type of intercellular regulation involving physical rather than chemical signals. These signals could be sonic. The effect of growth-promoting and sustaining signals can be seen in a mass of cells growing on the same surface of a nutrient-containing agar plate, even when in Petri dishes separated by a 1–2 mm-thick iron barrier (Matsuhashi et al., 1996). This newly discovered type of intercellular signaling appears to function in a variety of cells (Matsuhashi et al., 1996, 1997b).

In this paper, we report the response of a newly isolated bacteria, *Bacillus carboniphilus* strain Kasumi 6 (Fujita et al., 1996; Matsuhashi et al., 1995), to sound emitted from a speaker. *B. carboniphilus* requires carbon in the form of graphite or charcoal to grow under

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conditions that are ordinarily non-permissive. We chose this strain of bacteria for measuring bacterial growth-response to sound because this strain is the most sensitive signal recipient in tests of the remote growth-promoting effects of *B. subtilis* cells (Matsuhashi et al., 1996). Graphite is believed to convert external electromagnetic energy into sound (Matsuhashi et al., 1997a). We also report the detection of sonic signals produced by *B. subtilis* cells using a very sensitive, pressure-type condenser microphone system. *B. subtilis* was used for this study because this species is the most effective intercellular growth facilitator of the bacteria tested (Matsuhashi et al., 1996, 1997b).

Materials and Methods

Strains of bacteria and culturing conditions. *B. carboniphilus* strain Kasumi 6 (Fujita et al., 1996; Matsuhashi et al., 1995) was from our stock culture. The bacteria was cultured on an agar plate containing 15 g agar (Wako Chemical Co., Osaka, Japan, purest reagent grade) and Bacto Antibiotic Medium 3 (Difco Laboratories, Detroit, MI, USA) 8.75 g per liter at 37°C for several days, until spores developed. For the sonic signal recipient study, the spores were suspended in deionized water at a concentration of about 10^6 spores per milliliter. To measure the response of the bacteria to sound, $1–3\times10^3$ viable bacteria were inoculated onto an agar plate in a 90×15 mm polystyrene Petri dish. The medium used produced non-permissive stress conditions and consisted of 15 g of agar, 8.75 g of Bacto Antibiotic Medium 3 and 10 g of KCl per liter. The number of viable spores in the aqueous suspension was measured by inoculating an aliquot of the suspension onto an agar plate containing 15 g of agar and Trypticase Soy Broth (BBL, Becton Dickinson, Cockeysville, MD, USA) 30 g per liter, and incubating the plate at 37°C for 4 days.

Cells of *B. subtilis* Marburg strain JH642 (*phe-1 trpC2*) (Perego and Hoch, 1991), which were used to measure sound production, were cultured at 37°C for 1 day and then at 20°C for an additional day on an L-agar plate (Lennox, 1955). The medium was slightly modified. It consisted of 10 g of polypeptone, 5 g of yeast extract, 1 g of glucose, 5 g of sodium chloride and 20 mg of thymine per liter, and was adjusted to pH7.0 with NaOH to obtain maximum growth of the bacteria.

Measurement of the sonic response by B. carboniphilus. An artificial sound was produced by a function generator (Iwatsu FG330, 600-ohm output impedance) connected to a speaker (Ohm SP-88 full-range speaker, $65\times100\times65$ mm [$w\times h\times t$], 8-ohm impedance, 1.5 W output and 90dB intensity). The sound was a continuous sine wave produced at distinct frequencies between 1 and 50 kHz. The function generator was used at a fixed internal voltage setting of 10 V. The actual wattage was measured as 22 mW at 10 kHz, 38 mW at 20 kHz, 50 mW at 30 kHz and 55 mW at 40 kHz. The intensity of the sound, at the surface of the agar plate 0.5 cm from the speaker, measured through a 1 mm-thick polystyrene Petri dish cover was approximately 360 mV or 0.070 Pa at 1 kHz, 6.9 mV or 0.007 Pa at 20 kHz and undetectable at 49.8 kHz after correction for the sensitivity of the microphone. The intensity of the sound measured at 5 cm above the speaker (omitting the dish cover) was approximately 390 mV or 0.07 Pa at 1 kHz, 240 mV or 0.096 Pa at 10 kHz, 152 mV or 0.16 Pa at 20 kHz, 7.9 mV or 0.015 Pa at 30 kHz, 1.0 mV or 2.78×10^{-3} Pa at 40 kHz and 0.35 mV or 1.36×10^{-3} Pa at 50 kHz.

Apparatus for detecting the sonic waves produced by B. subtilis. The sound waves generated by *B. subtilis* cells were detected using a sensitive pressure condenser microphone (Type 4144, Brüel & Kjaer, Denmark) connected to a preamplifier (Type 2819, Brüel & Kjaer) with a pressure sensitivity of 52.5 mV/Pa below 8.2 kHz (-3dB). The acoustic signal intensity was measured using an apparatus for photoacoustic measurement (Yoshinaga et al., 1989) but this time with using no external light source. The internal volume of the photoacoustic cell was 0.5 cm^3. The microphone output was fed into a 40dB amplifier (Type LI-75A, NF Corporation, Yokohama, Japan) and the signal of every two microsecond interval was converted during 64 k-long words by a 12-bit A/D converter. The recorded signals were pooled in 4096-word datasets. Each set was analyzed by a Fourier transformation using a Hanning window, and the power spectra were averaged over the 64 k-word samples. Sensitivity of the detection system depended on the acoustic frequency, because there was acoustic resonance within the apparatus (sample-microphone room), and because the sensitivity of the microphone decreased at frequencies higher than 8.2 kHz. The resonance frequencies observed were 0.68, 6.0, 7.7, 17, 28, and 50 kHz. The overall sensitivity of the microphone to the sonic signal generated inside the photoacoustic cell was calibrated experimentally using a photoacoustic signal source. The sensitivity was 0 dB below 1 kHz, −30 dB from 5 to 15 kHz, −10 dB from 15 to 18 kHz, −25 dB from 20 to 32 kHz, −50 dB from 35 to 45 kHz and −30 dB from 47 to 50 kHz, with 0 dB referring to 52.5 mV/Pa.

Results

Response of B. carboniphilus to sound

A Petri dish containing a non-permissive stress agar

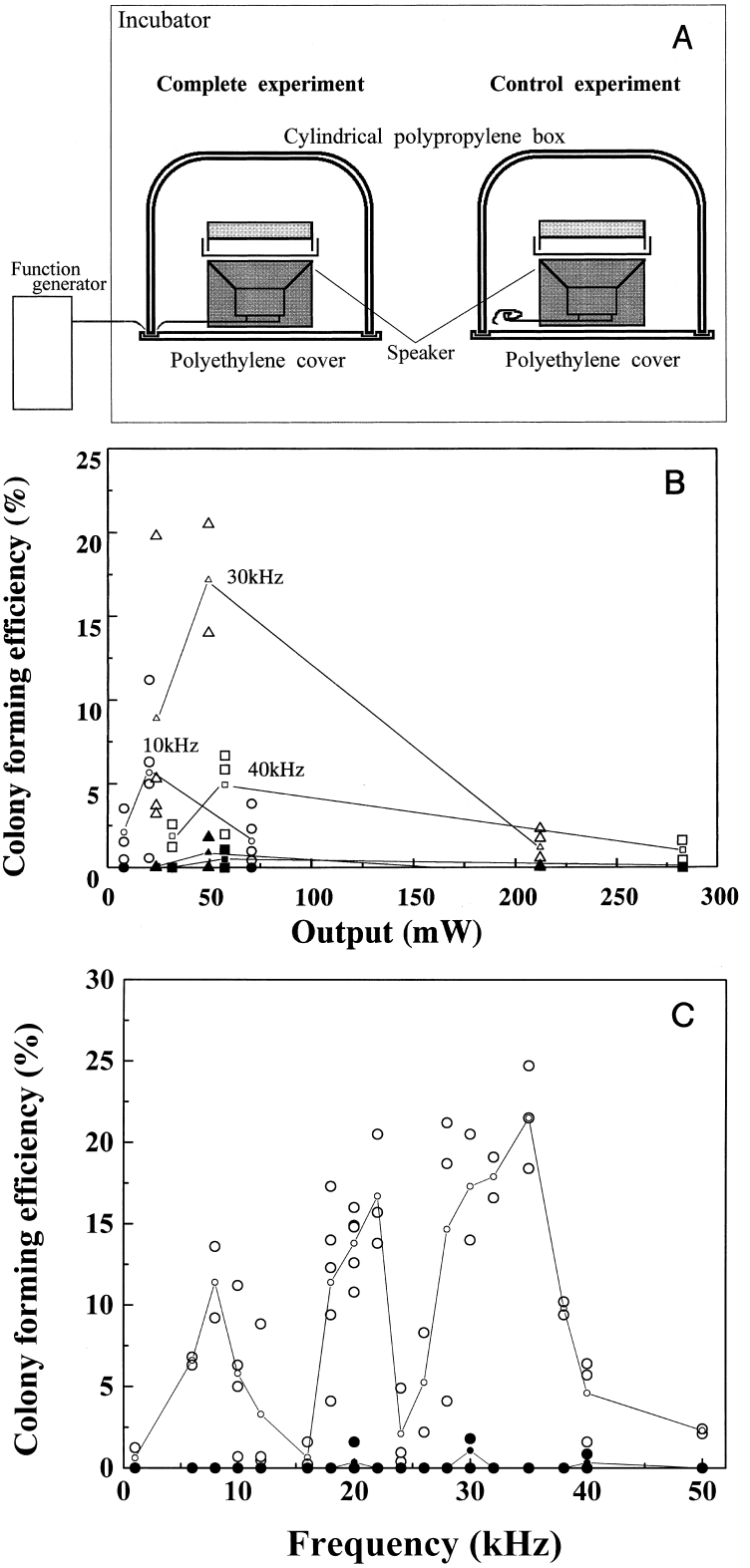

Fig. 1. Promotion of the growth of *Bacillus carboniphilus* by artificially produced sound.

(A) The experimental apparatus. (B) The effect of wattage on the efficiency of colony formation induced by sounds at 10 (○), 30 (△), and 40 kHz (□). For experimental details, see the legend to C. (C) The efficiency of *B. carboniphilus* colony formation (%) growing under KCl stress (8.75 g of Bacto Antibiotic Medium 3, 10 g of KCl and 15 g of agar per liter) plotted at various frequencies at 2 kHz intervals. The sounds were emitted from a speaker connected to a function generator set at 10 V. The open symbols represent the values obtained in experiments repeated 2–5 times, and the closed symbols correspond to control experiments run simultaneously. The small symbols indicate the mean values. See the text for experimental details.

plate inoculated with *B. carboniphilus* was inverted and placed on a speaker (Fig. 1A). The dish and speaker were placed in a cylindrical polypropylene container, which had dimensions of 142×92×2 mm [*w*×*h*×*t*] and was inverted with the polyethylene cover on the bottom. This was incubated at 43.5–44°C for 2 days. The number of colonies was counted and the efficiency of colony formation was determined. Control experiments were run with the speaker not connected to the function generator (Fig. 1A) or with a similar speaker connected to the function generator, in which the paper speaker cone was destroyed (Table 1).

Table 1. Disappearance of the growth-promoting effect of an artificially generated 35 kHz sound after destroying the paper cone of the speaker.

	Normal cone	Ruined cone	No power
Average of 5 experiments	16.6	3.3	0.5
Standard error	7.4	3.3	0.7

Numbers are the efficiency of colony formation expressed as a percentage. The details of the experiment are described in MATERIALS AND METHODS. Power 53 mW.

Figure 1B shows the effect of wattage on the efficiency of colony formation induced by sounds at 10, 30, and 40 kHz. With the output voltage of the function generator fixed at 5, 10 and 20 V, the most efficient colony formation occurred at 10 V in three experiments (i.e., 22 mW at 10 kHz, 50 mW at 30 kHz and 55 mW at 40 kHz). Therefore, all subsequent experiments at different frequencies were performed using this fixed voltage (Fig. 1C). Figure 1C shows that *B. carboniphilus* responded to continuous single sine wave sounds around 6–10, 18–22, and 28–38 kHz by forming colonies under the non-permissive conditions of KCl stress and high temperatures. The measure-

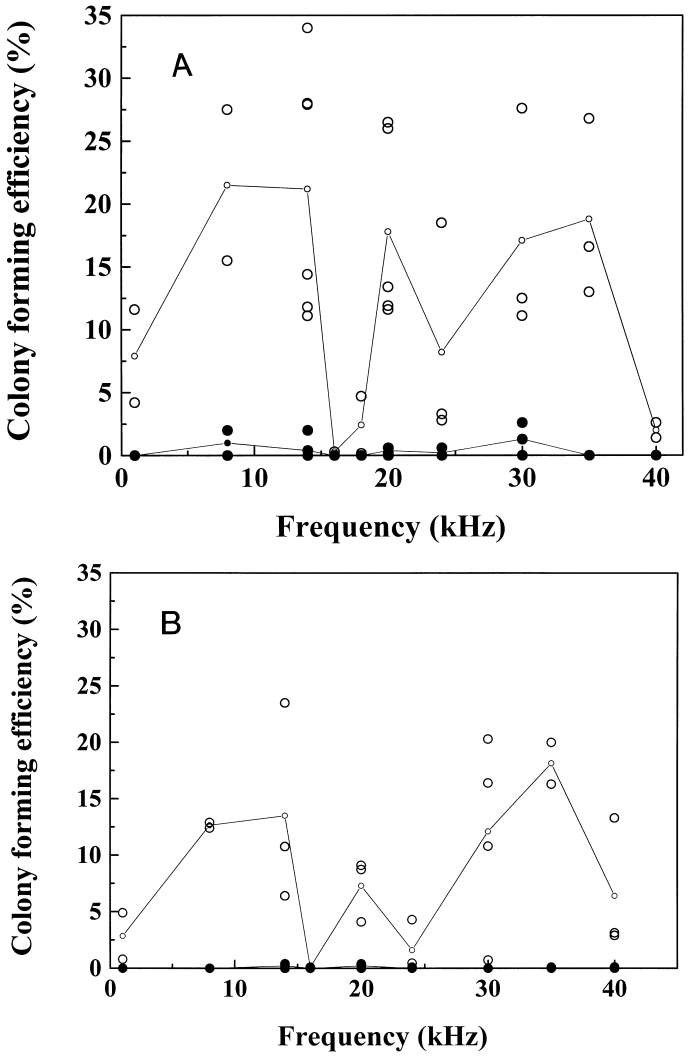

Fig. 2. Effect of altering the size of the polypropylene container (A) or Petri dish (B).
The experimental setup and spectra are similar to those shown in Fig. 1. (A) A larger polypropylene container (190×145×2 mm [*h*×*w*×*t*]) or (B) a smaller Petri dish (60×15 mm) was used. For symbols see the legend to Fig. 1C.

ments were repeated 2 to 5 times (large symbols), and the mean values are shown with small symbols.

Similar results were obtained when the Petri dish was placed on pieces of polystyrene foam so that it was 1 cm above the speaker. The colony-forming efficiency in triplicate experiments was (in percent) 1.3, 5.2, and 7.5 in the Petri dish placed directly on the speaker, and 3.5, 5.4, and 7.6 in the Petri dish placed above the speaker; when different sized Petri dishes were used (60×15 mm and 90×15 mm), when the Petri dish and speaker were enclosed in a larger cylindrical polypropylene container (190×145×2 mm [$w\times h\times t$]), when a different sized speaker was used (Tokyo Mark TS-150, 60×85×25 mm [$w\times h\times t$], 8-ohm impedance), or when vegetative cells were used instead of spores. Some of these results are shown below.

Figure 2 shows that varying the size of the plastic container (A) and Petri dish (B) did not have a discernible effect on the frequencies that promoted colony formation by *B. carboniphilus*. Colony formation also occurred at frequencies of 8, 15, 20, and 35 kHz when a smaller speaker (Tokyo Mark TS-150) was used.

Sound production by B. subtilis

Approximately 10^{10} freshly grown cells of *B. subtilis* Marburg strain JH642, with a wet weight of 50 mg, were mounted on a 1.5% agar disk 7 mm in diameter and 1 mm thick and placed at the bottom of a sample holder (inside diameter, 8×2 mm [$w\times h$]).

Figure 3 shows the spectra of sonic emission as a function of the frequency in a typical experiment. The sonic emission was scanned at frequencies between 1 and 50 kHz. The background sound levels were measured in a similar manner when there were no cells in the apparatus. In the presence of cells, three broad peaks were observed at 8 to 10 kHz, 18 to 22 kHz and 27 to 43 kHz. A sharp peak was seen at 49.5 kHz both in the presence and absence of cells. This sharp peak may have been caused by electronic noises throughout the apparatus used. Three other sharp peaks at 16, 25, and 48 kHz were detected only when cells were present, and not in background measurements taken before and after measurements with the cells.

The measurements were repeated under different climatic and physiological conditions, and with different preparations of *B. subtilis* cells at different stages of growth. The results shown in Fig. 3 were reproduced two more times when measured at an interval. Two other samples gave multiple broad peaks at 9, 14, 18, 29 (major peak), 32, and 34 kHz in 70 measurements. Other samples gave no significant signals. There were no positive results with heat-killed *B. subtilis* cells. No significant sound production by *B. carboniphilus*, *Escherichia coli* or *Saccharomyces cerevisiae* cells has yet been detected.

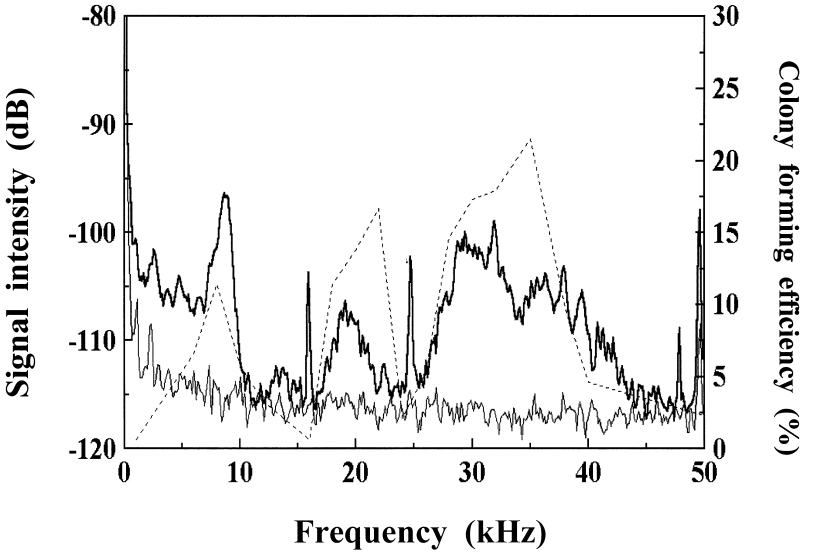

Fig. 3. Sounds emitted by *B. subtilis* cells.

About 50 mg of a cell paste from freshly grown cultures on an agar plate was placed on a small L-agar disk (8×1 mm [$d\times t$]) mounted in a quartz sample holder. In control experiments, the cell paste was omitted. The room temperature was about 20°C. The thick line is the intensity (dB) of sound detected by the microphone. The thin line is the result of the control experiments. The ordinate is the acoustic intensity at 52.5 mV/Pa at 0 dB, and the abscissa is the frequency in kilohertz. The data are the average of 31 overlapping measurements with 2,048 points each. For comparison, the data of the experiment of Fig. 1C is also shown with a thin dotted line.

Discussion

The sound emitted from billions of cells is probably a cacophony. It is a mixture of a vast number of sounds at slightly different frequencies producing a spectrum with broad peaks. Such a complex sound may be so-called white noise, with a non-repetitive waveform. It is difficult to isolate the sound produced by individual cells to measure the intensity. The spectra of the sonic waves shown in Fig. 3 consist of complex waves with rather broad peaks around a fundamental wave at a frequency of 8.5–9 kHz and its harmonics. Three sharp peaks at 16, 25, and 48 kHz were detected among the broad peaks. These sharp peaks are thought to be the result of the resonance of sounds produced by the bacteria within the photoacoustic cell.

The frequencies of sound that stimulated the growth of *B. carboniphilus* under KCl-stress resembled the pattern of broad peaks of sounds produced by *B. subtilis* cells. Although there were enormous differences in the intensities of the sounds produced by *B. subtilis* and the speaker used to stimulate *B. carboniphilus*, the similarity of their spectra (broad peaks) is noteworthy. Previous results suggested that the signals emitted by *B. subtilis* promote the growth of *B. carboniphilus* (Matsuhashi et al., 1996). This further supports the relationship between the sound emitted by one strain of bacteria and response to the sound by a related strain. We propose that these growth-regulating signals be called "biosonics."

B. subtilis emitted the strongest sound detected so far, however, it did not respond to artificially generated sounds under the present experimental conditions. *B. subtilis* is resistant to high temperature (52°C) and salt concentrations (15% KCl). So far, we have been unable to optimize the experimental conditions for measuring the sonic response of this bacterium.

The mechanisms involved in the production and perception of sound waves at specific frequencies are unknown. Cells emit and perceive sounds at wavelengths that exceed the size of the cell. Sounds might be produced by repeated expansion and contraction. This might be accomplished by using intracellular structures, such as membranes, cytoskeleton-like structures (e.g., Casarégola et al., 1990; Okada et al., 1994) or chromosomes, through mechanisms that involve the conversion of ATP or membrane potentials to movement. Activation of an ion channel might be the mechanism for the perception of sound. Such ion channels might have evolved into the acoustic sensing apparatus of higher animals.

In this paper, we report that the growth of *B. carboniphilus* is markedly enhanced by sounds at appropriate frequencies between 6 and 40 kHz. The growth of KCl-stressed *E. coli*, however, was found to be inhibited by sounds at lower frequencies (Matsuhashi et al., unpubl.).

The presence of sonic signaling might not be limited to bacteria. Unpublished studies suggest the existence of a similar mechanism in the yeast *S. cerevisiae*, bean seedlings (*Vigna mungo*) and developing fish eggs (Matsuhashi et al., unpubl.). Microwaves (Grundler et al., 1977) have also been suspected of affecting the growth of yeast cells and the magnetic fields of yeast and bacterial cells (Kimball, 1937; Moore, 1979).

Previously, we observed that carbon, in the form of graphite and activated charcoal, exerted a remote growth-promoting effect on bacterial cells. It is postulated that this effect results from the conversion of electromagnetic radiation into sonic waves (Matsuhashi et al., 1997a), presumably by a mechanism similar to that of photoacoustic emission (Bell, 1880). Natural materials, such as wood, soil, sand, minerals and metals, also exert similar growth-regulating effects on various living cells (Ohshima et al., unpubl.). Electromagnetic oscillations striking the cells directly might be converted into sonic waves intracellularly. The opposite effect, the intracellular conversion of sound into electromagnetic waves, is also theoretically possible (Norris and Hyland, 1997).

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