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Effects of Reduced Capsular Polysaccharide on Kanamycin Resistance in *Escherichia coli* B23 Cells

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Capsular polysaccharides found in members of the *Enterobacteriaceae* family play an important role in mediating the interactions between bacteria and their environment. Studies have shown that *Escherichia coli* B23 pretreated with sub lethal doses of kanamycin show increased capsular polysaccharide production. In addition, it has been suggested that bacterial capsules prevent uptake of aminoglycosides to confer resistance and protect the cell. This study attempted to investigate the effects of reduced capsular polysaccharide on kanamycin resistance. Kanamycin pretreated *E. coli* B23 were grown in sodium salicylate to decrease capsule production, then treated with 20 µg/ml kanamycin, a sub lethal concentration. It was observed that cultures grown in the presence of 60 µg/ml sodium salicylate, which had thinner capsules and lower detectable levels of capsular polysaccharide, were more susceptible to kanamycin relative to cells grown in Luria broth alone.

All members of the *Enterobacteriaceae* are able to elaborate a layer of surface-associated polysaccharides called the capsule. While the composition of these capsular polysaccharides is strain dependent, it is widely recognized that they play important roles in the mediation of bacterial interactions with the environment (13). Capsular polysaccharides function as virulence determinants that also provide protection against desiccation, attack from phages, and antibiotic activity (5, 13).

The aminoglycoside antibiotics, which include kanamycin, streptomycin, neomycin and gentamycin, are known to bind to the 30S bacterial ribosomal subunits to inhibit protein synthesis (3). Aminoglycoside resistance has been seen in *Pseudomonas aeruginosa* and *Enterobacteriaceae* and can be caused by one or more of the following mechanisms: inactivation of the drug by aminoglycoside-modifying enzymes produced by the bacteria, ribosomal alterations that prevent the drug from binding to its site of action, or loss of permeability of the bacterial cell to the drug. Membrane impermeablilization commonly occurs when cellular aminoglycoside entry is prevented by a thick bacterial capsule, which binds the antibiotic through charged interactions (3).

Ganal *et al*. have demonstrated that capsular polysaccharide production increases when *Escherichia coli* B23 cells are exposed to sub lethal doses of kanamycin and streptomycin (4). It has been observed that kanamycin pretreated cells develop increased antibiotic resistance relative to untreated cells, and that this resistance correlates with a significant increase in capsular polysaccharide production (8). However, the causation of kanamycin resistance by increased capsular polysaccharide has not been demonstrated.

We hypothesize that capsular polysaccharide confers resistance to aminoglycoside antibiotics in *E. coli* B23 cells, in a concentration dependent manner. To examine whether aminoglycoside resistance is due to the presence of bacterial capsule, we use sodium salicylate to inhibit capsule production and measure resultant *E. coli* growth. Aminoglycoside susceptible cultures are expected to grow more slowly than resistant cultures in sublethal levels of antibiotic. Investigations were carried out by pretreating *E. coli* B23 cultures with sub lethal levels of kanamycin, effectively increasing capsular polysaccharide to easily detectable levels. Cultures were then grown in the presence of sodium salicylate to knock down capsule production. Kanamycin was added to cultures following capsule knock down, and growth curves were generated to examine kanamycin resistance in capsular polysaccharide reduced cells. Capsule levels of cultures growing in kanamycin were measured by anthrone total carbohydrate assay and were visualized by capsule staining.

MATERIALS AND METHODS

Culture conditions. *Escherichia coli* B23 was obtained from the MICB 421 culture stock in the Microbiology and Immunology Department of the University of British Columbia. Sodium salicylate (Sigma, #057K0696) was mixed with distilled water to give a final concentration of 50 mg/ml. Then it was autoclaved and stored at room temperature. Two overnight cultures were prepared by inoculating 300 ml of Luria broth (LB) (5 g of yeast extract, 10 g of tryptone, and

10 g of sodium chloride per 1 L of distilled water) with a loopeful of *E. coli* B23 and were incubated on a shaker at 180 rpm and 37 °C.

Sub lethal antibiotic pretreatment. Each overnight culture was split into five flasks such that 40 ml of the overnight culture were transferred into a flask containing 940 ml of LB, and incubated for one hour on a shaker at 180 rpm at 37 °C. Kanamycin monosulfate (Sigma #K4000) was mixed with distilled water to give a final concentration of 20 mg/ml and then filter-sterilized. Then it was added to all five flasks to a final concentration of 10 □g/ml and they were incubated for 1 hour on a shaker at 180 rpm at 37 °C. Turbidity measurements were taken before and after the splitting of the culture, and after addition of antibiotic pretreatment using a spectrophotometer (Spectronic 20D+) at 460 nm wavelength.

Determination of effective sodium salicylate concentration. One hour after the antibiotic pretreatment (time 0), sodium salicylate stock solution (50 mg/ml) was added to each of the five flasks to the final concentration of 0, 10, 20, 40, and 60 µg/ml. At 0, 20, 40, 60, 80, 100, and 120 minutes after the addition of sodium salicylate, 100 ml of culture sample was taken from each flask for turbidity measurements, capsule isolation and capsule staining.

Sodium salicylate treatment and kanamycin treatment. One hour after the antibiotic pretreatment (time 0), five different conditions were setup in each flask, and we measured turbidity every 20 minutes for a duration of 3 hours at 460 nm wavelength using a Spectronic20D+ spectrophotometer. Flask 1 was our double negative control; neither sodium salicylate nor post antibiotic treatment was added. Flask 2 was our sodium salicylate control; only final concentration of 60 □g/ml of sodium salicylate was added at time 0. Flask 3 was our post antibiotic treatment control; only kanamycin was added to a final concentration of 20 □g/ml one hour after time 0. Flask 4 was our first test variable; sodium salicylate was added to a final concentration of 60 □g/ml at time 0, and of kanamycin was added to a final concentration of 20 □g/ml after one hour. Flask 5 was our second test variable; final concentration of 60 □g/ml of sodium salicylate and final concentration of 20 □g/ml of kanamycin were both added at time 0. In every 40 minutes (including time 0), 100 ml of sample culture from each flask was collected for a total duration of 2 hours in duplicates for capsule isolation and they were stored on ice.

Capsule isolation (6)**.** One hundred milliliters of sample collected from each time point was centrifuged in a Beckman J2-21 centrifuge using the JA-14 rotor at 17,000 x g for 15 min. The supernatants were discarded and the pellet was resuspended in 5 ml of PBS (137 mM NaCl, 2.7 mM KCl 1.4mM Na_2HPO_4, 1.8 mM KH_2PO_4). The resuspended pellet was vigorously mixed using a vortex and 10 ml of ice-cold acetone was added in order to precipitate the exopolymers. The well-mixed suspension was centrifuged using a Beckman J2-21 centrifuge with the JA-21 rotor at 6,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 2 ml of sterile distilled water and transferred to Spectra/Por® molecularporus membrane dialysis tubes (Spectra/Por®, #13260) with a molecular weight cut-off of 6,000-8,000 kDa. These samples were dialyzed in 4L of distilled water with a magnetic stir bar on a stir plate at 4°C for overnight. Then dialyzed exopolymers were lyophilized overnight using a lyophilizer (Virtis company, Inc.) at - 48°C with pressure level at 70 millitorr. The resultant crude exopolymers were dissolved in 2 ml of 10 mM $MgCl_2$ and then Deoxyribonuclease I (DNaseI) (Sigma #37F-9700) and ribonuclease A (RNaseA) (Sigma #063K1174) were added to each sample to final concentrations of 5 µg/ml and 0.1 mg/ml, respectively. The samples were incubated on a shaker at 37°C for five hours. After 5 h incubation, pronase (Calbiochem, #000875) was added to the samples to a final concentration of 0.1 mg/ml and the samples were incubated overnight on a shaker at 37°C. Then these samples were heated in a stationary water bath at 80°C for 30 min and centrifuged in a Beckman J2-21 centrifuge using the JA-14 rotor at 17,000 x g for 15 min. The resulting supernatants were dialyzed and lyophilized as described above. The partially purified and dried exopolysaccharides were dissolved in 5 ml of 0.05 M Tris base containing 0.1 M NaCl and 0.5 ml of 15 mM sodium deoxycholate was added to a final concentration of 1.5 mM to each sample. Sample mixtures were heated at 65°C for 15 min, and cooled to room temperature on ice. Then 0.275 ml of 20% acetic acid in distilled water was added to final concentration of 1%. Samples were subject to centrifugation in a Beckman J2-21 centrifuge using the JA-14 rotor at 16,000 x g for 5 min to separate the supernatant containing purified capsule from the pellets of lipopolysaccharides (LPS) and deoxycholate. Supernatant was stored at 4°C until further use.

Anthrone total carbohydrate assay (3)**.** Anthrone reagent was prepared by dissolving 200 mg of anthrone (Matheson Coleman & Bell, #CAX1655) in 5 ml of absolute ethanol. The solution was then made up to 100 ml using concentrated (18M) sulfuric acid (JT Baker, #331049) and chilled on ice. 0.5 ml of each purified capsule sample were transferred into glass test tubes and chilled on ice for 3 min. Then 2.5 ml of anthrone reagent was added to each chilled sample and was carefully mixed using a vortex. The test tubes were covered with aluminum foil and boiled in stationary 100°C water bath for 10 minutes. After the incubation, the tubes were removed from the water bath and cooled to the room temperature for 5 min and then stored on ice. When the test tubes were cooled, 3 ml of each sample were read at 625 nm wavelength using a Spectronic20D+ spectrophotometer.

Preparation of Glucose Standards (3)**.** 10 g/L of α-D (+) glucose stock solution was prepared by dissolving 200 mg of α-D (+) glucose (Sigma, #100F-0106) in 20 ml of distilled water. The glucose standard were made, in duplicates, using α-D(+) glucose stock solution (10 g/L) to a final concentration of 0, 10, 20, 40, 60, 80, and 100 □g/ml and added distilled water up to 0.5 ml. Then 2.5 ml of anthrone reagent was added to each glucose standard, covered with aluminum foil and boiled in stationary 100°C water bath for 10 minutes. After the incubation, the tubes were removed from the water bath and cooled to the room temperature for 5 min and then stored on ice. When the test tubes were cooled, 3 ml of each sample were read at 625 nm wavelength using a Spectronic20D+ spectrophotometer. A standard curve for total carbohydrate assay was generated.

Preparation of capsule staining solutions (9)**.** The 1% congo red was prepared by dissolving 0.10 g of congo red (Sigma, #72F-0612) in 10 mL of distilled water. The staining solution 2 was made by adding 15 ml of 5% aqueous phenol solution, 4.5 ml of 20% aqueous glacial acetic acid, 2 ml of 30% aqueous ferric chloride, and 0.75 ml of 1% aqueous solution of acid fuchsin.

Capsule Staining (9)**.** One side of the glass slide was flamed, and cooled to room temperature. One drop of 1% congo red solution was placed on the slide, and a loop of collected *E. coli* culture was placed on the congo red solution. The liquid was spread with an inoculation loop, and was air-dried. A few drops of staining solution 2 were added onto the dried smear. After 3 minutes, the slides were carefully rinsed with distilled water, blot dried and examined under oil-immersion microscope. Three cells were used to measure the relative thickness of the capsule as the ratio of capsule thickness to cell width. And ratio from a flask sample was compared with that of control.

RESULTS

Optimal concentration of sodium salicylate. Sodium salicylate concentration was determined in a separate experiment. 60 µg/ml sodium salicylate caused an observable reduction in capsular polysaccharide production without affecting bacterial growth and was thus chosen as the treatment concentration.

Turbidity Measurement of growth of *E. coli* B23 cells. All samples have undergone pre-treatment (PT) with kanamycin to induce capsule production and five test conditions were analyzed: Control (PT only), PT with sodium salicylate added at 0 min (PT + SS), PT with kanamycin (Kan) added at 60 min (PT + Kan

(60')), PT plus SS added at 0min and Kan added at 60 min (PT + SS + Kan(60')), PT plus SS and Kan added at 0 min.

Figure 1 displays the effect of sodium salicylate and kanamycin on cell growth of *E. coli* B23. The growth rate at log phase was obtained by measuring the slope of growth curves between 60 to 120 minutes. During log phase, the growth rate was identical for the growth conditions without or with sodium salicylate, as shown in figure 1 for growth rate of cells with control, and (PT + SS) respectively. The growth rates for (PT + Kan (60')), (PT + SS + Kan (60')), and (PT + SS + Kan (0')) were similar with 2% of difference between each other; however, they were lower than the growth rate for control and (PT + SS) by 20%. The growth rate for control and (PT + Kan (60')), which had no sodium salicylate treatment, did not alter till the end of the experiment period. The growth rate for (PT + SS) was

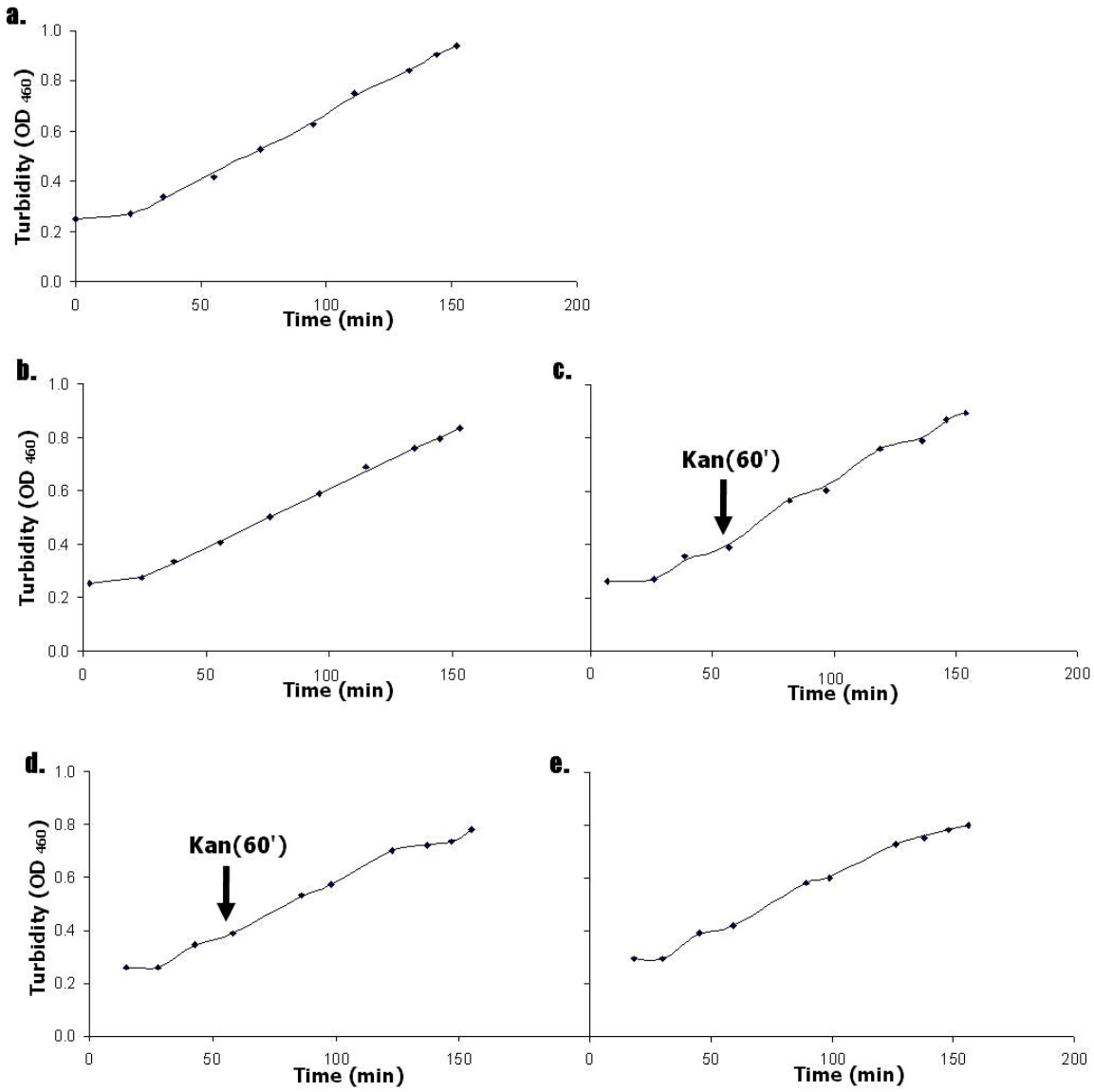
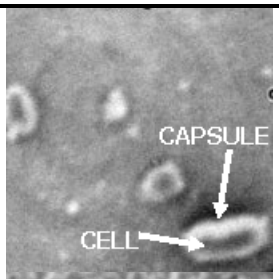
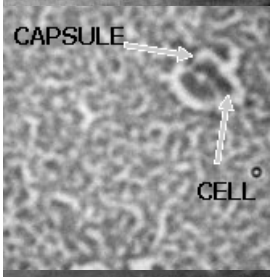
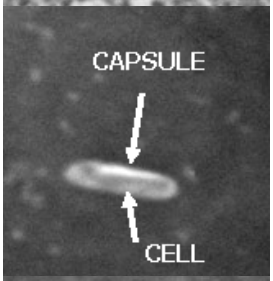
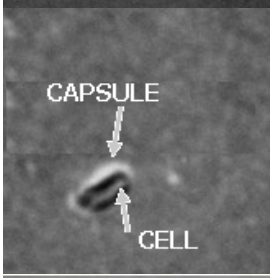
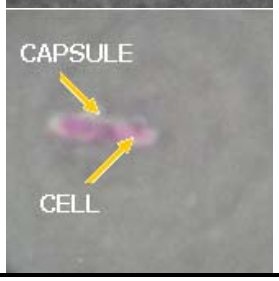


FIG. 1. Effect of kanamycin and sodium salicylate on the growth of *E .coli* B23. A) Pre-treatment (PT) only, Control, B) PT + sodium salicylate added at 0 min, C) PT + kanamycin added at 60 min, D) PT + sodium salicylate added at 0min and kanamycin added at 60 min, E) PT + Sodium salicylate and kanamycin added at 0 min.

TABLE 1. Effect of sodium salicylate and post kanamycin treatment on cell growth of *E. coli* B23 in LB and total capsular carbohydrate level at 80 minutes. The background was stained as grey, the cells were stained as red, and the capsules were clear, halo circles around the red cells.

Flask #	Cell growth conditions in LB^+*	Relative growth (%)**	Capsule staining picture at 80 minutes
1	Control (PT only)	100	
2	PT + SS	99	
3	PT + Kan (60')	95	
4	PT + SS + Kan (60')	92	
5	PT + SS + Kan (0')	95	

reduced after 120 minutes. Yet, more data points are needed to confirm if this curve actually started to flatten. After 140 minutes, the growth rate for (PT + SS + Kan (60')), and (PT + SS + Kan (0')), which had both sodium salicylate and kanamycin treatments but with at different time points, decreased gradually, while the slope of each growth curve was still parallel to each other.

Anthrone total carbohydrate assay and capsule staining. As shown in table 1, the relative growth was obtained by comparing the turbidity measurements from four test conditions with the turbidity measurement of control at 80 minutes. While the relative growth rates differed by 1% for control and (PT + SS) at 80 minutes, (PT + SS), (PT + SS + Kan (60')), and (PT + SS + Kan (0')) showed up to 8% of reduction in growth. The difference between the three conditions was approximately 2-3%. In contrast, control and (PT + Kan (60')) showed up to 3 times higher capsular carbohydrate concentration than the carbohydrate concentration of other three conditions.

The pictures in table 1 showed results of cell capsule staining method. As a result, the cells treated with sodium salicylate was 30% lower in capsule level than the control salicylate as shown in table 1 for control and (PT + SS). The highest capsule level was obtained from (PT + Kan (60')), which was without sodium salicylate treatment, while the lowest capsule level was obtained from (PT + SS + Kan (0')). The effect of sodium salicylate on capsular carbohydrate over time was confirmed by figure 2, which showed the relative total carbohydrate level from (PT + SS), (PT + Kan (60')), (PT + SS + Kan (60')), and (PT + SS + Kan (0')) were compared with the ratio of capsule thickness to width of cell from the control. Cells treated with sodium salicylate had a significant reduction in capsule production. In contrast, (PT + Kan (60')) showed increase in capsule level. Figure 2 also showed that (PT + SS + Kan (0')) had the most reduction in capsule level as its relative reduction at 80 minutes is 3 and 5 times higher than (PT + SS) and (PT + SS + Kan (60')) respectively.

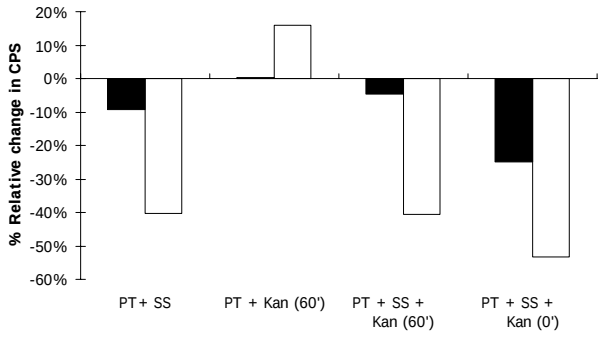


FIG. 2. Effect of a kanamycin pretreatment on *E. coli* B23 capsule concentration after a treatment with kanamycin and sodium salicylate on the CPS concentration compared to the control. Black bars refer to the samples collected at 80 minutes of incubation, and white bars refer to the samples collected at 120 minutes. PT refers to pre-treatment with kanamycin. SS indicates addition of sodium salicylate at time 0 min. Kan (0') indicates addition of kanamycin at 0 min. Kan (60') indicates addition of at 60 min.

DISCUSSION

Sodium salicylate has been shown to reduce capsular polysaccharide production in *E. coli* (2), but it was not clear whether effect was independent of bacterial growth. To evaluate the effects of sodium salicylate on growth, we grew *E. coli* B23 in Luria broth while manipulating sodium salicylate presence. We found that that the addition of 60 μg/ml sodium salicylate affects *E. coli* B23 growth rates only minimally after 120 minutes (Fig. 1), in line with findings by Pomposiello et al. (10). In addition, we show that 60 μg/ml sodium salicylate observably reduces the production of total capsular carbohydrate in *E. coli* (Fig. 3, flask 2). As table 2b shows, the thickness of the bacterial capsule was markedly reduced by sodium salicylate addition, while cell growth was generally unaffected. Together, these results suggest that sodium salicylate is an effective compound to use for the reduction of capsular polysaccharide production when growth independent manipulation of capsule levels is desired.

In order to observe conferred aminoglycoside resistance in *E. coli*, we subjected an *E. coli* B23 culture to sublethal levels of kanamycin and observed growth after a one hour incubation period. It was hypothesized that this incubation allowed bacteria to express the physiological changes needed to for antibiotic protection. Lebrun et al. have shown that cultures in the presence of sublethal antibiotic have altered bacterial growth kinetics, although killing is not observable on a macroscopic level (7), and we confirmed this in figure 1. Other studies have shown that sublethal levels of kanamycin stress bacteria to increase capsule production (2), which we confirmed in figure 2. Thus, antibiotic treatment served two purposes in our experiment- kanamycin pre-treatment increases capsule production to more easily detectable levels, while kanamycin's continued presence allowed us to observe whether aminoglycoside resistance was conferred as a function of capsular polysaccharide levels.

Suggestions have been raised that increased capsule levels protect *E. coli* from the effects of kanamycin (8). We tested this hypothesis by growing kanamycin pre-treated bacteria in the presence of sodium salicylate, which effectively reduced capsule, and then later adding additional kanamycin increasing antibiotic pressure. We expected to see slower bacterial growth in this condition, since capsular protection would be less available. This is indeed what we observed (Fig. 1). Figure 3 confirms that capsular polysaccharide levels were indeed reduced in cultures with sodium salicylate and kanamycin treatment. It appears that sublethal concentrations of kanamycin cause more pronounced inhibition of bacterial growth when sodium salicylate induced capsular polysaccharide production reduction

is present. From this, we propose that capsular polysaccharide confers aminoglycoside resistance in *E. coli*, and that a reduction in capsule levels caused the observed increase in antibiotic activity.

It is possible that the enhanced effect of kanamycin on sodium salicylate treated cultures was caused by an interaction of the two compounds, rather than an effect of sodium salicylate on the capsular polysaccharide. Any capsular knockdown that is seen could be a side effect unrelated to capsule conferred antibiotic resistance. To address this, we grew pre-stressed bacteria in media containing sodium salicylate and kanamycin, in flask 5. It was expected that any chemical interaction between the compounds would be initiated immediately, and that the effects, on growth, of the interacting compounds on the *E. coli* culture would be exposed by differential growth curves between flask 5 and flask 4.

The similarity of the growth curves generated for the culture co-incubated with sodium salicylate and kanamycin, and the culture incubated with sodium salicylate for an hour before kanamycin treatment (Fig. 1) suggests that any chemical interactions between sodium salicylate and kanamycin do not affect bacterial growth. In essence, we believe that growth-affecting interactions between sodium salicylate and kanamycin were minor in this experiment, and that the increased antibiotic effect of kanamycin was predominantly caused by reduced *E. coli* capsule levels.

However, it must be noted that other studies have suggested that some properties of salicylate are responsible for the potentiation of aminoglycoside susceptibility. As a weak acid, salicylate increases the membrane potential of cells at low pHs (1). Since the low membrane potentials limit aminoglycoside susceptibility, salicylate would facilitate kanamycin uptake by cells if the environment was acidic. This would enhance the observed effects of kanamycin in a capsule-independent manner, blurring the validity of the results obtained. Since we did not measure pH in our cultures, we can not be certain if the phenomenon reported by Aumercier et al. (1) applies in our case.

After 120 minutes, the growth rate of cultures treated with both sodium salicylate and kanamycin declined, while cultures treated with neither of the compounds, or only one of the compounds, did not. This suggests that an interaction between the two compounds may exist without observable effect on bacterial growth kinetics until 120 minutes have past. The nature of this interaction and its molecular effects on the cell are unknown, and warrant further investigation if sodium salicylate is to be used in bacterial growth media in concert with aminoglycoside antibiotics in the future. While it was small, the inhibitory effect of sodium salicylate on bacterial growth existed, implying that this method was not ideal for the study of capsular polysaccharide production on aminoglycoside resistance. In the future, it may be advisable to use *E. coli* strains genetically modified to produce reduced capsular polysaccharide, or strains modified to produce no observable capsule at all.

Our results suggest that the addition of 20 μg/ml kanamycin to an *E. coli* B23 culture in Luria broth (LB) slows bacterial growth, and that this slowing effect is intensified when bacterial capsular polysaccharide production is prevented through the addition of 60 g/ml sodium salicylate. These results suggest that reduced capsular polysaccharide levels contribute to a decrease in aminoglycoside antibiotic resistance.

FUTURE EXPERIMENTS

To overcome the uncertain synergistic effects of kanamycin and sodim salicylate, it would be helpful to repeat the experiment using an agent that reduces capsular polysaccharide production without affecting membrane permeability in any way. Should such an agent be used, it would allow for a much more reliable understanding of capsular contribution to aminoglycoside resistance. Unfortunately, thorough searches of the literature have not revealed to us an agent that reduces capsular polysaccharide without affecting membrane permeability. It is known that the chelating agents, ethylenediamine tetra-acetic acid and ethylene bis-tetraacetic acid have capsular polysaccharide reducing properties similar to that of sodium salicylate, making these compounds possible alternatives. However, these positively charged molecules may bind the negatively charged cell wall, reducing the cell wall's ability to interact with positively charged aminoglycoside antibiotics to prevent cellular entry. This possibility will have to be considered if such compounds are used.

It will also be important to examine the effects of higher, but sublethal antibiotic concentrations on cells with lowered capsule levels. These cells may show slower growth rates under higher antibiotic stress, giving stronger results that more clearly outline the role of capsule in aminoglycoside resistance.

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