Interaction of Silver(I) Ions with the Respiratory Chain of *Escherichia coli*: An Electrochemical and Scanning Electrochemical Microscopy Study of the Antimicrobial Mechanism of Micromolar Ag^{+}†

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ABSTRACT: Electrochemical techniques were used to study the behavior of *Escherichia coli* on the addition of ≤10 μM $AgNO_3$. Respiration in the presence of glucose was measured using a Clark ultramicroelectrode to determine the oxygen concentration as a function of time. The rate of respiration increased initially upon the addition of silver(I) because of the uncoupling of the respiratory chain, followed by cessation of respiration. The toxicity of 1 μM $AgNO_3$, as determined by the time until respiration ceased, increased in the absence of glucose and in the presence of K^+. The uptake of 1 μM Ag^+, measured from the stripping peak height of Ag electrodeposited on a 25 μm Pt ultramicroelectrode, showed a fastest uptake in the presence of glucose, although glucose was not required for uptake. Efflux of Ag^+ from cells was also detected in the presence of glucose. Scanning electrochemical microscopy (SECM) was used to follow the uptake of 1 μM Ag^+ by living and dead *E. coli* immobilized on a glass slide coated with poly-L-lysine. Ferricyanide was used as an alternative electron acceptor to oxygen to probe the site of inhibition of Ag^+ in the respiratory chain.

The antibacterial effect of the silver(I) ion has been noted for centuries (*1*), but the mechanism of action has been addressed only sporadically in the scientific literature (*2*−10). The subject has received renewed interest in recent years because of the commercial introduction of novel silver-containing materials. With the onset of multidrug resistance in many bacterial strains, new treatment methodologies are increasingly being sought, and the powerful antibacterial properties of silver(I), at low concentrations, make it an attractive proposal. Reports of antimicrobial silver-containing films (*11*), dressings (*12*), textiles (*13*), composite coatings (*14*), and dendrimers (*15*) are readily available in the literature. However, despite the increased interest in novel methods of silver delivery, the quantitative aspects and mechanism of antimicrobial action remain relatively obscure.

Submillimolar concentrations of $AgNO_3$ are reported to be lethal to a range of bacterial species, both Gram-positive and Gram-negative (*2*−*5*). The exact mode of action is unknown, but Ag^+-treated bacteria exhibit a characteristic initial stimulation in respiration before cell death occurs (*2*, *3*). Ag^+ has been variously reported to uncouple the respiratory chain from oxidative phosphorylation (*4*), collapse the proton-motive force across the cytoplasmic membrane (*9*), or interact with thiol groups of membrane-bound enzymes and proteins (*7*, *10*). It is unclear whether there is one major site of action or whether the silver(I) interacts detrimentally with the cell at many sites. The site most vulnerable to attack by biocidal species is the cytoplasmic membrane, where many important proteins reside, including enzymes of the respiratory chain and vital transport channels. Previous observations suggest the cytoplasmic membrane as the primary target site for biocidal activity of Ag^+ (*3*, *4*, *10*), especially at low concentrations, although at higher concentrations silver has been observed to interact with cytoplasmic components within the cell (*8*, *10*). Most strains of bacteria have not yet developed resistances to Ag^+ (*16*); however, recent molecular biology studies have isolated the genes believed to code for Cu^I and Ag^I transport proteins in *Enterococcus hirae*, responsible for both uptake and efflux (*17*, *18*). In addition, silver(I)-resistant *Escherichia coli* show active efflux of Ag^+ and are deficient in outer membrane porins (*19*). Recently, genes conferring copper and silver resistance to *E. coli* have been found to encode the CusCFBA protein complex, which spans the cytoplasmic and outer membrane of the bacterium and is responsible for the efflux of Cu^I and Ag^I (*20*). Deletion of this gene has been found to increase the sensitivity of *E. coli* to silver(I) (*21*). In addition, plasmid-encoded silver resistance, mediating silver efflux, has also been discovered for *E. coli* previously exposed to silver (*22*). It is therefore important to study the mechanism of silver antimicrobial action to further understand the development of silver resistance.

This paper uses electrochemical methodology to study the antimicrobial effect of micromolar concentrations of $AgNO_3$ on *E. coli*. The respiratory characteristics of bulk and immobilized bacteria were used as a diagnostic tool to determine the effect of the silver(I) ion on the respiratory chain. Silver uptake and efflux were also measured at low Ag^+ concentrations using an ultramicroelectrode.

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EXPERIMENTAL PROCEDURES

Materials, Chemicals, and Instrumentation. All chemicals were reagent-grade and used as received. All solutions were prepared with deionized water (Milli-Q, Millipore Corp.). D-Glucose, poly-L-lysine, $AgNO_3$, Na-citrate, and NH_4NO_3 were obtained from Aldrich; NaCl, KCN, $MgSO_4$, $CuSO_4$, $Fe_2(SO_4)_3$, $K_4Fe(CN)_6$, and $K_3Fe(CN)_6$ were obtained from Fisher; K_2HPO_4 and KH_2PO_4 were purchased from J. T. Baker. All electrochemical and scanning electrochemical microscopy (SECM)[1] measurements were carried out using a Model 900 SECM (CH Instruments, Austin, TX).

Growth and Preparation of E. coli. E. coli (ATCC number 25922) were obtained as lyophilized pellets from MicroBio-Logics and grown on minimal agar plates (37 °C for 48 h) using standard bacterial culture techniques. Cells taken from the plates were then grown aerobically in broths containing 30 mM K_2HPO_4, 3.3 mM KH_2PO_4, 20 mM NaCl, 19 mM NH_4NO_3, 1.7 mM Na-citrate, 1 mM $MgSO_4$, 0.1 M D-glucose, and trace amounts of $Fe_2(SO_4)_3$, ZnO, $CuSO_4$, $Co(NO_3)_2$, H_3BO_3, and $(NH_4)_6Mo_7O_{24}$. All stock solutions were autoclaved prior to use. Growth broths were vigorously stirred to ensure aeration, and cells were grown for 18 h at room temperature, until the stationary phase. Cells were harvested by centrifugation at 10 000 rpm for 3 min and resuspended in fresh 0.2 M phosphate buffer solution at pH 7 to allow accumulation of phosphate. After 2 h, the solution was centrifuged, the buffer solution was removed, and the cells were washed with 0.1 M $NaNO_3$. The cells were then recentrifuged and suspended in 2.5 mL of 0.1 M $NaNO_3$ to give a final concentration of $\sim 2 \times 10^{10}$ cells/mL. This procedure was followed to ensure a solution free of phosphate, because most of the experiments were carried out in the presence of silver(I), which precipitates with phosphate. All experiments were carried out in chloride-free solutions for the same reason.

Measurement of Respiration of E. coli in Solution. The respiration of the cells in solution was measured using a Clark ultramicroelectrode, constructed as described previously (*23*). The Clark ultramicroelectrode consists of a 25 μm Pt-disk, Ag-ring electrode, immobilized behind an oxygen-permeable high-density polyethylene membrane, and measures the solution oxygen concentration using the current for oxygen reduction. The membrane prevents the deactivation of the Pt electrode surface by adsorption of biological molecules. In addition, any contact between the bacteria and the outer silver coating of the electrode is prevented. However, the extent of dissolution of Ag^+ from the silver coating was found to be sufficiently negligible to have no effect on the outcome of these experiments. In contrast to a Clark electrode of more traditional dimensions, the ultramicroelectrode draws currents in the nanoamp range. The diffusion layer (region of depletion of electroactive species at the electrode surface, in this case oxygen) of such an electrode is also much thinner. Not only can the ultramicroelectrode be used to undertake measurements in much smaller volumes (10−1000 μL) because of its small dimensions, but the formation of concentration gradients at the electrode/solution interface is also negligible in contrast to larger electrodes, removing the necessity for constant mixing. A 1 mL electrolyte solution containing $\sim 5 \times 10^9$ *E. coli* cells was used in each experiment. The exact composition of solution used in the experiments is described in the text. The solution was mixed thoroughly before measurements were started and also upon addition of the silver nitrate but not covered or deaerated during measurement; therefore, oxygen was free to enter the solution from the atmosphere. The solution was not mixed during the measurement because this was found to disturb the membrane of the Clark electrode and give unacceptably noisy readings. The electrode was placed in the solution to a depth of 5−7 mm, and settling of cells during the time of the experiment was assumed to not affect the measurements. The oxygen reduction current was measured over time by cyclic voltammetry (CV), and the limiting current for oxygen reduction at −0.8 V versus silver was then plotted against time. All respiration measurements were repeated approximately 6 times for each condition to ensure reproducibility. For experiments carried out on the same day, using the same Clark ultramicroelectrode, almost identical results were obtained for experiments under the same conditions. However, when the membrane of the electrode was changed, absolute values of the currents could change by up to 10% despite the same trends being observed. Therefore, relative limiting current values (*y* axis) between figures may vary by up to 10%, if the experiment was carried out using a different electrode. However, data presented within each figure is that from an experiment carried out (in triplicate) on the same day, using the same electrode; therefore, it can be considered as self-consistent and reproducible.

Measurement of Silver(I) Uptake/Efflux by E. coli in Solution. For the measurement of silver(I) uptake, 1 mL electrolyte solutions, each containing 5×10^9 *E. coli* cells, were treated with 1 μL of 1 mM $AgNO_3$, to give a final concentration of 1 μM Ag^+. Different electrolyte compositions were used as described in the text. After intervals of time, from 0 to 30 min, the solutions were centrifuged for 3 min at 10 000 rpm and the solution was separated from the cells. The solutions were then electrochemically analyzed for silver using stripping voltammetry. A freshly polished 25 μm Pt disk−Ag ring electrode was used for the analysis. The tip potential was held at −0.3 V versus Ag for 60 s to preconcentrate electrodeposited silver onto the electrode surface. The tip potential was then swept from −0.3 to +0.35 V versus Ag at 0.1 V s^{-1} to strip the silver from the electrode. A stripping peak was obtained whose height was proportional to the concentration of silver(I) in solution.

For the efflux experiments, 1 mL solutions containing 5×10^9 *E. coli*, 0.1 M $NaNO_3$, and 0.05 M glucose were treated with 1 μM $AgNO_3$ for 2 min, to allow the cells to accumulate silver(I), before being centrifuged for 3 min at 10 000 rpm. The solution was removed from the cell pellet and discarded, and the cells were resuspended in 1 mL of fresh electrolyte solution (composition described in the text). After different intervals of time, from 0 to 20 min, the solutions were recentrifuged, and the resulting clear solution was removed from the cells and analyzed using stripping voltammetry, as described above.

SECM Experiments: Measurement of Respiration of Immobilized E. coli. E. coli immobilized on glass microscope slides was used in SECM experiments. The slides were first

[1] Abbreviations: CV, cyclic voltammetry; ROS, reactive oxygen species; SECM, scanning electrochemical microscopy.

washed carefully with 70% ethanol, rinsed thoroughly with water, and dried with argon. Spots of 10% poly-L-lysine solution were then placed on the slide and allowed to dry at room temperature for 1 h. The poly-L-lysine-modified slide was then rinsed and dried again, and drops of solution containing $\sim 10^{10}$ *E. coli*/mL were placed on the poly-L-lysine spots. After 60 min, the solution was removed and the slides were rinsed in water and dried with argon. This procedure allowed the immobilization of *E. coli* on the slide at a density of 5×10^7 cells/cm^2.

For SECM experiments, a 25 μm diameter Pt tip was used, with an Ag paint coating acting as the counter/reference electrode. Using this ring−disk electrode, only a small 10 μL volume of electrolyte was required to carry out electrochemistry. The tip was approached to 25 μm above a nonmodified area of the glass slide using the negative-feedback approach curve for oxygen reduction. The solution drop was then removed; the electrode was rinsed and dried with argon, to ensure that a film of electrolyte was not trapped between the electrode and the glass surface; and the tip was moved over the immobilized *E. coli*. A 10 μL drop of 0.1 M $NaNO_3$ electrolyte was then placed over the cells, and the oxygen concentration of the solution, in the region above the cells, was continuously monitored by CV by measuring the limiting current for oxygen reduction at −0.8 V versus silver. Experiments were carried out in air with no deaeration.

SECM: Silver(I) Uptake by Immobilized E. coli Cells. The uptake of 1 μM $AgNO_3$ in 0.1 M $NaNO_3$ by *E. coli* immobilized on a glass slide with poly-L-lysine was measured using the same 25 μM Pt disk−Ag ring electrode, as described previously. The electrode was held at −0.3 V versus Ag for 60 s to deposit silver and then swept from −0.3 to 0.35 V at 0.1 V s^{-1} to strip the silver. The concentration of silver(I) in the solution was proportional to stripping peak height. In preliminary experiments with the electrode located 100 μm above the cells, no Ag^+ could be detected, because the depletion was so fast. Instead, the electrode was located 1 mm above the cells, where the concentration of silver(I) could be measured by stripping voltammetry. Uptake experiments were carried out above live cells and cells previously treated with 10 mM KCN for 2 h to compare uptake by dead and live cells, i.e., to determine the proportion of silver(I) that complexes with the outside of the cell rather than entering the cell.

RESULTS

Measurement of Respiration of E. coli Using the Oxygen Reduction Current: Respiration of Bacteria in the Presence of Glucose: Effect of 1−10 μM $AgNO_3$. The respiration of 5×10^9 suspended bacteria was followed by measuring the solution oxygen concentration over time, using a Clark ultramicroelectrode, as described in the Experimental Procedures. Figure 1 shows typical respiration of *E. coli* in a 1 mL solution containing 0.1 M $NaNO_3$ and 0.1 M glucose. The *y*-axis values are the limiting current for oxygen reduction at time *t*, divided by the limiting current at $t = 0$ and correlate with the concentration of oxygen in the solution relative to time $t = 0$. The respiration rate for the first 10 min after addition of the cells to the glucose-containing solution is fast, but after approximately 10 min, the rate of respiration slows.

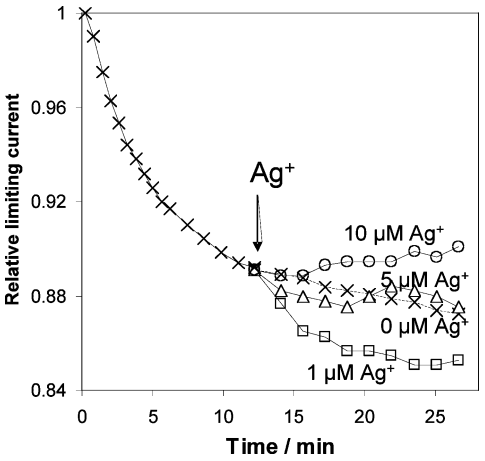

FIGURE 1: Respiration of suspended *E. coli* in 1 mL solutions initially containing 0.1 M $NaNO_3$ and 0.1 M glucose, plotted as limiting current for oxygen reduction at −0.8 V versus silver paint relative to limiting current at $t = 0$ min, as measured by Clark ultramicroelectrode. (×) 0 μM $AgNO_3$ added at $t = 12$ min. (○) 10 μM $AgNO_3$ added at $t = 12$ min. (△) 5 μM $AgNO_3$ added at $t = 12$ min. (□) 1 μM $AgNO_3$ added at $t = 12$ min.

At $t = 12$ min, $AgNO_3$ was added to produce different concentrations of Ag^+ (1−10 μM) to the normally respiring *E. coli* solutions, and the change in respiration rate compared to that containing 0 μM Ag^+ was measured. For 1 and 5 μM Ag^+, there was a rapid increase in the rate of oxygen depletion, indicating an increase in the bacterial respiration rate. For 10 μM Ag^+, this period of stimulated respiration was not observed, and instead, there was an immediate increase in the solution oxygen concentration because of cessation of respiration coupled with dissolution of oxygen from the atmosphere. *E. coli* treated with 10 μM $AgNO_3$ do not grow when placed overnight in well-aerated nutrient broths, which correlates this inhibition of respiration with the loss of cell viability. The time after the addition of silver at which the solution oxygen concentration begins to rise decreased with increasing silver(I) concentration: 3.5 min for 10 μM, 6.5 min for 5 μM, and >15 min for 1 μM. At $AgNO_3$ concentrations of 1 μM, the oxygen depletion because of stimulated respiration is fastest, but within the time scale of the experiment, no increase in oxygen concentration is observed. Although silver(I) at this concentration interferes with normal respiration, it appears that the cells are still respiring after 15 min. However, when cells treated with 1 μM $AgNO_3$ are placed in a nutrient medium overnight, no growth is observed. These data are consistent with previously reported studies where concentrations of $AgNO_3$ as low as 1 μM are found to inhibit bacterial growth (*5*).

Respiration of Cells without Glucose: Effect of 1 μM $AgNO_3$. The requirement of glucose for cell respiration and silver uptake was studied for cells suspended in solution. Figure 2a shows normal respiration, from $t = 0$ to 12 min, of 5×10^9 *E. coli* cells in a 1 mL solution of $NaNO_3$, with and without added glucose. The figure shows that the initial respiration rate in the absence of glucose was faster than with glucose. The reason for this is unclear, but the cells had previously been suspended in phosphate buffer solution for up to 2 h and then in $NaNO_3$; therefore, respiration can only be powered by any remaining intracellular reserves. The increased respiration rate compared to that observed in the

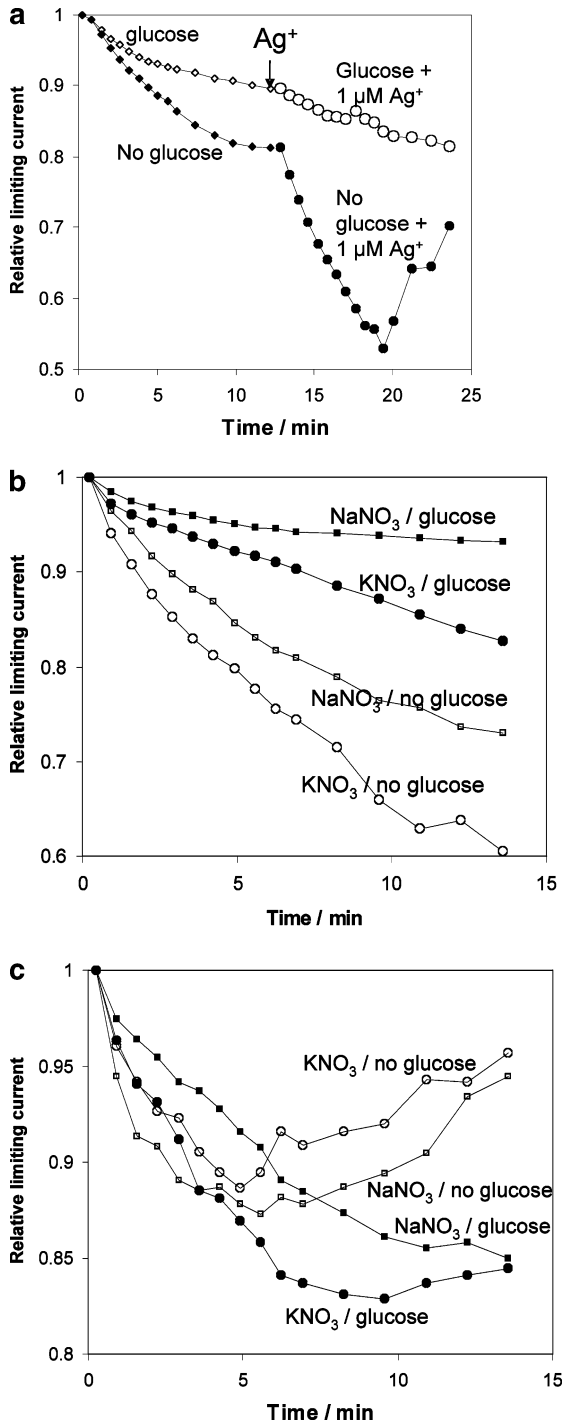

FIGURE 2: Respiration of suspended *E. coli* determined by solution oxygen concentration with time, measured by Clark ultramicroelectrode using limiting current for oxygen reduction (relative to that at $t = 0$) at -0.8 V versus Ag. (a) (◇) Solution initially containing *E. coli*, 0.1 M $NaNO_3$, and 0.1 M glucose. (○) 1 μM $AgNO_3$ added to the same solution at $t = 12$ min. (◆) Solution initially containing *E. coli* and 0.1 M $NaNO_3$ only. (●) 1 μM $AgNO_3$ added to the same solution at $t = 12$ min. (b) (■) Solution containing *E. coli*, 0.1 M $NaNO_3$, and 0.1 M glucose. (●) Solution containing *E. coli*, 0.1 M KNO_3, and 0.1 M glucose. (□) Solution containing *E. coli* and 0.1 M $NaNO_3$. (○) Solution containing *E. coli* and 0.1 M KNO_3. (c) (■) Solution containing *E. coli*, 0.1 M $NaNO_3$, 0.1 M glucose, and 1 μM $AgNO_3$. (●) Solution containing *E. coli*, 0.1 M KNO_3, 0.1 M glucose, and 1 μM $AgNO_3$. (□) Solution containing *E. coli*, 0.1 M $NaNO_3$, and 1 μM $AgNO_3$. (○) Solution containing *E. coli*, 0.1 M KNO_3, and 1 μM $AgNO_3$.

presence of glucose may be due to trace silver(I) in the experimental apparatus, even after careful cleaning. Very small concentrations of silver(I) are capable of inducing stimulated respiration, and the effect of silver appears to be more pronounced in the absence of glucose (*vida infra*). At $t = 12$ min, 1 μM $AgNO_3$ was added to the solutions and stimulated respiration was observed both in the presence and absence of glucose. The extent of initial stimulated respiration is greater in the absence of glucose. Moreover, silver(I) taken up during glucose-deficient conditions appears to have a greater inhibiting effect as shown by the increase of oxygen in the solution after about 7 min as the cells stop respiring. In comparison, the solution containing glucose does not show an increase in oxygen concentration within the time scale of the experiment, indicating that the bacteria are still respiring.

Effect of Supporting Electrolyte on Respiration of E. coli in the Presence of Silver(I): $NaNO_3$ versus KNO_3. The effect of alkali metal cations on the toxicity of 1 μM $AgNO_3$ to *E. coli* was investigated by replacing the 0.1 M $NaNO_3$ electrolyte with 0.1 M KNO_3. Figure 2b shows respiration of *E. coli* in the absence and presence of glucose, with 0.1 M $NaNO_3$ or 0.1 M KNO_3. Both solutions containing K^+ show faster respiration than the corresponding solution containing Na^+. Figure 2c shows respiration in the same solutions after the addition of 1 μM $AgNO_3$. The data for Na^+ shows the same trend as described previously; the initial rate of stimulated respiration is greater, and the time until cessation of respiration is shorter in the absence of glucose (>15 min with glucose versus 5.5 min without glucose). For K^+, a different behavior is observed; the initial rate of stimulated respiration is the same in the absence or presence of glucose, and in both cases, the time for inhibition is shorter than that observed with Na^+ (9.5 min with glucose versus 5 min without glucose).

Measurement of Silver(I) Uptake and Efflux by E. coli Using Stripping Voltammetry: Silver(I) Uptake in the Presence and Absence of Glucose. Silver uptake was determined by measuring the concentration of Ag^+ present in a 1 mL electrolyte solution, by stripping voltammetry, after different times of contact with 5×10^9 *E. coli* cells, as described in detail in the Experimental Procedures. In the first experiment, the uptake of 1 μM Ag^+ by the bacteria was measured, at 3 min intervals, in the presence of 0.1 M $NaNO_3$ and 0.05 M glucose. A lower concentration of glucose was used in this experiment, compared to the respiration experiment, because glucose can adsorb to the bare electrode surface during stripping analysis and reduce the sensitivity when measuring very low concentrations of silver. The same experiment was then repeated under the same conditions but with 0.1 M $NaNO_3$ only. The silver stripping peaks obtained after 0, 3, 6, 9, 15, and 20 min are shown in parts a and b of Figure 3, where stripping peak heights are proportional to the concentration of Ag^+ in solution. The experiments were repeated twice for each condition, and the averaged data for the concentration of silver(I) taken up by the cells is summarized in Figure 3c. The uptake of silver(I) within the first 3 min was very fast, with 0.55 μM of the silver(I) being taken up in the absence of glucose and 0.65 μM in the presence of glucose. Silver continues to be taken up faster with glucose, until $\sim$0.85 μM of the Ag^+ has been taken up after 9 min. In contrast, in the absence of glucose, the initial uptake rate is slower, especially over the first 9 min. However, the uptake of Ag^+

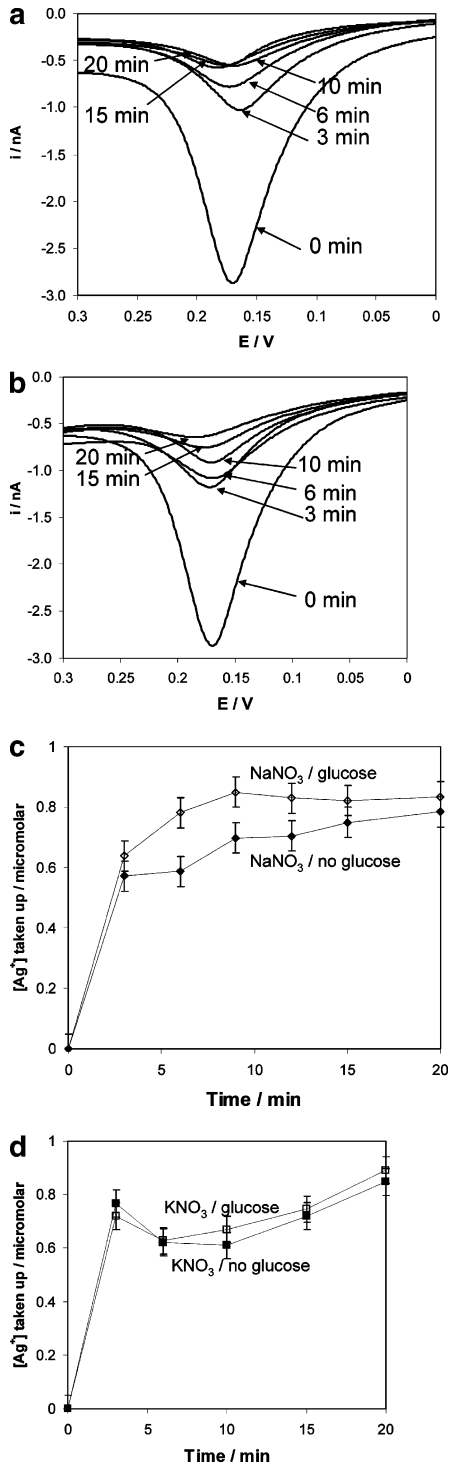

FIGURE 3: Stripping voltammetry peaks obtained from analysis of the uptake of 1 μM $AgNO_3$ by suspended *E. coli* in solutions containing (a) 0.1 M $NaNO_3$ and 0.05 M glucose and (b) 0.1 M $NaNO_3$ only. The peak height is proportional to the concentration of silver(I) remaining in solution after $t = 0$, 3, 6, 9, 15, and 20 min, determined at a 25 μm Pt−Ag ring ultramicroelectrode by applying a potential of −0.3 V versus silver for 60 s to electrodeposit silver and then sweeping −0.3 to 0.35 V (0.1 V s^{-1}) to obtain a stripping peak. (c) Average data from two experiments, for the concentration of silver(I) taken up by bacteria in solution [initial concentration (1 μm) − concentration at time, t] containing 0.1 M $NaNO_3$ only (◆) and 0.1 M $NaNO_3$ and 0.05 M glucose (◇). (d) Average data from two experiments, for the concentration of silver(I) taken up by bacteria in solution containing 0.1 M KNO_3 only (■) and 0.1 M KNO_3 and 0.05 M glucose (□).

continues at a regular rate after this time and shows no evidence of leveling off or decreasing. The uptake of silver(I) by the cells in the presence of glucose is 13 nmol of $Ag^+/10^9$ cells after 3 min.

Silver Uptake in the Presence of Potassium Ions. The same uptake experiments were repeated as in parts a and b of Figure 3 but with the $NaNO_3$ electrolyte solution replaced with KNO_3. The concentration of Ag^+ taken up by the *E. coli* over time, in the presence and absence of glucose, is summarized in Figure 3d (data is the average of two experiments). Unlike the Na^+ experiments, there is not a statistically significant difference between the uptake behavior with and without glucose. Initial uptake is again fast, with 0.75 μM of the silver taken up in the first 3 min, but after 6−10 min, the silver(I) uptake decreases. However, this process appears to be short-lived, because after 10 min the rapid uptake of Ag^+ continues.

Detection of Silver(I) Efflux from E. coli. The quantity of Ag^+ effluxed from *E. coli* in different media was measured using stripping voltammetry, as described in the Experimental Procedures. First, 5×10^9 *E. coli* cells were preincubated in 1 mL of 0.1 M $NaNO_3$ and 0.05 M glucose in a centrifuge tube, for 15 min, before 1 μM $AgNO_3$ was added. The cells were allowed to accumulate silver(I) for 2 min before being centrifuged. The supernatant solution was then removed from the cells and discarded, and the cells were resuspended in 1 mL of a new "efflux medium" solution, containing either 0.1 M $NaNO_3$ or 0.1 M KNO_3, with or without 0.05 M glucose. After different lengths of time, the solution was once again separated from the *E. coli* by centrifugation and analyzed for Ag^+. Because there was no rinsing stage, trace amounts of silver(I) may remain in solution by sticking to the outside of the cells, resulting in a small background silver concentration. Any silver(I) present in solution above this concentration was assumed to have been effluxed from the cells.

Silver-stripping results obtained after 3, 6, 10, and 20 min in an efflux medium containing 0.1 M $NaNO_3$ and 0.05 M glucose are shown in Figure 4a. The stripping peak height increases with time as the accumulated silver(I) concentration increases, confirming that the efflux of Ag^+ from *E. coli* is taking place in the presence of Na^+ and glucose. Figure 4b shows data for the same experiment but with no glucose in the efflux medium (0.1 M $NaNO_3$ only), showing no or very small silver-stripping peaks, indicating that only trace amounts of Ag^+ are present in the solution.

The concentration of effluxed silver(I) can be calculated approximately by comparing the stripping peak heights to those obtained in $AgNO_3$ solutions of known concentration. The concentration of silver(I) effluxed over time is summarized in Figure 4c, for the 0.1 M $NaNO_3$ solutions, along with data obtained from analyzing efflux solutions composed of 0.1 M KNO_3, with and without 0.05 M glucose (data is averaged from two sets of experiments). The background silver concentration, because of lack of rinsing, is assumed to be 0.01 μM, as shown by a dotted line in the figure. Only silver concentrations above this level are attributed to the efflux. The concentrations measured are all below 0.1 μM; therefore, only very small amounts of Ag^+ are effluxed by the cells under these conditions. This is illustrated by comparing the stripping peaks obtained in parts a and b of Figure 3 with those in parts a and b of Figure 4, where clearly

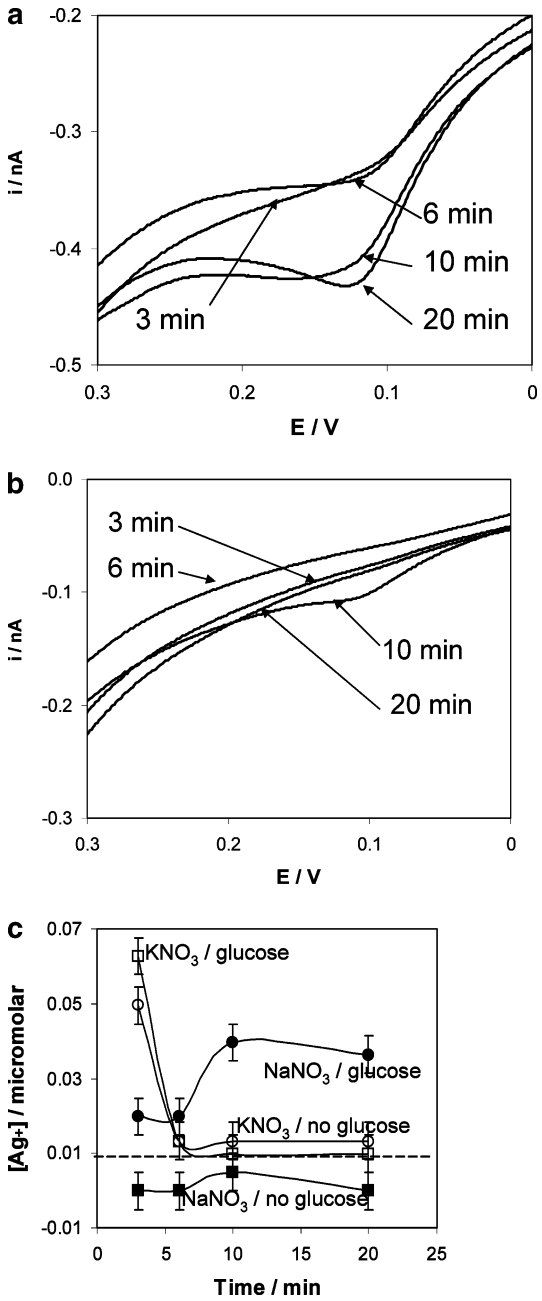

FIGURE 4: Stripping voltammetry peaks obtained from analysis of the efflux of Ag^+ by suspended *E. coli* in solutions containing (a) 0.1 M $NaNO_3$ and 0.05 M glucose and (b) 0.1 M $NaNO_3$ only. The peak height is proportional to the concentration of silver(I) effluxed from the cells after 3, 6, 10, and 20 min, determined at a 25 μm Pt−Ag ring ultramicroelectrode by applying a potential of −0.3 V versus silver for 60 s to electrodeposit silver and then sweeping −0.3 to 0.35 V (0.1 V s^{-1}) to obtain a stripping peak. Bacteria were initially incubated for 2 min in solution containing 0.1 M $NaNO_3$, 0.05 M glucose, and 1 μM $AgNO_3$ before centrifuging and resuspending in efflux medium. (c) Summary of the concentration of Ag^+ effluxed from cells in solutions containing (●) 0.1 M $NaNO_3$ and 0.05 M glucose, (○) 0.1 M KNO_3 and 0.05 M glucose, (■) 0.1 M $NaNO_3$ only, and (□) 0.1 M KNO_3 only (data average of two experiments).

the stripping peaks for effluxed silver are an order of magnitude smaller than those for the silver uptake. The cells in $NaNO_3$ show efflux only in the presence of glucose, corresponding to 8×10^{-3} nmol of $Ag^+/10^9$ cells. If it is assumed that, during the 2 min preincubation with Ag^+, the cells accumulate ~13 nmol of $Ag^+/10^9$ cells (see the uptake section above), then this efflux corresponds to less than 0.1% of the Ag^+ originally accumulated. In KNO_3, considerable efflux is detected, with and without glucose, after 3 min. However, at longer times, the silver(I) concentration drops close to the background level. This is also consistent with the silver uptake data in Figure 3d, which showed that the concentration of silver(I) taken up decreased during 3−10 min before uptake resumed. The efflux data is also consistent with the respiration experiments, where long-term efflux is only observed in the $NaNO_3$/glucose solution, which is also the only condition where respiration of the bacteria does not cease within 15 min.

SECM Experiments with Immobilized E. coli: Respiration of Immobilized Cells: Effect of 0.1 μM $AgNO_3$. Respiration and silver uptake experiments were repeated using cells immobilized onto the surface of a glass slide. There are several advantages to performing experiments in this manner compared to using cells suspended in solution: (i) experiments can be performed using fewer cells, in a smaller volume; (ii) electrode surface fouling is minimized because the immobilized cells cannot adsorb onto the surface of the electrode: real-time silver uptake experiments can then be performed without having to remove the cells from solution by centrifugation prior to analysis; and (iii) the precise positioning of the electrode above the surface of the cells achievable through this method allows the future extraction of more quantitative data and theoretical modeling of the uptake kinetics.

Experiments to follow the respiration of *E. coli*, immobilized on a glass slide using poly-L-lysine, were carried out using a 25 μm Pt disk−Ag ring electrode (with no membrane to improve sensitivity of oxygen detection) located 25 μm above the cells. A 10 μL drop of electrolyte solution, containing 0.1 M $NaNO_3$ and 0.1 M glucose, was placed over the cells ($t = 0$), and respiration followed by measuring the depletion of oxygen from the solution above the cells using CVs for oxygen reduction. Because the ultramicroelectrode draws such small currents (nA) within the time scale of this experiment (less than 10 min), the electrode causes little depletion of the oxygen concentration in the region above the cells. Likewise, the accumulation of hydroxide ions, the product of the oxygen reduction reaction at the electrode, is assumed to be negligible and have no effect on the measured respiration rate. The limiting current for oxygen reduction at time t relative to that at $t = 0$ was plotted versus time, and the results obtained are shown in Figure 5a. The initial rate of respiration is fast and nonlinear, becoming slower after 2 min. At this point, the oxygen concentration above the cells is depleted only slowly and then begins to increase again slightly after 3 min, because oxygen is continually diffusing into the small electrolyte drop and into the depleted region between the tip and bacteria and the rate at which it is removed by the respiring bacteria is no longer fast enough to compensate. At $t = 4$ min, a 1 μL drop of 1 μM $AgNO_3$ was added to the electrolyte drop, to give a silver(I) concentration of 0.1 μM Ag^+. As observed in the bulk solution, the respiration rate increased upon addition of low concentrations of silver(I), until about 2 min after the addition of the silver(I), when the oxygen concentration began to increase slightly again, signifying a decrease in the respiration rate. Although because the oxygen increase is very slight, it appears the cells are still respiring, albeit

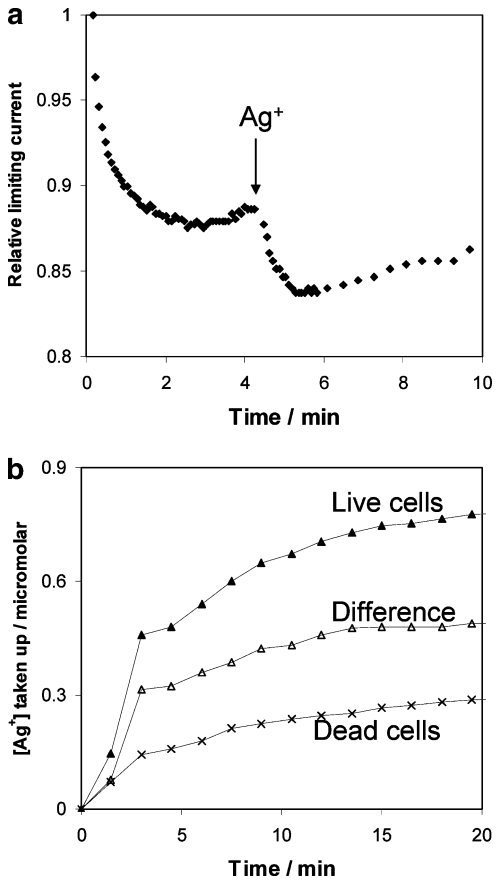

FIGURE 5: (a) Limiting current for oxygen reduction, relative to that at $t = 0$, at -0.8 V versus Ag paint, obtained at 25 μm Pt disk−Ag ring electrode located 25 μm above immobilized *E. coli* over time. Electrolyte initially was 10 μL drop of 0.1 M $NaNO_3$. At $t = 4$ min, a 1 μL drop of 1 μM $AgNO_3$ was added. (b) Concentration of Ag^+ taken up by immobilized *E. coli*, measured using the stripping peak height obtained using a 25 μm Pt disk electrode located 1000 μm above the cells, held at -0.3 V versus silver paint for 60 s before sweeping -0.3 to 0.35 V at 0.1 V s^{-1} to obtain stripping peak. (▲) Live cells. (×) Dead cells previously treated with 10 mM KCN for 2 h. (△) Difference in Ag^+ taken up by dead and alive cells.

more slowly. Higher concentrations of $AgNO_3$ would be required to completely inhibit respiration; however, it was not possible to use higher concentrations of Ag^+ with this experimental technique, because at higher concentrations, silver deposits on the electrode surface during oxygen reduction, which changes the behavior of the electrode. These results confirm that the immobilization process has little effect on the behavior of the cells, and in the future, more quantitative studies can be undertaken using this methodology.

Uptake of Silver(I) by Immobilized E. coli. Uptake of silver(I) by immobilized *E. coli* was measured using stripping voltammetry as described in the Experimental Procedures. A solution drop of 1 μM $AgNO_3$ in 0.1 M $NaNO_3$ was placed above the cells, and the silver(I) concentration was measured over time. Preliminary experiments showed that, if the electrode was located 100 μm above the cells, no Ag^+ was detected, because silver depletion from the region directly above the cells was so fast. However, if the electrode was placed 1 mm above the cells, the decrease in the $AgNO_3$ concentration over time could be measured. Experiments were also repeated over dead cells that had been previously treated with 10 mM KCN for 2 h to determine the amount of Ag^+ that complexed with the outside of the dead cells, without being transported into the cell. The polysaccharide outer membrane of Gram-negative bacteria, such as *E. coli*, often incorporates inorganic (poly)phosphates, which readily complex with Ag^+ (*24*).

Figure 5b shows the total amount of silver(I) taken up by the live and dead *E. coli* (average of two experiments in each case). The data shows that almost 0.3 μM Ag^+ was depleted by the dead cells after 20 min, presumably by complexation to inorganic functional groups on the outside of the cells. In contrast, the live cells consumed almost 0.8 μM of the 1 μM Ag^+ originally in solution. Most of this uptake took place within the first 10 min, with more gradual uptake thereafter. The difference in uptake between the dead and live cells (uptake live − uptake dead) is also shown in Figure 5b and corresponds to the amount of Ag^+ accumulated *within* the live cells, rather than complexing to the outside. This gives an approximate value of 0.5 μM for the concentration of silver transported into the cells after 20 min, corresponding to ∼60% of the total silver(I) depleted from solution. Approximately 40% of the silver(I) taken up is then assumed to complex to the outside of the cell without passing through the membrane. Uptake into the cell appears to cease after about 13 min, according to the difference data (ii), while further silver(I) depletion can be attributed to continued binding to the outside of the cells. This observation is generally consistent with the bulk respiration and silver uptake experiments where, under similar conditions, respiration ceased at around 8 min (Figure 2a) but silver uptake continued (Figure 3c). Uptake of Ag^+ through the membrane into the cell may cease once the bacteria stop respiring, but silver continues to bind to the outside of the cells.

Site of Interaction of Ag^+ with the Respiratory Chain of E. coli: Use of Ferricyanide as an Electron Acceptor: Reduction of Ferricyanide to Ferrocyanide by Respiring Bacteria. Ferricyanide has previously been used as a mediator to follow the respiration of *E. coli* using SECM by measuring the production of ferrocyanide (*25*), but the site of interaction with the respiratory chain was undetermined. Ferricyanide is a hydrophilic molecule, making transport through the cytoplasmic membrane unlikely; therefore, to scavenge electrons from the respiratory chain, it probably interacts with a membrane-spanning protein, such as complex I or III, at some point in the periplasmic space, on the outside of the cytoplasmic membrane. While ferricyanide is reduced by the respiratory chain, its reduction is not coupled to ATP synthesis (*26*).

To measure the reduction of ferricyanide by suspended *E. coli*, the reduction product ferrocyanide, $Fe(CN)_6^{4-}$, was detected at a 25 μm Pt electrode, by holding the potential at +0.5 V versus Ag and recording the current with time. The experiment was carried out in a 1 mL volume of electrolyte solution, containing 5×10^9 *E. coli* cells, 0.1 M $NaNO_3$, 0.1 M glucose, and 50 mM potassium ferricyanide. Figure 6 shows the current obtained for the oxidation of ferrocyanide versus time, with the current being proportional to the quantity of ferricyanide reduced by the bacteria.

Effect of Treatment with $AgNO_3$ on the Reduction of Ferricyanide. The experiment described above was repeated but with *E. coli* that had been preincubated with 1 μM $AgNO_3$ for 10 s in 0.1 M $NaNO_3$. The cells were then

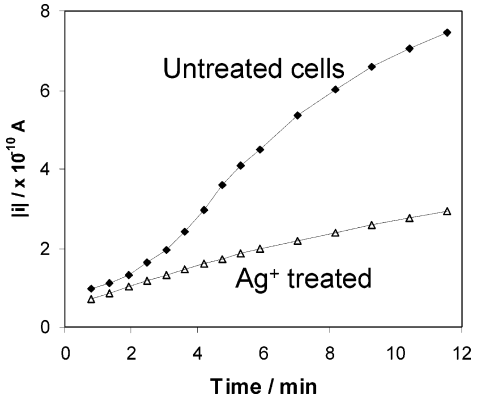

FIGURE 6: (a) Limiting current versus time for the oxidation of ferrocyanide produced by the reduction of 50 mM potassium ferricyanide by respiring *E. coli* in the presence of 0.1 M $NaNO_3$ and 0.1 M glucose. (◆) Untreated *E. coli*. (△) *E. coli* that had been preincubated with 1 μM $AgNO_3$ for 10 s in 0.1 M $NaNO_3$. The cells were then centrifuged and rinsed to remove the excess Ag^+ and resuspended in clean 0.1 M $NaNO_3$. A 25 μm Pt disc−Ag ring electrode held at 0.5 V versus silver.

centrifuged and rinsed to remove the excess Ag^+ and resuspended in clean 0.1 M $NaNO_3$. The respiration of the cells in 0.1 M $NaNO_3$ and 0.1 M glucose was measured using a Clark electrode, and the reduction of 50 mM ferricyanide was also measured under the same conditions, as described above. The Ag^+-treated bacteria showed stimulated respiration (when measuring the oxygen reduction current), similar to those treated with 1 μM $AgNO_3$ in Figure 1, which we speculate because of uncoupling of respiration from ATP synthesis (data not shown). In contrast, the reduction of ferricyanide was inhibited by the treatment with Ag^+, as shown in Figure 6. The uncoupling action of Ag^+ cannot therefore be attributed solely to an increase in the proton permeability of the cytoplasmic membrane (as observed for traditional uncouplers such as FCCP) because in that case there would be no inhibition of electron transport to ferricyanide, as discussed further below.

DISCUSSION

It might be useful to put these results in perspective based on previous studies of the effects of Ag^+ on bacteria. The stimulated respiration observed upon addition of Ag^+ to bacteria (Figure 1) has been noted previously (*2*, *3*). The same effect is reported for rabbit brain mitochondria, along with an uncoupling of respiration from ATP synthesis (*27*). The action of Ag^+ has been proposed to render the cytoplasmic membrane permeable to protons and to collapse the proton gradient. To compensate for the loss of the proton concentration gradient, the cell respires faster (stimulated respiration), in an attempt to expel more protons via the respiratory chain. The loss of the proton-motive force means that the respiration becomes independent of ATP synthesis and its rate is no longer determined by the availability of ADP, as it is during normal respiration. This so-called "uncoupling" effect is diagnostic of the collapse of the proton-motive force, with the loss of vital concentration gradients that provide the energy for membrane-transport processes. Ag^+ is unlikely to induce cytoplasmic membrane permeability by interacting with the lipid bilayer portions of the membrane, similar to traditional uncoupling compounds (protonophores) such as FCCP. Instead, it is more likely to interact with the many membrane-bound proteins found in the cytoplasmic membrane, such as transport or respiratory chain enzymes. If inhibition occurs at a coupling site (or sites) on the respiratory chain, this will prevent the efficient pumping of protons across the membrane and the maintenance of the proton gradient.

The ferricyanide reduction data (Figure 6) suggest that treatment with Ag^+ results in damage to the respiratory chain, which prevents the efficient passage of electrons from complex I to the ferricyanide molecules on the outside of the cytoplasmic membrane. As the respiration rate with oxygen as a final electron acceptor is increased under the same conditions, as observed in Figure 1, there must be sites on the cytoplasmic side of the chain where oxygen reduction can still take place. In addition to the terminal oxidase, oxygen can also accept electrons at lower potential sites of the chain. However, such uncontrolled respiration results in the formation of superoxide and hydroxyl radicals that are extremely damaging to the cell.

Surface-enhanced Raman spectroscopy of bacteria treated with $AgNO_3$ and sodium borohydride suggests that Ag binds preferentially at sites associated with flavin groups (*10*). This is consistent with a major site of inhibition being the NADH-binding site of complex I. *E. coli* possesses two different NADH dehydrogenases: the proton pumping NDH-I, containing FMN (flavin mononucleotide) and up to 9 FeS clusters, and the noncoupled NDH-II, which contains FAD (flavin adenine dinucleotide) only (*28*). Both dehydrogenases appear as possible sites for Ag^+ binding, containing cysteine residues that have a high affinity for silver(I) (*7*). Ag^+ is also known to inhibit other dehydrogenase-type enzymes, such as glucose oxidase (*29*), which have similar mechanisms of reaction, relying on flavin coenzyme groups. Dehydrogenase enzymes typically exhibit a "ping-pong" type mechanism where, after oxidation of the substrate, the reduced functional group of the enzyme must be reoxidized by another entity before further substrate molecules can be oxidized. In the case of glucose oxidase, Ag^+ is believed to inhibit this process by complexing with the reduced form of the enzyme and forming an inactive complex, preventing its reoxidation (*28*).

Binding of Ag^+ to low-potential enzymes of the bacterial respiratory chain and the resulting production of large quantities of reactive oxygen species (ROS), because of the inefficient passage of electrons to oxygen at the terminal oxidase, may be an explanation as to why the silver(I) ion is toxic to bacteria in such low concentrations, although experiments to detect the presence of ROS would be required to test this hypothesis.

Ag^+ appears to be more toxic to *E. coli* in the absence of glucose (Figure 2a). The reason for this is unclear, but the apparent protective effect conferred by glucose may be due to partial complexation of this large molecule with the silver cation, reducing its availability to the *E. coli*. Additionally, Figure 4 suggests that *E. coli* can actively efflux Ag^+ from the cell when provided with glucose. Silver-resistant *E. coli* have been reported to show active efflux, although it was unclear whether the energy for the process derives from ATP hydrolysis or the proton-motive force (*19*). The possibility that nonresistant *E. coli* may also possess this efflux route was discussed but not determined. The protection that glucose appears to offer *E. coli* at $AgNO_3$ concentrations of 1 μM

may be attributed to an Ag^+ efflux process that requires ATP hydrolysis. Silver(I) uptake experiments (Figure 3) show that at least 10% less silver is taken up by the *E. coli* in the absence of glucose. An interpretation of this observation is that, in the absence of glucose, less silver is taken up into the cell but instead interacts on the outside of the membrane or in the periplasm. In this region, there are protein targets, such as transport and respiratory enzymes that are vital to the cell function. In the presence of glucose, even though more silver is taken up, it may be transported away from these vulnerable sites, instead interacting with proteins in the cytoplasm.

These data suggest that 1 μM $AgNO_3$ is more toxic to *E. coli* in the presence of KNO_3 (with and without glucose), as shown in Figure 2c. An explanation for this behavior may be the requirement for asymmetric distribution of K^+ and Na^+ cations across the cytoplasmic membrane. *E. coli* cells require $[K^+]_{in} > [K^+]_{out}$ and preserve an internal K^+ concentration of about 0.25 M to maintain osmotic pressure in the cell above that of the medium. An increase in the ionic strength (osmolarity) or the K^+ concentration of the medium stimulates potassium uptake. Sodium, on the other hand, is actively expelled from bacteria to maintain $[Na^+]_{in} < [Na^+]_{out}$. As shown in Figure 2b, respiration is faster with K^+ in the medium, because the cells are stimulated to take up the potassium against a concentration gradient. This requires energy in the form of ATP hydrolysis or the proton-motive force, both of which are coupled to respiration. Upon the addition of 1 μM Ag^+, cell respiration ceases more quickly in the presence of K^+. The increased respiration rate in the presence of K^+ may induce the faster uptake of Ag^+, although why this should be is unclear. There is some evidence of Ag^+ efflux at short (<10 min) times in the presence of KNO_3, but in comparison with the $NaNO_3$/glucose medium, any efflux seems to be short-lived. A comparison with the respiration data in Figure 2c shows that cell respiration ceases at about the same time that the efflux process stops (approximately 5 min). When respiration can no longer be maintained, then the resulting concentration-gradient-driven transport mechanisms also cease, with the result that the cell can no longer protect itself and Ag^+ efflux stops. Figure 3 shows that uptake of silver in the $NaNO_3$/no glucose, KNO_3/glucose, and KNO_3/no glucose media continues even after respiration ceases (by comparison to Figure 2c), perhaps by interaction of the Ag^+ with the outside of the dead cells, as was also observed in the SECM experiments (Figure 5b).

CONCLUSIONS

The addition of $\leq$10 μM $AgNO_3$ to suspended or immobilized *E. coli* results in stimulated respiration before death, signifying uncoupling of respiratory control from ATP synthesis. This is symptomatic of the interaction of Ag^+ with enzymes of the respiratory chain. Use of ferricyanide as an alternative electron acceptor to oxygen suggests that Ag^+ inhibits at a low potential point of the chain, possibly the NADH dehydrogenase stage. When high concentrations of potassium are present in the medium, there is an increase in the apparent toxicity of silver(I). The presence of glucose affords some short-term protection against Ag^+ in Na^+-containing media, perhaps because of the existence of efflux pathways requiring ATP. The measurement of Ag^+ uptake by immobilized cells showed that approximately 60% of the silver(I) taken up is transported into the cell and the remaining 40% binds to the outside of the cell.

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