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Rapid Biosensor for Detection of Antibiotic-Selective Growth of *Escherichia coli*

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A rapid biosensor for the detection of bacterial growth was developed using micromechanical oscillators coated in common nutritive layers. The change in resonance frequency as a function of the increasing mass on a cantilever array forms the basis of the detection scheme. The calculated mass sensitivity according to the mechanical properties of the cantilever sensor is ~50 pg/Hz; this mass corresponds to an approximate sensitivity of ~100 *Escherichia coli* cells. The sensor is able to detect active growth of *E. coli* cells within 1 h. The starting number of *E. coli* cells initially attached to the sensor cantilever was, on average, ~1,000 cells. Furthermore, this method allows the detection of selective growth of *E. coli* within only 2 h by adding antibiotics to the nutritive layers. The growth of *E. coli* was confirmed by scanning electron microscopy. This new sensing method for the detection of selective bacterial growth allows future applications in, e.g., rapid antibiotic susceptibility testing.

In recent years, nanomechanical oscillators have been used as a new class of biological and chemical sensors (14). Such cantilever sensors are successfully applied in the fields of genomics (8, 11, 16), gas sensing (2), and proteomics (1, 15, 17). The elegance of these sensing methods is that the detection of an analyte requires no labeling, as well as that the various application fields differ only in the functional layers on the cantilever interface. The detection scheme remains common for all the different applications. In principle, any detection method which is based on molecular recognition is able to be implemented (3). The building of this biological sensor is based on oscillating cantilevers, where additional mass loading onto the cantilever interface results in a change of its resonance frequency. With the help of these micromechanical mass sensors, single bacterial cells and virus particles could be detected in a dry environment (10, 13). A first attempt at the application of micromechanical oscillators as growing cell sensors was made by Prescesky and coworkers in 1992 (21).

Bacterial infections are common and involved in many forms of disease, ranging from inflammation to food poisoning. Of much concern are nosocomial infections and especially the increasing resistance of bacteria to antibiotics. Rapid reporting of susceptibility can significantly improve the outcome for infected patients by enabling fast adjustment of antibiotic treatment, leading to decreased mortality and lower hospitalization cost (20, 23). Conventional susceptibility testing methods, such as the agar dilution method described in the National Committee for Clinical Laboratory standards guidelines, take at least 16 h before an interpretation of a result is feasible (9, 18).

In this work, detection of active bacterial growth using a resonance frequency-based mass sensor has been accomplished. Furthermore, the detection of selective bacterial growth on antibiotic-containing media in a bacterial adapted environment (high relative humidity and ideal growth temperature) will be presented.

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MATERIALS AND METHODS

Reagents. All reagents were purchased from Fluka (Buchs, Switzerland), unless otherwise stated, and were of analytical grade.

Bacteria and culture method. *Escherichia coli* XL1-Blue was obtained from Stratagene Inc. *E. coli* was grown in Luria-Bertani (LB) broth (22) overnight at 37°C before use. Cells from 1 ml of broth were harvested by centrifugation (14,000 rpm, 20 min, 4°C) (Genofuge 16 M; Techne), and the resulting pellet was resuspended in 500 μl LB broth supplemented with or without an appropriate antibiotic, depending on the experiment. The antibiotics used were purchased from Sigma.

Cantilever functionalization. Eight cantilevers were linearly arranged in an array at a pitch of 250 μm, each of them 500 μm long, 100 μm wide, and 7 μm thick. The cantilever arrays were obtained from the microfabrication group of IBM Zurich Research Laboratory. To remove contamination from the surfaces, the cantilever arrays were cleaned for 20 min in Piranha solution (95 to 97% H_2SO_4 in 30% H_2O_2; 1:1) and subsequently rinsed twice with sterile filtrated water and once with deionized water. The cleaning procedure was performed twice. The cantilever arrays were then immediately immersed in silylating solution [1% (3-glycidyloxypropyl)-trimethoxysilane–0.5% *N*-ethyldiisopropylamine in water-free toluene] for about 4 h at room temperature to form a self-assembled monolayer, which provided a hydroxyl-reactive surface towards primary hydroxyl groups (e.g., agarose). After the silanization, the cantilevers were rinsed twice with toluene for 20 min and dried under argon.

Agarose was dissolved in deionized water at a concentration of 1% (wt/vol), melted, and stored at 60°C. Just prior to use, the pH of the agarose solution was adjusted to a pH of ~11 with 5 M NaOH. The agarose coating of each cantilever was applied in quartz microcapillaries (outer diameter, 250 μm; inner diameter, 150 μm; Garner Glass, Inc., Clarmont, CA). The cantilevers were immersed into the microcapillaries using micromanipulators as described previously (5) and exposed to the agarose solution for 10 s. Longer incubation of the cantilever led to agarose collar formation at the base of the oscillators. The agarose layer thickness was determined by scanning force microscopy (MultiMode; Digital Instruments, Santa Barbara, CA). After the agarose coating is applied, the arrays can be stored in a dry environment for up to 1 month.

The bacterial inoculation of single cantilevers and the swelling of the agarose layer in the nutritive LB broth were performed simultaneously in the above-mentioned quartz capillaries. The sensor cantilevers were exposed to an *E. coli* suspension in LB broth (see "Bacteria and culture method") either containing 10 μg/ml of tetracycline or kanamycin or containing no antibiotic for 10 min, depending on the experiment. The reference cantilevers were immersed for 10 min in pure LB broth without *E. coli* cells. The functionalized cantilevers had to be used immediately for the measurement.

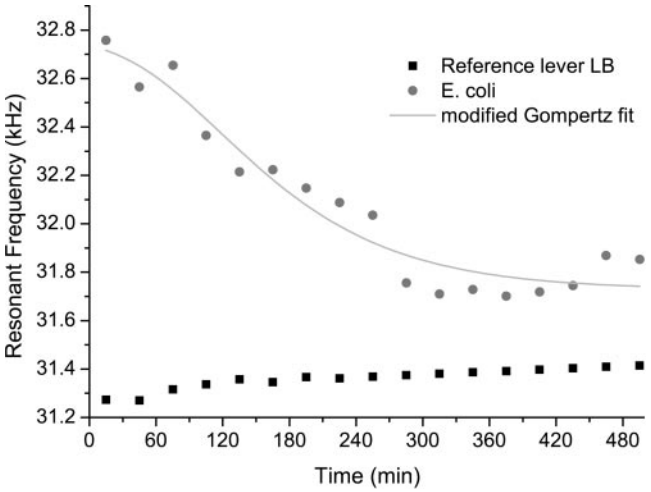

FIG. 1. Typical experiment showing detection of bacterial growth due to resonance frequency decrease where the resonance frequency of the reference lever (no bacteria) remains stable. The curve shown corresponds to a modified Gompertz model fit (4).

Experimental setup. Experiments were performed in a scanning probe MultiMode head with NanoScope control software (Digital Instruments). The measurement principle is as follows. A laser is focused onto the tip of an oscillating cantilever. The reflecting laser beam is then deflected onto a position-sensitive detector, where changes in the frequency can be measured.

To obtain suitable conditions for bacterial growth, the MultiMode head was placed inside a temperature- and humidity-controlled box. Humidity and temperature were measured using a HygroClip-SC05 sensor (Rotronic, Bassersdorf, Switzerland). LabView software and a data acquisition board (6036E; National Instruments, Austin, TX) were used to control the humidity handling system of the setup, i.e., mass flow meters and controllers (EL-Flow and FlowDDE; Bronkhorst HI-TEC, Reinach, Switzerland), as well as the temperature setting of the cooling/heating box (Intertronic; Interdiscount, Switzerland). The measurements were performed at 37°C and ≥93% relative humidity. For ideal measurement it was important to keep the temperature and relative humidity constant (change in temperature, ~0.2°C; change in relative humidity, ~2%).

In order to determine a frequency change due to bacterial growth, frequency spectra of the cantilever were taken every 30 min. For differential measurements the laser had to be switched manually between the sensor and reference cantilevers.

RESULTS

Detection of active *E. coli* growth. Figure 1 shows a typical frequency response curve obtained by incubating a sensor cantilever and a reference cantilever in a humid and warm environment (37°C, 93% relative humidity). For this experiment both levers were coated in a nutritive layer, but only the sensor lever was exposed to *E. coli* XL1-Blue cells. The average number of *E. coli* cells attached to the sensor lever surface was estimated to be ~1,000 by counting on scanning electron microscopy (SEM) micrographs (data not shown). No decrease of the resonance frequency of the reference lever, which was not exposed to bacterial cells, was detected over a period of 8 h. In contrast to this, the resonance frequency of the bacteria-containing lever dropped exponentially during the first 5 h and then slowly leveled out to a constant value. After the completed measurement the levers were examined with SEM in order to confirm bacterial growth. To exclude any undesired environmental changes (e.g., temperature and relative-humidity shifts in the humidity box), the frequency curves of the reference levers were always subtracted from the curves of the sensor levers in the following experiments. Figure 2 shows the SEM micrographs of the sensor (B) and reference (A) levers after exposure to a humid and warm environment. It can be clearly seen that bacterial cells were attached to the sensor lever as well as that they started to form small colonies. The bacterial cells are distributed randomly over the whole lever surface. On the other hand, no bacterial cells can be detected on the reference lever. As the cantilevers had to be completely dry for SEM micrographs, the salt crystallized as a result of the drying procedure. These crystals can be seen on the micrographs, but they were not present during the active growth experiment. The blisters which can be seen on Fig. 2B were caused by a much longer exposure of the sensor lever than the

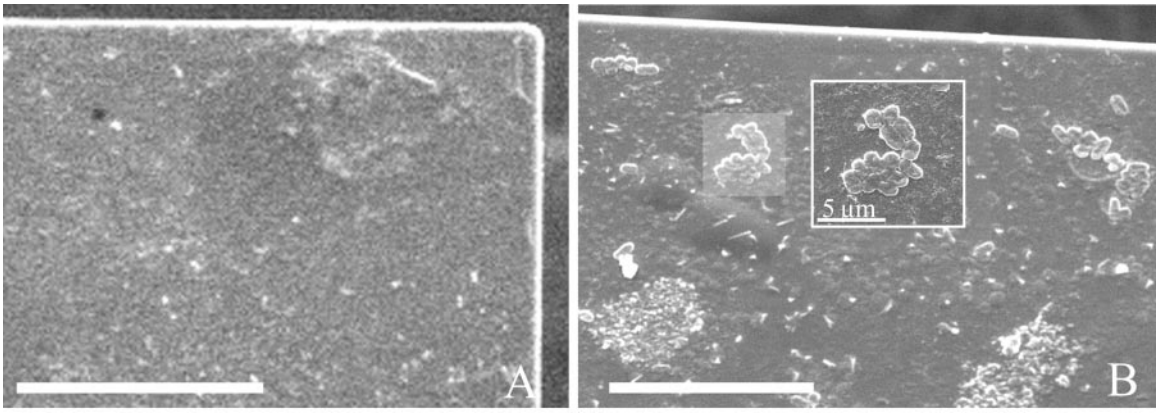

FIG. 2. SEM micrograph of agarose-coated cantilevers after 24 h of exposure to a warm, humid environment (93% relative humidity; 37°C). (A) Reference lever coated only with a nutritive medium (LB). No bacterial cells were observed. (B) Sensor lever coated with *E. coli* XL1-Blue and a nutritive medium (LB). Accumulations of *E. coli* XL1-Blue cells were observed. The inlay shows a close-up of *E. coli* XL1-Blue cells on the cantilever. The grainy salt pattern visible on both surfaces originates from drying required for SEM imaging. During sensing measurement salt precipitation can be neglected due to humid environment. In order to reduce the charging effects during SEM imaging, samples were prepared by evaporating a thin layer of Au. Scale bars correspond to 20 μm, if not otherwise stated.

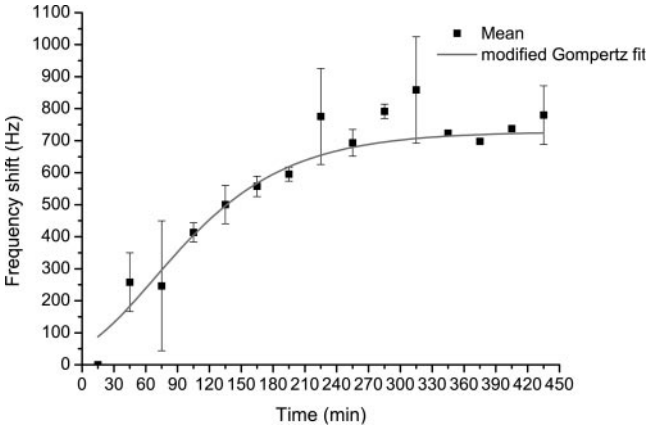

FIG. 3. Mean values of resonance frequency shifts due to bacterial growth of the sensor cantilever of multiple measurements as a function of time. The cantilevers were coated with agarose within the same batch to obtain similar mechanical properties. The curve shown corresponds to a modified Gompertz model fit (4). Bars correspond to the standard deviation.

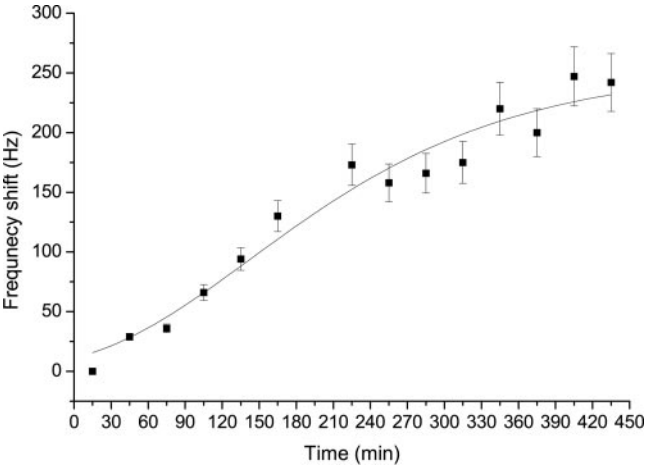

FIG. 4. Mean values of resonance frequency shifts due to selective bacterial growth as a function of time. The growth-supporting cantilevers were coated by nutritive layers which contained 10 μg/ml tetracycline, to which *E. coli* XL1-Blue is resistant. The curve shown corresponds to a modified Gompertz model fit (4). Bars correspond to the standard deviation.

reference lever to the electron beam during SEM imaging, shown in Fig. 2A.

Additional mass loading onto a cantilever causes a shift in its resonance frequency (Fig. 1). Assuming that the added mass is much less than the mass of the cantilever, the approximate resonant frequency shift due to additional mass loading (Δf) is given in reference 12 as

$$\Delta f = \frac{(1.875)^2}{4\pi} \cdot \sqrt{\frac{EI}{12}} \cdot \frac{m_{\text{load}}}{m_c^{1.5}}$$

where I is the moment of inertia of the cantilever, E is the Young's modulus of the oscillator, m_{load} is the added cell mass, and m_c is the mass of the cantilever before bacterial growth. For deeper insight into the mass/frequency dependence we refer to the work of Chen and coworkers (7). The calculated mass sensitivity according to the mechanical properties of the cantilever sensor is ~50 pg/Hz. This theoretically allows the detection of ~100 *E. coli* cells at best. In addition to the equation given above, the resonance frequency depends also on the damping of the oscillating system (24). As we observed no change in the quality factor Q, which is equal to the reciprocal of the damping, no change in the damping of our system was detected during the measurements (data not shown). Thus, the measured frequency shifts were caused by additional mass loading onto the cantilevers.

For the following experiment the cantilevers were all coated with agarose within the same batch in order to obtain similar layer thicknesses and, furthermore, comparable mechanical properties. In Fig. 3 the mean values of the resonance frequency shifts of multiple measurements are plotted as a function of time. The obtained data were fitted using the modified Gompertz model (4), which describes the bacterial growth. According to the equation shown above, different researchers observed a linear dependence of the frequency shift to cell number (*E. coli*, vaccinia virus particle) bound to the cantilever (10, 13). As an outcome of this, we compare the fitted curve with a conventional bacterial growth curve. The growth curve is usually divided into four phases. The adaptation period, in which newly inoculated cells in a fresh medium typically show a lag phase, is followed by an exponential growing phase, at which the bacteria cells double at each doubling time. The third phase, the stationary phase, starts when the cell density reaches its maximum. The growth is partly inhibited as a fact of the limitation of nutrition. The last phase is the cell death, where the bacteria start to die due to accumulation of acids (e.g., *Lactobacillus*) or the action of their own enzymes (e.g., autolysis). The cell death phase cannot always be observed. In the curve plotted in Fig. 3 the first three growing phases mentioned can be detected. During the mid-exponential growth phase the frequency shifted at a rate of ~200 Hz per hour. The stationary phase was already reached after 5 h of measurement. The short exponential growing phase can be explained by the shortage of nutrition, as the nutritive layer on our sensors had a thickness of approximately 200 nm only (an ordinary *E. coli* cell has a diameter of 500 nm). The agarose layer thickness was determined with the help of scanning force microscopy (data not shown). The very short lag phase resulted in bacterial growth which could be detected easily after 1 hour of measurement as a differential shift in the resonance frequency.

Detection of antibiotic-selective growth of *E. coli*. Figure 4 shows a typical shift in the resonance frequency of the oscillator after selective growth of *E. coli* XL1-Blue in a warm and humid environment (37°C, 93% relative humidity) as a function of time. For this experiment two cantilevers were coated differently. The growth-inhibiting lever was coated by a nutritive layer containing 10 μg/ml kanamycin, an antibiotic, which inhibits the growth of *E. coli* XL1-Blue. On the other hand, the growth-supporting lever was coated by the same nutritive layer, but tetracycline (10 μg/ml) was added. *E. coli* XL1-Blue is resistant to the antibiotic tetracycline and is therefore able to grow on such a coating. Both levers were then exposed to equal amounts of *E. coli* XL1-Blue cells (~1,000 *E. coli* cells per

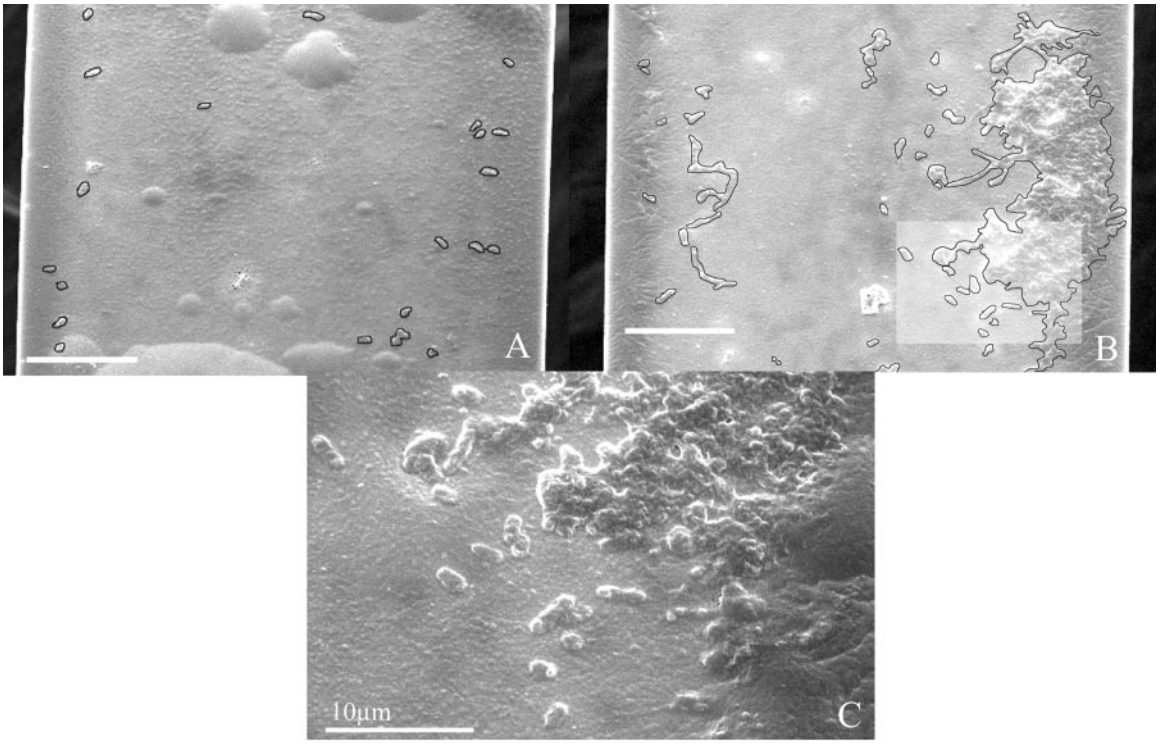

FIG. 5. SEM micrographs of agarose-coated cantilevers after 24 h of exposure to a warm, humid environment (93% relative humidity; 37°C). (A) The oscillator was incubated in LB broth containing 10 μg/ml kanamycin and *E. coli* XL1-Blue cells. The added antibiotic inhibited bacterial growth. (B) Micrograph of growing *E. coli* cells on top of a cantilever incubated in an *E. coli* XL1-Blue cell suspension containing the selective antibiotic tetracycline. (C). Close-up of *E. coli* XL1-Blue cells on a cantilever. For better visibility, the bacterial cells were bordered by a black line (A and B). In order to reduce the charging effects during SEM imaging, samples were prepared by evaporating a thin layer of Au. Scale bars correspond to 20 μm, if not otherwise stated.

cantilever surface). No change in the resonance frequency of the growth-inhibiting levers could be observed (data not shown). On the contrary, the frequency shifts of the growth-supporting levers increased over the first 4 hours of measurement and then slowly leveled out to a constant value. After the completed experiment the levers were examined with SEM to confirm selective bacterial growth on the growth-supporting levers. Figure 5 shows the SEM micrographs of a growth-inhibiting/kanamycin lever (panel A) and a growth-supporting/tetracycline lever (panel B) after exposure to a humid and warm environment. It can be clearly seen that there were *E. coli* cells settled on the growth-supporting lever as well as on the growth-inhibiting lever, but only on the growth-supporting lever did *E. coli* start to grow and form colonies. This can be detected on the magnified picture of the growth-supporting lever (Fig. 5C).

The curve plotted in Fig. 4 can be understood as a bacterial growth curve where the increasing mass is plotted versus time. In this experiment we again observed three distinct growth phases. Due to the sensitivity of our sensor, resulting in a very short lag phase, it was possible to detect selective growth of *E. coli* due to antibiotics in less than 2 h. The detection time of selective growth was slightly longer than the one of the active growth reported previously. Tetracycline in the nutritive layer led to slowed-down growth of *E. coli*, since the bacterial cells had to overcome the bactericidal effect of the antimicrobial agent. Since we used antibiotics as selective agents, we were able to detect the antibiotic resistance of *E. coli* XL1-Blue to tetracycline in less than 2 h.

DISCUSSION

The utility of micromechanical oscillators for the detection of bacterial cells and virus particles has been demonstrated previously (10, 13). However, this is the first use of an oscillating cantilever as a sensor for active bacterial growth. This new detection system provides numerous advantages, such as rapid real-time detection, label-free and small analyte volume, and high sensitivity, compared to conventional bacterial detection methods.

The measured frequency shifts were caused by additional mass loading onto the cantilevers, although the nutrition which the bacteria assimilate out of the layer stays inside the bacterial cell, and as a fact of this the mass balance between the bacteria and the thin nutrition layer (~200 nm) on the cantilever is zero. Consequentially, no decrease in frequency due to bacterial growth should be observed. Furthermore, the loss of carbon dioxide during the aerobic metabolism would lead to an increase of the resonance frequency as the cantilevers get lighter. In spite of these facts, we still detect a resonance frequency decrease caused by active bacterial growth. We interpret the detected frequency shifts due to mass loading as follows (Fig. 6). The absorbed water in the nutritive layer stays in equilibrium with the humid environment (Fig. 6A). Bacteria

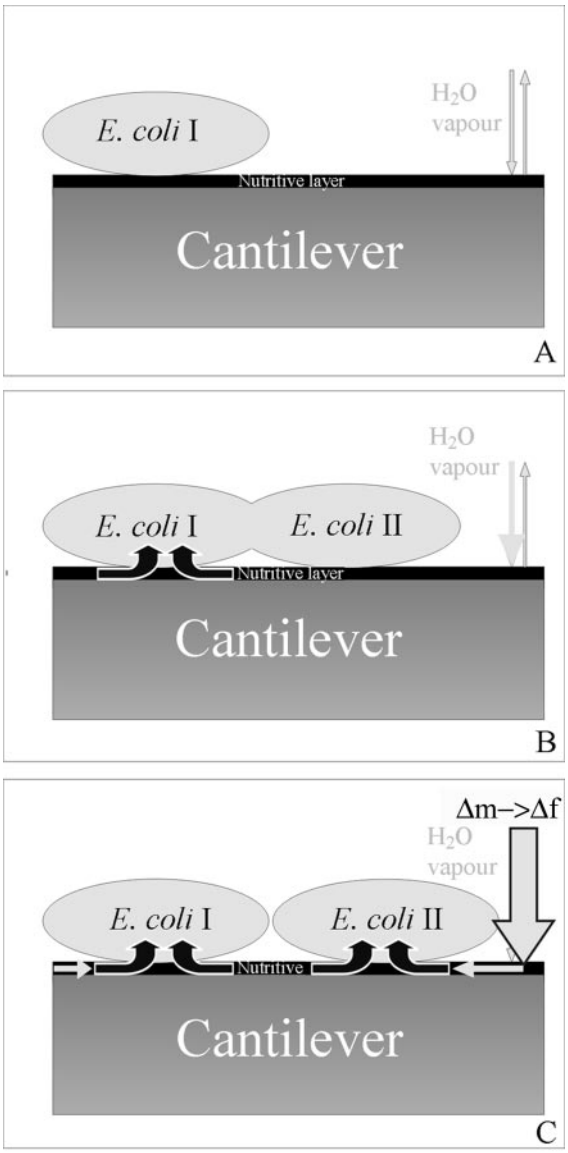

FIG. 6. Scheme of mass increase due to bacterial growth on agarose-coated cantilever. (A) Freshly deposited *E. coli* cells on top of coated cantilever. The thin nutritive layer (~200 nm) stays in equilibrium with the humid environment. (B) The bacterial cells start to grow and assimilate water, protein, salts, and carbohydrates out of the nutritive layer. (C) To regain equilibrium with the humid environment, the nutritive layer absorbs water. This compensation leads to additional mass loading onto the cantilever.

consist mostly of water (~70% [6]). When the bacterial cells start to grow they assimilate mainly water but also salt, carbohydrates, and proteins. These compounds will be diminished in the nutritive layer (Fig. 6B). This leads to a compensating adsorption of water back into the nutritive layer on top of the cantilever to regain the equilibrium with the environment (Fig. 6C).

We compared the observed frequency shifts due to additional mass loading onto the cantilevers with a conventional bacterial growth curve. All the characteristic growing phases can be observed. The very short lag phase and the quickly starting exponential growth result in bacterial growth which can be detected easily as a differential shift in the resonance frequency after 1 hour of measurement. Due to our initial cell count we estimate that the total number of *E. coli* cells rises to ~8,000 cells attached to the sensor cantilever surface after 1 h of measurement. This estimation correlates with the observed resonant frequency shift of ~80 Hz after 1 h (Fig. 1), since a single *E. coli* cells weighs ~500 fg (19). Therefore, we demonstrate for the first time the use of a micromechanical sensing device for the detection of active bacterial growth in less than 1 hour.

Furthermore, we have proven the ability of such a device to detect antibiotic resistance in less than 2 hours. Conventional antibiotic resistance testing methods, such as, e.g., the agar dilution method described by the National Committee for Clinical Laboratory Standards (18), are based on the optical detection of bacterial growth or nongrowth on antibiotic-containing agar plates. Depending on the investigated bacteria this test takes from 16 h up to 2 days (9). The fastest commercially available susceptibility testing device (Vitek 2, Biomerieux, France), based on conventional testing methods, needs more than 8 h to detect antibiotic resistances in bacteria using fluorescence-based technologies (25). We exclude other detection methods using real-time PCR methods for the amplification of antibiotic resistance genes, as these methods may detect genes of dead bacteria. To our knowledge this is the fastest susceptibility testing method reported until now. In addition, the system can easily be expanded to continuous multiplex detection of multiple antibiotic resistances and is therefore a candidate for fast susceptibility measurements in clinical environments. Furthermore, by altering the nutritive layer as well as the gas phase in which the measurement take place, a wide range of different bacterial species can be detected. Moreover, the mass sensitivity of future sensors will be increased by downsizing the cantilever dimensions. This will speed up the detection time for selective bacterial growth.

In the future, surface coupled antibodies can be implemented as an additional feature to identify clinical relevant *E. coli* serotypes (e.g., *E. coli* O157:H7). After selective fishing of *E. coli* cells, the measurement can proceed in a humid and temperature-controlled environment as reported in this paper to determine the viability of the attached cells.

ACKNOWLEDGMENTS

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