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Specific Growth Rate Determines the Sensitivity of *Escherichia coli* to Thermal, UVA, and Solar Disinfection

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Knowledge about the sensitivity of the test organism is essential for the evaluation of any disinfection method. In this work we show that sensitivity of *Escherichia coli* MG1655 to three physical stresses (mild heat, UVA light, and sunlight) that are relevant in the disinfection of drinking water with solar radiation is determined by the specific growth rate of the culture. Batch- and chemostat-cultivated cells from cultures with similar specific growth rates showed similar stress sensitivities. Generally, fast-growing cells were more sensitive to the stresses than slow-growing cells. For example, slow-growing chemostat-cultivated cells ($D = 0.08\ h^{-1}$) and stationary-phase bacteria from batch culture that were exposed to mild heat had very similar T_{90} (time until 90% of the population is inactivated) values ($T_{90,\ chemostat} = 2.66$ h; $T_{90,\ batch} = 2.62$ h), whereas T_{90} for cells growing at a μ of $0.9\ h^{-1}$ was 0.2 h. We present evidence that the stress sensitivity of *E. coli* is correlated with the intracellular level of the alternative sigma factor RpoS. This is also supported by the fact that *E. coli rpoS* mutant cells were more stress sensitive than the parent strain by factors of 4.9 (mild heat), 5.3 (UVA light), and 4.1 (sunlight). Furthermore, modeling of inactivation curves with GInaFiT revealed that the shape of inactivation curves changed depending on the specific growth rate. Inactivation curves of cells from fast-growing cultures ($\mu = 1.0\ h^{-1}$) that were irradiated with UVA light showed a tailing effect, while for slow-growing cultures ($\mu = 0.3\ h^{-1}$), inactivation curves with shoulders were obtained. Our findings emphasize the need for accurate reporting of specific growth rates and detailed culture conditions in disinfection studies to allow comparison of data from different studies and laboratories and sound interpretation of the data obtained.

Effective disinfection of water is extremely important in developing countries, where access to safe drinking water is often difficult. In such regions, enteric bacteria, such as *Salmonella* spp., *Shigella* spp., or *Vibrio cholerae*, are still the major drinking water health threats (20). An estimated four billion cases of diarrheal disease occur every year worldwide, causing three to four million deaths, mostly among children. This situation can be improved only by effective household water treatment systems that meet local financial and practical needs. Solar disinfection (SODIS) is such a method (1, 39). Exposing drinking water in polyethylene terephthalate (PET) bottles to sunlight (ca. 6 h) inactivates enteric bacteria present in the water. The two primary causes for bacterial inactivation in this method are believed to be mild heat and UVA light (39).

Disinfection methods like SODIS are assessed with living test microorganisms in order to ensure efficacy and sound application. In most cases, the resistance of a particular indicator organism to different quantities of chemical disinfectant or amounts of physical stresses is investigated. However, a key point often overlooked is that the resistance of bacteria can vary significantly as a function of growth conditions. Bacteria change their physiology or even their phenotype under different growth conditions (6, 8, 37). For example, the expression of heat shock proteins (HSPs) in *Escherichia coli* was shown to be affected by the composition of the growth medium and growth conditions (41). Moreover, it was shown that induction of the general stress response in *E. coli*, which is controlled by the global regulator RpoS, increases with decreasing specific growth rate (23, 33). Recently, it was found that the concentration of the DNA-binding protein Dps in *E. coli*, which is believed to protect DNA from oxidative stress, increases with decreasing specific growth rate (2). Therefore, it is very important to define precisely the growth conditions and physiological state of bacteria used for disinfection experiments. In most disinfection studies, the growth conditions of the test organisms are not properly specified and, where mentioned, mostly ill-defined states like "late mid-log" or "early stationary phase" are given. This makes proper comparisons among different studies difficult, if not impossible.

In light of the widespread use of disinfection methods, it is surprising how few studies have addressed the dependence of disinfection (or stress) resistance on the physiological state of bacterial cells. In most studies the scope of different physiological states investigated is confined to exponential and stationary phase. Sensitivity of *E. coli* to disinfectants like ClO_2, UVC, mild heat, and sunlight were shown to be higher during exponential phase than during stationary phase (16, 28, 32, 34), but only Berg (6) showed a correlation of specific growth rate and the sensitivity to ClO_2 for *E. coli* and *Legionella pneumophila*.

In the present study we tested the hypothesis that sensitivity of *E. coli* K-12 MG1655 to physical stresses like mild heat,

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UVA light, and sunlight increases with increasing specific growth rate (μ) and that μ can be used as a key parameter to compare different disinfection experiments and ensure reproducibility. Furthermore, the role of RpoS as a possible key link between specific growth rate and sensitivity to the respective stresses was investigated by using an *rpoS* mutant of *E. coli* and by measuring the RpoS-dependent catalase activity.

MATERIALS AND METHODS

Bacterial strains. For most experiments, *E. coli* K-12 MG1655 (genotype $F^- \lambda^-$ *rph-1*) was used (42). For selected experiments, an isogenic *rpoS* mutant of *E. coli* K-12 MG1655 (*rpoS13*::Tn*10*) (43) was used for comparisons.

Growth media and cultivation conditions. (i) Batch cultivation. Luria-Bertani (LB) broth (10 g tryptone per liter, 5 g yeast extract per liter, 10 g NaCl per liter) was used for batch cultivation (31). LB medium was always filter sterilized with Millipore syringe filters (Millex GP; 0.22 μm) and diluted to 25% (vol/vol) of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon filtered). Precultures were prepared for each individual batch experiment from the same cryovial stored at −80°C by streaking onto LB agar plates. After 15 to 18 h of incubation at 37°C, one colony was picked and loop inoculated into a 125-ml Erlenmeyer flask containing 20 ml of LB broth. This flask was incubated at 37°C on a rotary shaker at 200 rpm. At an optical density at 546 nm (OD_{546}) between 0.1 and 0.2 (measured spectrophotometrically in glass cuvettes with a 1-cm light path using a JASCO V550 UV/visible spectrophotometer [JASCO, Tokyo, Japan]), cells were transferred into 500-ml Erlenmeyer flasks containing 50 ml of LB broth. This way no lag phase was observed. The culture volume to be inoculated into the fresh medium was calculated beforehand to attain an initial OD_{546} of 0.002. These flasks were then shaken at 200 rpm in a temperature-controlled water bath (SBK 25D; Salvis AG, Reussbühl, Switzerland) at 37°C, and OD_{546} was monitored every 20 min. The specific growth rate μ was calculated from five consecutive OD_{546} measurements ($\mu = \Delta \ln OD_{546}/\Delta t$, where t is time). For the measurement of dissolved oxygen concentration (pO_2) in batch culture experiments, a pO_2 probe (Ingold Electrodes Inc., Wilmington, MA) with a Bioengineering amplifier was used (Bioengineering AG, Wald, Switzerland).

(ii) Chemostat cultivation. Precultures were prepared as described above. For most continuous culture experiments, temperature-controlled and air-sparged 1-liter glass reactors (Schmizo AG, Zofingen, Switzerland) were used. For particular experiments, computer-controlled glass and stainless steel bioreactors (MBR, Wetzikon, Switzerland) were employed. The pH was kept constant at 7.0 ± 0.1 by automatic addition of 0.5 M NaOH–0.5 M KOH, and the temperature was maintained at 37 °C ± 0.1°C. Oxygen concentration was kept between 95 and 100% of air saturation at 37°C, and the stirrer speed was set to 800 rpm. For glucose-limited chemostat cultures, the medium was prepared as described earlier (23), and for LB chemostat cultures, 10% (vol/vol) LB broth was used.

Sample preparation and plating. Cells were harvested by centrifugation from batch or chemostat cultures (at 13,000 × *g* in a Biofuge fresco [Kendro, Zürich, Switzerland]), washed three times with filter-sterilized (Nuclepore Track-Etch membrane [0.22 μm] [Sterico AG, Dietikon, Switzerland]) commercially available bottled water (Evian; Groupe Danone, Paris, France), and diluted to an OD_{546} of 0.01 (corresponding to 1×10^7 to 5×10^7 cells/ml). Exposure of bacterial suspensions was started immediately after dilution to prevent the cells from adjusting to low-nutrient conditions. Aliquots were withdrawn at different time points and diluted in a series of dilutions (10^{-1} to 10^{-5}) with sterile-filtered (0.2-μm) bottled water (Evian). Following dilution, 1 ml of test solution was withdrawn and mixed with 7 ml of liquid tryptic soy agar (Biolife, Milano, Italy) at 40°C (pour plate method). After 20 min, the solidified agar was covered with another 4 ml of liquid tryptic soy agar (40°C). Plates were incubated for 48 h at 37°C until further analysis. Plate counts were determined with an automatic plate reader (aCOLyte; SYNBIOSIS, Cambridge, United Kingdom).

Mild heat exposure. A bacterial suspension of 20 ml (see above) was transferred into 30-ml quartz glass tubes (WISAG AG, Zürich, Switzerland) and placed in a temperature-controlled water bath at 48°C. Care was taken to completely submerge the vials to prevent temperature gradients in the vials. The final temperature was reached in ≤2 min. During exposure, samples were stirred with a magnetic stirrer. Unstressed control samples were kept in the dark at 37°C.

Sunlight exposure. Samples of 10 ml of bacterial suspension (see above) were exposed to solar light in 30-ml quartz glass tubes, which were placed in a temperature-controlled acrylic glass container with a quartz front glass, holding 25 tubes in total (Fig. 1A). A circulating water bath was used to control the temperature of the sample tubes in the container. The container was adjusted regularly so that sunlight met the tubes at an angle of 90° ± 2°. At each time point, one tube was withdrawn and its contents were immediately processed as described above. Irradiation intensity data were obtained from a weather station, which is located 300 m away from the exposure site (BUWAL/NABEL, EMPA, Dübendorf, Switzerland). The fluence rates for sunlight irradiation given in this work refer to the wavelength range of 350 to 450 nm, which reflects the wavelength range of the UVA lamps also used (see below). Conversion factors and calculations were as previously described by Wegelin and coworkers (39). The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-COR, Lincoln, Nebraska), 8-nm bandwidth, fitted with a model 1800-10 detector head (Fig. 1B).

UVA exposure. Samples of 10 ml of bacterial suspension (see above) were exposed to UVA light in 30-ml quartz tubes placed in a carousel reactor (holding 10 tubes) (adapted from that used by Wegelin et al. [39]) equipped with medium-pressure mercury lamps (Hanau TQ718 or TQ718 Z4), which were operated at 700 W or 500 W, respectively. The TQ718 Z4 mercury lamp exhibits a broader UVA spectrum and is more effective in disinfection than the lamp without mercury (Fig. 1B). The lamp was placed in a cooling jacket (Duran 50 borosilicate glass) in the center of the carousel reactor. The light emitted from the lamp passed through the glass jacket and through 35 mm of filter solution before reaching the cells in the quartz tubes. The temperature of the filter solution was maintained at 37°C and consisted of 12.75 g/liter sodium nitrate with a cutoff at 320 nm and a half maximum at 340 nm. The transmission property of the filter solution was measured before each experiment. Chemical actinometry with *p*-nitroanisole/pyridine was used to determine the fluence rate at the tube position (39). Bacterial solutions were mixed intermittently on a magnetic stirrer. At each time point, one tube was withdrawn and its contents immediately processed as described above.

Hydroperoxidase assay. The two *E. coli* hydroperoxidases (catalases) were used as "reporter genes" for RpoS-dependent transcription because both hydroperoxidase I (HPI; *katG* gene product, cytoplasmic membrane associated) and hydroperoxidase II (HPII; *katE* gene product, present in the cytosol) were shown to be regulated by σ^S (24), with hydroperoxidase II expression being entirely dependent on RpoS (36). HPI and HPII specific activities were measured in whole-cell assays. The activities of the two enzymes can be separated by a heat inactivation treatment step, because HPII is heat stable, whereas HPI is heat labile (36). Briefly, samples from chemostats were washed and diluted in phosphate-buffered saline (140 mM NaCl, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO_4) to an OD_{546} of 0.1. To measure total catalase activity, 1 ml of this solution was mixed with 1 μl of chloramphenicol solution (25 mg/ml) transferred into a quartz cuvette and placed into a temperature-controlled (37°C) spectrophotometer. Before the start of the assay, the cuvette was incubated for 2 min for the sample to adjust to the temperature. Absorption at 240 nm was recorded for 2 min after starting the assay by the addition of 2 μl of H_2O_2 (37%) and thorough mixing. For heat-stable catalase activity measurements, samples were incubated for 15 min in a heating block at 55°C to inactivate HPI prior to analysis (36). Catalase activity was calculated from the first 60 s of recorded absorption where the rate of $\Delta E_{240}/\Delta$time decrease was linear.

Modeling with GInaFiT. The Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT) was used to test six different types of microbial survival models on our data (14). The following models were used: log-linear regression (7), log-linear regression plus tail (13), log-linear regression plus shoulder (13), log-linear regression plus shoulder plus tail (13), biphasic model (9), and biphasic-plus-shoulder model (14). All models were run for each inactivation curve, and the values of the root mean sum of squared errors (RMSE) were compared. The RMSE is considered to be the most simple and most informative measure of goodness of fit for linear and nonlinear models (14). The model with the smallest RMSE was considered the best fit for the respective inactivation curve. When the magnitude of RMSE was much smaller than experimental precision, the model was considered to overfit the data and a simpler model was chosen. If two models had the same or very similar RMSE values, the simpler model was considered to fit best. T_{90} values (time until 90% of the population is inactivated) were calculated using the best-fit model of GInaFiT.

Reproducibility. To ensure reproducibility, selected experiments were repeated. Analysis of inactivation behavior with GInaFiT modeling showed nearly identical results for two replicate experiments in all cases. This was based on comparison of specific parameters of the best-fitting model (e.g., the specific inactivation rate constants k, k_{max1}, and k_{max2} for the biphasic model [14]). The standard deviations of model parameters were less than 5% of the means. Only data from one representative experiment are displayed in the graphs. Periodically, the precision of pour plating with subsequent colony counting was analyzed. The standard deviation for the standardized plating and colony counting

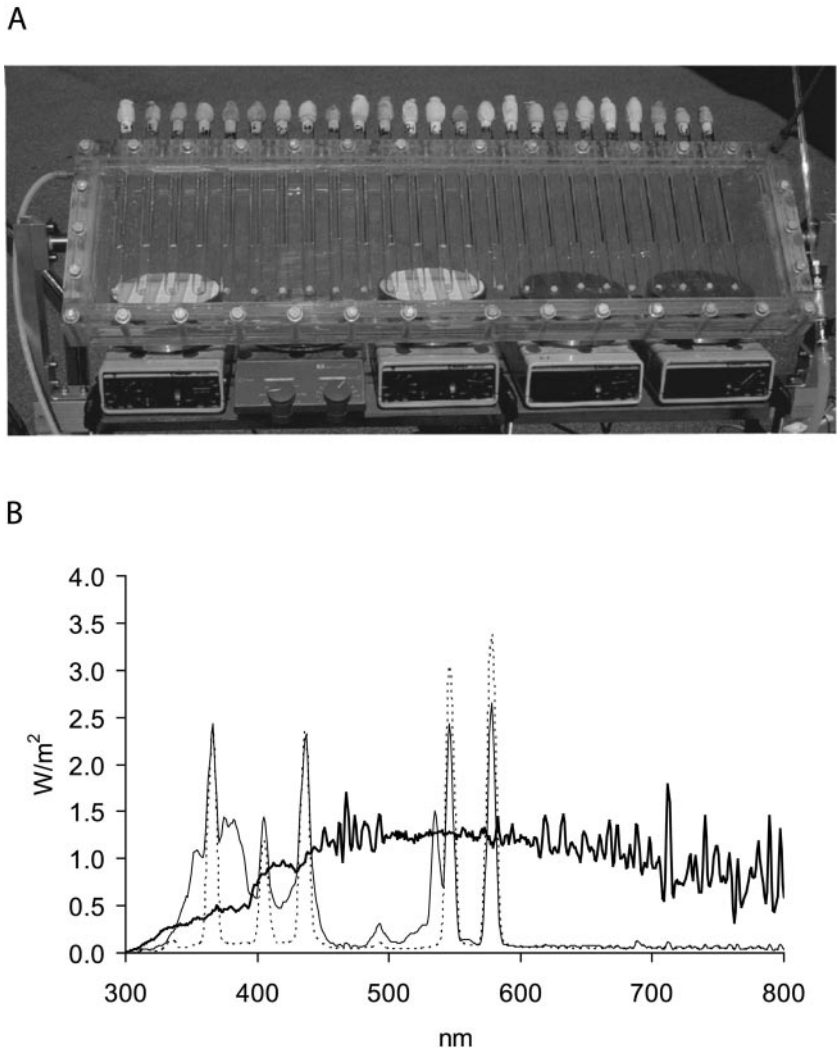

FIG. 1. (A) Field reactor for sunlight exposure of bacterial samples. The container is built from acrylic glass and has a quartz glass front window. Up to 25 tubes can be fitted into the container. Samples are stirred magnetically, and the temperature is controlled by a circulating water bath. The apparatus can be swiveled and turned in any direction. (B) Wavelength spectra of medium-pressure mercury lamps Hanau TQ718 (dashed line) and TQ718 Z4 (thin solid line) both corrected for filter solution $NaNO_3$ and the sunlight spectrum on a mid-summer day measured at 1 p.m. (fat solid line).

was within 15% at the maximum (calculated from three experiments). Catalase activity was measured in three experiments in all cases. In all experiments, an unstressed control sample was measured at the beginning and end of data acquisition. For convenience, these control samples are always displayed with the same *x*-axis value as the last data point of the respective curve.

RESULTS

Influence of cultivation conditions on batch growth pattern. The growth history of the test organism has a strong influence on the reproducibility of disinfection experiments. Therefore, for our laboratory setup, the influence of different cultivation variables on the bacterial growth pattern was investigated first. Because aeration is known to be a critical factor in shaking flask cultures, the oxygen concentration for different aeration regimens during batch culture cultivation was measured (Fig. 2). Cultures were grown in either stirred or shaken Erlenmeyer flasks and in a fully aerated and stirred bioreactor using the same growth medium. The pO_2 decreased rapidly in stirred Erlenmeyer flasks as the cell density increased (Fig. 2), and an abrupt decline in specific growth rate (μ) was observed as soon as the culture became oxygen limited. In contrast, cultivation of *E. coli* in a fully aerated and stirred bioreactor resulted in O_2 levels of $\geq$60% and a continuous decrease of specific growth rate with time. The growth pattern and O_2 levels of Erlenmeyer flask cultures that were shaken on a rotating shaker at 200 rpm were identical to those for the bioreactor (data not shown). This indicates that in our setup, shaking prevents oxygen depletion during growth; therefore, it was employed as the preferred method for all further experiments. Furthermore, we found that a typical batch growth curve of *E. coli* cultivated in LB medium shows no true exponential phase (i.e., a straight line of the growth curve in a semilogarithmic plot), but in fact, the specific growth rate decreases constantly until stationary phase is reached (Fig. 3). This stands in contrast to

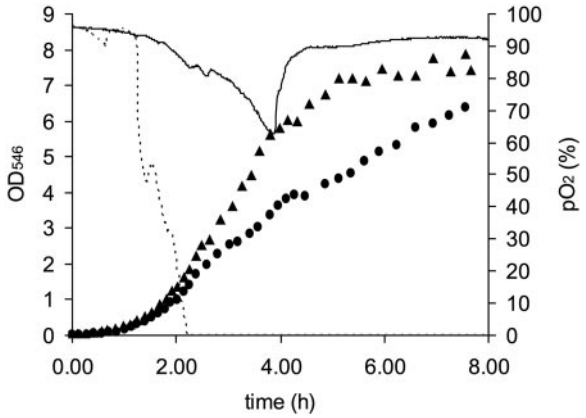

FIG. 2. *E. coli* growth curves and corresponding dissolved oxygen tension for different aeration systems in batch cultivation. The effect of stirring in Erlenmeyer flasks on the development of cell density (OD_{546}) and pO_2 was investigated and compared to growth in a fully aerated and stirred bioreactor. OD_{546} (●) and pO_2 (dashed line) in an Erlenmeyer flask and OD_{546} (▲) and pO_2 (solid line) in a bioreactor are shown.

the common assumption that during LB batch culture an exponential phase exists where the specific growth rate remains constant over an extended time period. Examination of the sterilization method of the growth medium revealed two interesting aspects. During the first 2 hours after inoculation from a exponentially growing inoculum ($\mu \geq 1.5\ h^{-1}$), filter-sterilized LB medium allowed a higher initial specific growth rate ($\sim 2.4\ h^{-1}$) than autoclaved LB medium did ($\sim 2\ h^{-1}$) (data not shown). Additionally, we observed that prolonged autoclaving lowered the initial μ. Therefore, only filter-sterilized LB medium was used for all experiments.

Specific growth rate and stress resistance. Comprehensive and detailed information about cultivation conditions are rarely given in the disinfection literature, although it has been known that cultivation conditions and growth phase can affect the sensitivity of bacteria to stress. This calls for a general growth parameter, which allows reproducibility and comparability among different disinfection experiments. Here we test the hypothesis that the specific growth rate of *E. coli* correlates with sensitivity to mild heat treatment, UVA light, and sunlight.

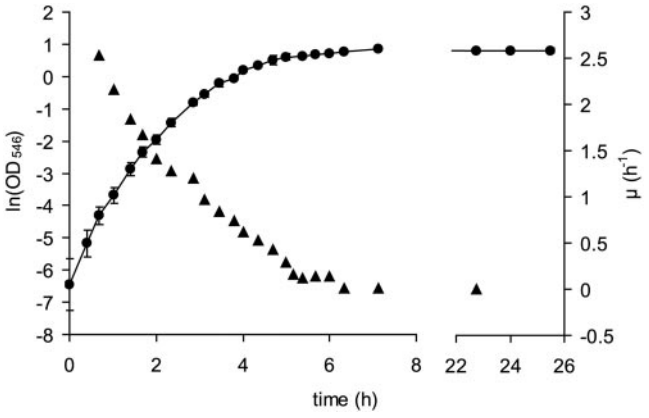

FIG. 3. Growth curve of *E. coli* K-12 MG1655 in LB batch culture (shaken Erlenmeyer flask) at 37°C (●, $\ln OD_{546}$). The specific growth rate μ (h^{-1}) was calculated as the slope of five adjacent points (▲). Values are means ± standard deviations (error bars) for three experiments.

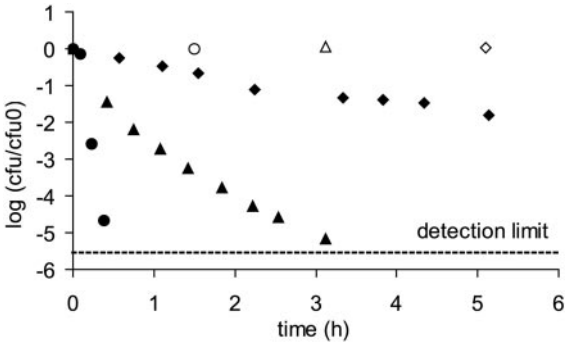

FIG. 4. Sensitivity to mild heat (48°C) of *E. coli* K-12 MG1655 harvested at three different specific growth rates from LB batch culture (0 h^{-1} [◆], 0.44 h^{-1} [▲], and 2 h^{-1} [●]). The values for unstressed control samples are displayed by open symbols. Sensitivity was determined as CFU/CFU at time zero.

(i) Mild heat. *E. coli* cells were harvested from LB batch cultures at three different specific growth rates and exposed to mild heat (48°C) (Fig. 4). Inactivation rates of bacterial cells harvested at different time points differed markedly. Cells with slow specific growth rates were significantly less sensitive to thermal stress than faster-growing cells. The correlation between sensitivity to mild heat and specific growth rate was confirmed when *E. coli* cells cultivated in the same medium in chemostat cultures, operated at five different dilution rates (D = 0.08 h^{-1}, 0.2 h^{-1}, 0.3 h^{-1}, 0.6 h^{-1}, and 0.9 h^{-1}), were exposed to the same stress (Fig. 5). Batch- and chemostat-grown cells from similar specific growth rates exhibited similar sensitivities. For example, chemostat-cultivated cells growing very slowly (D = 0.08 h^{-1}) exhibited the same sensitivity as stationary-phase cells from batch culture. Curve fitting with GInaFiT gave a good fit with the traditional log-linear decay

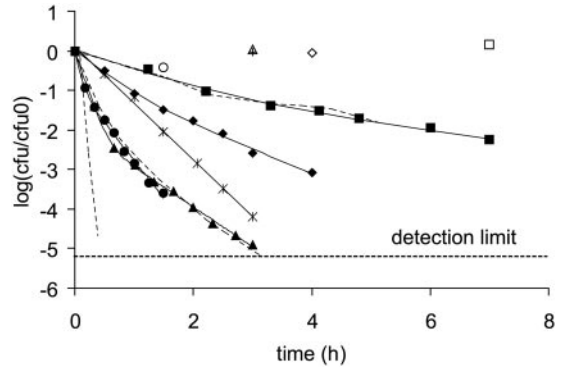

FIG. 5. Sensitivity to mild heat (48°C) of *E. coli* K-12 MG1655 cells cultivated in filter-sterilized LB medium in chemostat culture operated at different dilution rates (0.08 h^{-1} [■], 0.2 h^{-1} [◆], 0.3 h^{-1} ⁎, 0.6 h^{-1}[▲], and 0.9 h^{-1}[●]). Curves were fitted with GInaFiT (14). The best fit is displayed for each curve. Inactivation curves from Fig. 4 are displayed as dashed lines for comparison. The values for unstressed control samples are displayed by open symbols. Sensitivity was determined as CFU/CFU at time zero.

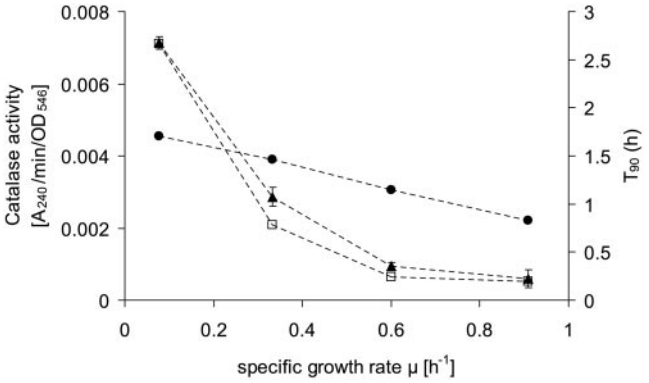

FIG. 6. Catalase specific activity (HPI [●] and HPII [▲]) of *E. coli* cells grown at different dilution rates in LB chemostat culture. Inactivation values T_{90} (□) (time when 90% of the population are inactivated measured by plate counting), derived from data in Fig. 5, are displayed. Catalase activity was not measured at the dilution rate 0.2 h^{-1}. Values are means ± standard deviations (error bars) for three experiments. HPI activity was calculated as the difference between total catalase activity (HPI and HPII) minus HPII activity; therefore, no error bars are displayed for HPI activity.

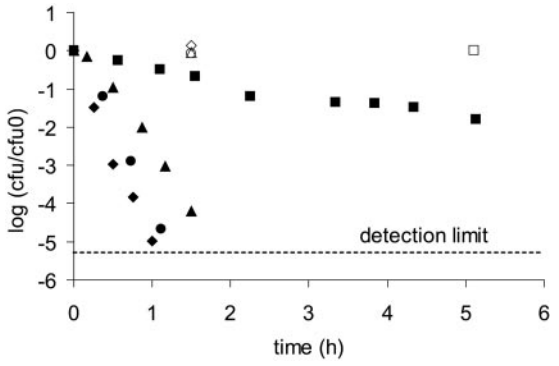

FIG. 7. Sensitivity to mild heat (48°C) of *E. coli rpoS* mutant cells harvested at different specific growth rates and cultivation conditions (0 h^{-1} in the LB batch [▲], 0.3 h^{-1} in the glucose mineral medium chemostat [●], and 1.5 h^{-1} in the LB batch [◆]) compared to wild-type *E. coli* cells from stationary phase (0 h^{-1} in the LB batch [■]). The values for unstressed controls are displayed by open symbols. Sensitivity was determined as CFU/CFU at time zero.

model for low specific growth rates (0.08 h^{-1}, 0.2 h^{-1}, and 0.3 h^{-1}). The inactivation pattern of cells with a higher specific growth rate (0.6 h^{-1} and 0.9 h^{-1}) showed a true biphasic behavior (fast inactivation followed by a slower part), suggesting that the population was heterogeneous with respect to sensitivity. Changes in initial inactivation rates displayed as T_{90} values became more pronounced, especially for slower dilution rates (Fig. 6). Total catalase activity (HPI and HPII) and T_{90} values followed a similar pattern in relation to dilution rates of chemostat cultures. Activities of the RpoS-dependent catalase HPII correlated well with T_{90} values ($R^2 = 0.988$), while with HPI activities the correlation was very poor ($R^2 = 0.727$). This result points to an important possible role of RpoS in the thermal stress resistance of *E. coli*. Mild heat treatment of *E. coli rpoS* mutant cells grown at three different specific growth rates (1.5 h^{-1}, 0.3 h^{-1}, and 0 h^{-1}) revealed that RpoS does indeed regulate protection from mild heat stress to some extent (Fig. 7). Although bacteria were sampled at different specific growth rates, differences in inactivation rates were very small compared to the growth rate for wild-type (wt) cells. Nevertheless, the pattern of decreasing sensitivity for bacteria with slower growth rates remained.

(ii) UVA light. UVA sensitivity of *E. coli* cells from chemostat cultures operated at three different dilution rates also showed a dependence on the specific growth rate (Fig. 8). Stationary-phase cells and chemostat cells that were grown at a low dilution rate ($D = 0.3$ h^{-1}) exhibited a log-linear inactivation behavior with a typical shoulder. This was confirmed with GInaFiT modeling. For bacterial cells with a specific growth rate of 0.7 h^{-1}, no shoulder effect was observed and the model log-linear regression fitted best. Cells cultivated at a D of 1.0 h^{-1} exhibited a true biphasic behavior. A small proportion of the culture (0.05%) seemed to be less sensitive to UVA stress. We speculated that this might be due to physiological changes during exposure or sample preparation resulting in increased protection against UVA injury. To test this hypothesis, *E. coli* cells from the same chemostat culture ($D = 1.0$ h^{-1}) were left in Evian water for 4 h before exposure to UVA (Fig. 9). The best fit for this curve was now obtained with the biphasic-plus-shoulder model. The appearance of a shoulder indicates that physiological changes took place during sample preparation and bacterial cells became more resistant to UVA irradiation. Obviously, growth at a μ of 1.0 h^{-1} and a subsequent change from a nutrient-rich medium (LB) to a nutrient-poor environment (Evian water) forces *E. coli* to enter a stationary-phase-like state. Still, the culture that was left to adapt to Evian water for 4 h also displays a biphasic inactivation pattern, indicating that we see a combination of cellular adaptation effects during preparation and exposure. Again, the *E. coli rpoS* mutant strain from stationary-phase batch culture was much more sensitive to UVA light than the parent wt strain was (Fig. 10A).

(iii) Sunlight. Sensitivity to sunlight was also dependent on the specific growth rate (Fig. 10B). A 4-log-unit inactivation of

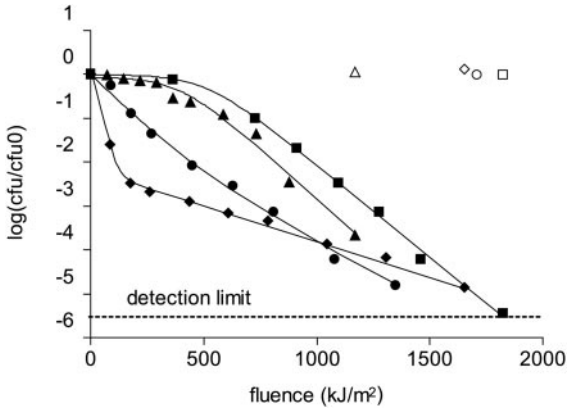

FIG. 8. Sensitivity to UVA irradiation (TQ718 Z4) at 37°C of *E. coli* K-12 MG1655 harvested from LB chemostat cultures run at different dilution rates (0.3 h^{-1} [▲], 0.7 h^{-1} [●], and 1.0 h^{-1} [◆]) compared to cells from stationary-phase batch cultures (μ = 0.0 h^{-1} [■]). The values for unstressed control samples are displayed by open symbols. Curves were fitted with GInaFiT (14). The best fit is displayed for each curve. Sensitivity was determined as CFU/CFU at time zero.

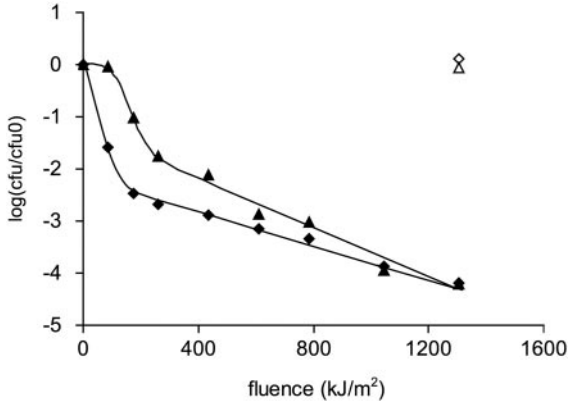

FIG. 9. Sensitivity to UVA irradiation (TQ718 Z4) at 37°C of *E. coli* K-12 MG1655 from LB chemostat culture run at a dilution rate of 1.0 h^{-1}. Cells were either irradiated immediately after harvesting (◆) or left to adjust to the new environment for 4 h before UV exposure (▲). The values for unstressed control samples are displayed by open symbols. Curves were fitted with GInaFiT (14). The best fit is displayed for each curve. Sensitivity was determined as CFU/CFU at time zero.

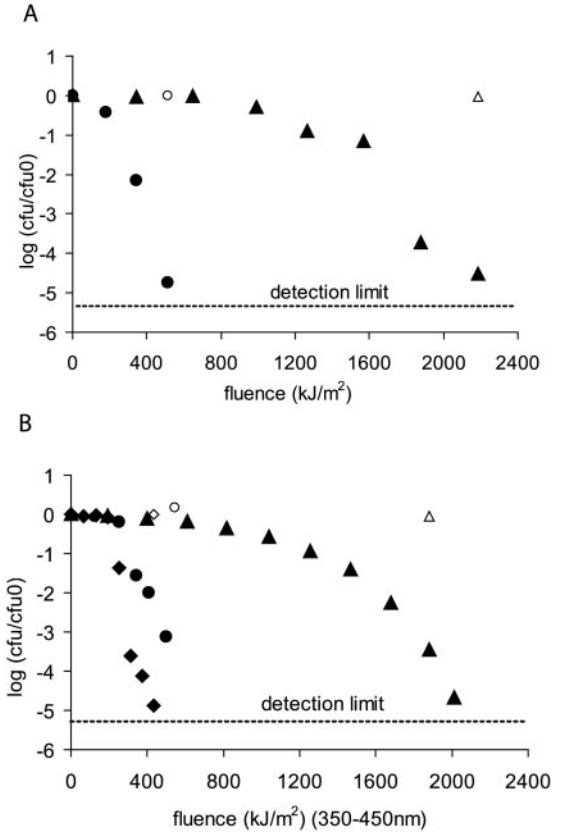

FIG. 10. Sensitivity of *E. coli* K-12 wt (▲) and *rpoS* mutant (●) to UVA light (TQ718) (A) and to sunlight (B). Bacterial cells were harvested from stationary-phase LB batch cultures. (B) An inactivation curve for *E. coli* cells harvested at a μ of 2 h^{-1} (◆) was added for comparison. The values for unstressed control samples are displayed by open symbols. Sensitivity was determined as CFU/CFU at time zero.

stationary-phase cells was observed at a threefold-higher fluence compared to fast-growing *E. coli* cells. Exposure to sunlight also resulted in inactivation curves exhibiting a shoulder effect. Interestingly, fast-growing cells also exhibited a small shoulder effect. The sensitivity of the *E. coli rpoS* mutant strain from stationary phase was similar to that of fast-growing cells from the parent wt strain.

DISCUSSION

This work clearly demonstrates that the sensitivity of *E. coli* to stresses like mild heat, UVA light, and sunlight is determined to a large part by the specific growth rate (Fig. 4 to 8). The decreasing sensitivity of progressively slower-growing bacterial cells strongly depends on RpoS, a global stress regulatory protein, which itself is increasingly expressed with decreasing specific growth rate (23). Furthermore, our results imply that detailed information on growth conditions and the specific growth rate of bacterial cells must be given in disinfection experiments to allow comparison and correct interpretation of reported data. We suggest that for testing a disinfection method it is not enough to choose the apparently most resistant organism but that one also has to produce cells of the most resistant state or of reproducible resistance. For solar disinfection, this would be cells from stationary phase ($\mu = 0\ h^{-1}$).

There have been a number of reports in the past where sensitivity to disinfection was related to culture age or growth phase, but almost all of them showed only differences between bacterial cells from stationary phase and (an insufficiently defined) exponential phase. To our knowledge, the only systematic study on specific growth rate and sensitivity to disinfection was conducted by Berg (6), who showed that the sensitivity of *E. coli* and *L. pneumophila* to ClO_2 increases with increasing dilution rate in chemostat experiments. Concerning sensitivity to UVA light, conflicting results have been reported. In one study, stationary-phase *E. coli* cells were more resistant than exponentially growing cells to both lethal and sublethal doses of UVA radiation (10). In contrast, Reed (34) showed only very minor differences between the inactivation rates for stationary-phase *E. coli* cells and their growing counterparts. In seawater microcosm experiments, *E. coli* cells from exponential phase were shown to be more susceptible to artificial visible light than stationary-phase cells were. However, in all three studies, the exponential phase was not well defined. For thermal inactivation, it was demonstrated that the age of the culture of *E. coli* or *Streptococcus faecalis* affects heat resistance and death kinetics (12, 17, 27, 40). During exponential phase, these bacteria exhibited high sensitivity to mild heat (different temperatures), while in stationary phase and during the lag phase, they were less sensitive. For UVC-irradiated *E. coli*, it was demonstrated that UVC resistance varies during growth in batch culture; however, it is not clear from the published data how the specific growth rate relates to UVC sensitivity (30, 32). Our results show that the sensitivity of *E. coli* to three different physical stress conditions relevant to the SODIS disinfection method (UVA light, sunlight, and mild heat) strongly correlates with the specific growth rate. Hence, the results presented here indicate a general pattern for bacterial sensitivity to these stresses.

RpoS, the σ^S subunit of RNA polymerase, seems to link stress response and specific growth rate (Fig. 6). T_{90} values

correlate well with HPII activity, which increases 12-fold for bacteria with lower specific growth rates, while HPI activity only doubles. These results confirm published reports of a mild increase in HPI expression as cells approach stationary phase and a major increase in the synthesis of the RpoS-dependent HPII (36). Another indication of the importance of RpoS is demonstrated in experiments with *E. coli rpoS* mutants that show largely increased sensitivities to all three physical stresses virtually independent of the specific growth rate. RpoS is a global regulator that enables *E. coli* to adapt to various stress conditions like osmotic stress, pH differences, or oxidative stress (19). It was reported to regulate over 480 different genes (38). Ihssen and Egli (23) proposed that as an intracellular signal, (p)ppGpp might link RpoS and the specific growth rate, because RpoS expression is positively regulated by this factor. In our experiments, HPII activity was measured as an indicator for RpoS levels because HPII is regulated primarily by RpoS (36). Although our results strongly suggest RpoS to be a main reason for differences in stress resistance, other mechanisms could still be important. The small differences in inactivation rates for *rpoS* mutants from different specific growth rates (Fig. 7) suggest that additional regulatory mechanisms (also dependent of the specific growth rate) are involved in stress protection against mild heat, and this might also be the case for sensitivity towards UVA light and sunlight.

A distinct shoulder effect is observed only for UVA- and sunlight-irradiated samples. In contrast, no such effect was detected during exposure to mild heat. Two models have been proposed in the literature to explain the shoulder effect: the multitarget and multihit scenarios (18). The multitarget scenario suggests that an organism contains *n* distinct (usually identical) targets, each of which must receive at least one hit before the cell is inactivated, while the multihit model is based on the assumption that a single target must be hit at least *n* times. In the case of sunlight and UVA, a combination of the two theories seems most likely. In contrast to UVC disinfection (254 nm), where only one target (DNA) exists, the broad wavelength spectrum of UVA can harm the cells in many different ways, such as membrane damage, DNA damage, or indirect damage by reactive oxygen species (11). Cells from chemostats run at higher dilution rates also exhibited initial first-order inactivation kinetics when the cells were irradiated with UVA light. When fast-growing cells were exposed to high-intensity UVA irradiation, probably enough targets are hit at once for the population to show first-order inactivation kinetics without a (visible) shoulder effect. In sunlight experiments, a shoulder effect was also observed for faster-growing cells. This was probably due to the longer incubation time before UV exposure was started, because the vials with the bacterial solution had to be carried outside. In contrast, *E. coli* cells exposed to mild heat seem to follow first-order inactivation kinetics. The mechanism of thermal inactivation of microorganisms has been widely investigated. Cellular sites of heat injury, such as membranes, nucleic acids, and certain enzymes, have been identified (4, 15, 26, 29). Evaluation of heat inactivation by differential scanning calorimetry suggested that a strong relationship exists between thermal death of bacteria and the first major peak in differential scanning calorimetry thermograms, which is attributed to the unfolding of ribosomes (26). However, it should be pointed out that these studies were conducted with cells preexposed to heat, which are generally known to be more resistant to thermal stress (25). To date, we are unable to come up with a coherent explanation for the first-order inactivation pattern during mild heat treatment.

The biphasic nature of the inactivation curves in UVA and mild heat disinfection experiments seems to occur only when bacterial cells from growing cultures are used. It indicates that two discrete populations were present during exposure as has been reported for heat-stressed *Salmonella enterica* serovar Enteritidis PT4 over a range of lethal temperatures (22). Humpheson et al. (22) suggested that heat shock protein production occurs in a small proportion of the culture and that this results in the tails of survivor curves during lethal heating. Allen et al. (3) demonstrated that a temperature shift from 30 to 45°C induced synthesis of at least 17 HSPs in *Pseudomonas aeruginosa* within approximately 1 min. In our mild heat experiments, the sample temperature increased from 37°C to 48°C in 2 min. During this time, some HSP production could have occurred. In the UVA light experiments, we have already seen that the handling of cells until UV exposure can influence their sensitivity. The change from a nutrient-rich to a nutrient-poor environment puts *E. coli* cells under starvation stress. This stress must be less pronounced for stationary-phase cells because they have already gone through a starvation-adaptation period. Apparently, the starvation stress imposed on growing bacteria leads to a general stress response similar to that of stationary-phase bacteria, which can include the induction of protective mechanisms against UVA stress. However, the cells do not become as resistant as stationary-phase cells because the adaptation process needs energy and this requires changes in cell composition, intracellular degradation of components, etc. Still the induction of a stress-specific protection during exposure cannot be ruled out. Such a response would be more specific for the stress imposed and therefore could produce cells which are more resistant than stationary-phase cells (as seen in Fig. 8). The mechanism of inactivation by UVA light is still not fully understood. Recent studies suggest that UVA light mediates its biological effects primarily via oxidative mechanisms that lead to reactive oxygen species (21).

In batch culture with defined media where only one carbon source is present, the microbial growth pattern can be clearly separated into an exponential and a stationary phase. However, in complex medium, where many different carbon sources are present, the change from exponential to stationary phase is not clear-cut (Fig. 3). The specific growth rate decreases steadily, which means that physiological changes take place throughout the whole growth cycle. This is supported by the work of Schaechter et al. (35) who examined variations in growth and composition of *Salmonella enterica* serovar Typhimurium cultures in different media and found that the cellular contents of DNA, RNA, and protein at a given temperature are highly dependent on the specific growth rate. Therefore, expressions, such as early exponential, late exponential, or early stationary phase, as often used in the literature, are inadequate to describe the physiological state of cells. Also, stating the harvesting time and optical density of a culture is insufficient because the growth history is a very important factor when it comes to reproducibility. For example, if bacterial cells are harvested at an OD of 0.3, a culture that was inoculated to an initial OD of 0.1 will be in a different physi-

ological state than a culture that started from an OD of 0.001. If a high bacterial cell concentration is present at the start, easily accessible nutrients in LB like amino acids will be used up very fast and the development of the specific growth rate will differ greatly from that of highly diluted cultures. Also, small changes in cultivation conditions like changing the aeration method (Fig. 2) or autoclaving the medium can lead to a big change for the development of the culture (5).

Our results corroborated previous reports (e.g., Berg [6]) on the direct link between increasing specific growth rate and increasing susceptibility to stress for bacteria. We confirmed our hypothesis that this concept also applies to physical stresses, like mild heat, UVA light, and sunlight, and that it is strongly related to cellular RpoS levels. This emphasizes the need for accurate reporting of specific growth rates and detailed culture conditions during disinfection studies, which would enable better comparison of data from different studies and sound interpretation of the data obtained.

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