Journal of Experimental Microbiology and Immunology (JEMI) Vol. 15: 90 – 95

Bacterial Plating is a Suitable Method for Determining the Effect of Alginate Lyase on *Pseudomonas aeruginosa* PAO1

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Many research efforts have attempted to elucidate the mechanisms by which biofilms confer resistance to antibiotic treatment; this is particularly relevant to opportunistic pathogens, such as *Pseudomonas aeruginosa*. In previous studies, treatment of *P. aeruginosa* PAO1 biofilms with alginate lyase was correlated with an increase in cell division based on colony counts observed from plate counting. Given that the alginate cleaving activity of alginate lyase could contribute to artificially increased colony counts, which may be more accurately attributed to better dispersal of biofilm cells, the reliability of using bacterial plating to assay alginate lyase-treated biofilms was investigated. Physically disrupted alginate lyase-treated cells were concurrently treated with a reversible cell division inhibitor, sodium azide, for four hours. Plate counts were taken at regular intervals to monitor changes in cell counts. Alginate lyase-treated cells were observed to enter exponential phase growth sooner than untreated cells, resulting in higher initial plate counts. However, no difference in plate counts was observed between cells treated with alginate lyase and sodium azide, compared to sodium azide alone, thus ruling out enhanced dispersion of biofilms as the reason for increased counts. These results suggest that plate counting is a sufficient method for discerning increased cell division of *P. aeruginosa* PAO1 biofilms as induced by alginate lyase. Additionally, liberation of cells from the biofilm matrix by this enzyme points to increased growth rate likely due to their release from the biofilm, and subsequently, increased susceptibility to killing by bacteriostatic antibiotics compared to physical disruption.

Pseudomonas aeruginosa is the causative agent of many nosocomial infections. As such, much work has focused on studying its ability to form and maintain biofilms, which confers enhanced resistance to killing by both immune cells and antibiotics (2, 5, 14). Studies of *P. aeruginosa* PAO1 biofilms have established the role of alginate, a polysaccharide polymer of ß-1-4 linked D-mannuronic and L-guluronic acids, as a structural component of the biofilm matrix (7, 13). Alginate is known to confer protection against antibiotic killing, and the mechanism of this effect was investigated in a study by Duncan *et al*. (5, 6), which implicated the role of alginate lyase in inducing cell division in *P. aeruginosa* PAO1 based on plate count enumeration of cells (6). Furthermore, alginate lyase has been shown to cleave alginate polymers in the construction and deconstruction of biofilms, allowing for cell dispersal from the matrix (2, 7). The study by Duncan *et al*. proposed that the induction of cell division due to the liberation of cells from the biofilm matrix by alginate lyase increased susceptibility to bacteriostatic antibiotics known to work effectively on actively growing bacteria (6).

Plate counting is a well-established technique in microbiology and is commonly applied to enumerate *P. aeruginosa* and other bacteria (4, 5, 6). However, the reliability of this method depends on several factors, including the adequate dispersal of cells prior to plating and the assumption that each colony forming unit (CFU) is representative of a single cell that has undergone exponential rounds of binary fission to become a visible colony (11). Despite homogenization, cells contained within a biofilm are liable to clump; in treating cells with any enzyme or compound that encourages dispersal, increased liberation from biofilms may be mistaken for increased cell division.

In this study, we aimed to clarify the link between alginate lyase and increased cell division in *P. aeruginosa* PAO1 as suggested by Duncan *et al.* (6). We hypothesized that the effects of alginate lyase on the dispersal of biofilms may produce higher plate counts than untreated cells and that the reversible cell division inhibitor, sodium azide, could be used to distinguish the effect of alginate lyase induced cell division from alginate lyase induced dispersal by plate count enumeration. The results of this investigation have shown that alginate lyase increases cell division in comparison to untreated cells, and this result is important because alginate lyase-enhanced cell division may increase biofilm susceptibility to antibiotic

treatment, as proposed by Duncan *et al.* (6).The results also provide validation for the use of the common methodology of bacterial plating in investigating the effect of alginate lyase on *P. aeruginosa* biofilms.

MATERIALS AND METHODS

Bacterial strains, media and chemicals. *P. aeruginosa* PAO1 was obtained from the MICB 421 Culture Collection in the Department of Microbiology and Immunology at the University of British Columbia (8). For both routine culturing and experimental assays, cells were grown in tryptic soy broth (TSB) (EMD, Cat. # 361021348) at 37 ºC with shaking at 150 rpm, and cell plating was carried out on tryptic soy agar (TSB with Select Agar, Invitrogen, Cat. # 30391-023). For the inhibition of *P. aeruginosa* PAO1 growth, sodium azide (BDH, Cat. # B30111), tetracycline (Sigma-Aldrich, Cat. # T-3383) and chloramphenicol (Sigma-Aldrich, Cat. # C-0378) stock solutions were prepared according to the manufacturer's protocols. Alginate lyase from *Flavobacterium sp.* (Sigma-Aldrich, Cat. # A1603) stock solution was prepared immediately prior to use according to the manufacturer's protocol.

Active, reversible *P. aeruginosa* PAO1 cell division inhibitor assay. Cells were harvested from overnight cultures of *P. aeruginosa* PAO1 by centrifugation at 18,900 x g for 15 min. The pellet was resuspended in 7 ml of supernatant from the overnight culture and homogenized with a 7 ml Pyrex dounce homogenizer with its corresponding pestle for 2 min using a push/twist motion, and homogenized cultures were resuspended into 5 ml cultures at an OD_{600} between 0.1 and 0.2. Inhibitors were then added to each culture at one of the following concentrations: sodium azide, 0.02% or 0.03% (w/v); tetracycline, 8 or 16 ug/ml; chloramphenicol, 64 or 128 ug/ml, or left untreated as a control. Cultures were incubated for 4.5 hr with the turbidity monitored at 30 min intervals. Inhibitors were then removed by centrifugation at 8500 x g for 15 min, and the pellets were then resuspended in 5 ml of fresh TSB, and incubated for an additional 60 min.

Biofilm preparation, isolation and physical homogenization. Biofilms were cultured and prepared according to the protocol outlined by Duncan *et al.* (6). Briefly, 1:100 dilutions of *P. aeruginosa* PAO1 overnight culture were prepared in 16 x 125 mm test tubes. Mature biofilms were obtained by growing these cultures for an additional 5 days. Isolation and homogenization were done as described by Duncan *et al.* (6) with slight modifications. Briefly, each mature biofilm was divided into two 1.5 ml microfuge tubes and centrifuged for 15 min at 16,100 x g. Biofilms in the supernatants were poured onto a 47 mm, 0.45 micron metrical membrane filter and vacuum filtered. The biofilms on the filter were washed with TSB in between the addition of each tube of biofilm. Filtered biofilms were pooled and homogenized in a 7 ml Pyrex dounce homogenizer with its corresponding pestle for 2 min using a push/twist motion, and resuspended in fresh TSB.

Alginate lyase and sodium azide treatment of cultures. Homogenized cultures were divided into four equal volumes to serve as the following four treatments: negative control, alginate lyase (20 units/ml) treatment, sodium azide (0.03% (w/v)) treatment, and alginate lyase + sodium azide treatment. Each treatment was then divided into nine 500 ul aliquots to minimize the effects of potential biofilm reformation, and then incubated for 4 hr.

Quantification of viable cells in the biofilms. Aliquots from each treatment were used for plating at 30 min intervals. The removed cultures were homogenized with disposable pestles (Nalge Nunc International, Rochester, NY) for 1 min using a push/twist motion. Each homogenized culture was then serially diluted in TSB to two or more of the following final plated dilutions: 10^{-6}, 10^{-7}, and 10^{-8}. Samples were plated in duplicate, and plates were incubated simultaneously in a 37 ºC incubator at the end of the 4 hr time course experiment. Colony counts were recorded after approximately 16 hr of incubation.

RESULTS

Determination of a suitable growth inhibitor. In order to determine which inhibitor would be best suited for subsequent assays, the growth of planktonic cells in the presence - of each inhibitor, and after the removal of each inhibitor was examined. In this initial test, the determining factors for an appropriate cell division inhibitor were that it was fast-acting and it was reversible. The concentrations chosen for each inhibitor (sodium azide, tetracycline, chloramphenicol) were based on the MIC values determined in previous studies . (1, 9, 10) There was a significant difference between the growth of the untreated sample and the growth of samples incubated with various inhibitory agents throughout the 4.5 hour time course experiment, as indicated by Figure 1.

However, the degree of inhibition and recovery of cell growth after removal of the inhibitors varied with the use of each inhibitor. Although OD_{600} readings correlate with the amount of biomass in a given sample, an assumption was made so that increases and decreases in OD_{600} were interpreted as cell growth and cell death, respectively. As seen in Figure 1A, the addition of 0.02 % w/v and 0.03 % w/v sodium azide resulted in minimal increases in turbidity of 1.7-fold (0.190 to 0.325) and 1.5-fold (0.190 to 0.280), respectively, throughout the 4.5 hour time course. Comparing these results to the 3.8-fold increase in OD_{600} of the untreated sample indicates that the sodium azide treatment was successful in limiting the growth of *P. aeruginosa* cells. Furthermore, after the removal of the inhibitor at 4.5 hours, the cells appeared to have resumed growth at a rate comparable to the untreated sample; however, this conclusion was based on an extrapolation of the limited number of data points taken after the removal of inhibitors. Upon examination of the results, 0.03 % w/v sodium azide was chosen as the inhibitor to be used in conjunction with alginate lyase treatment of biofilm samples.

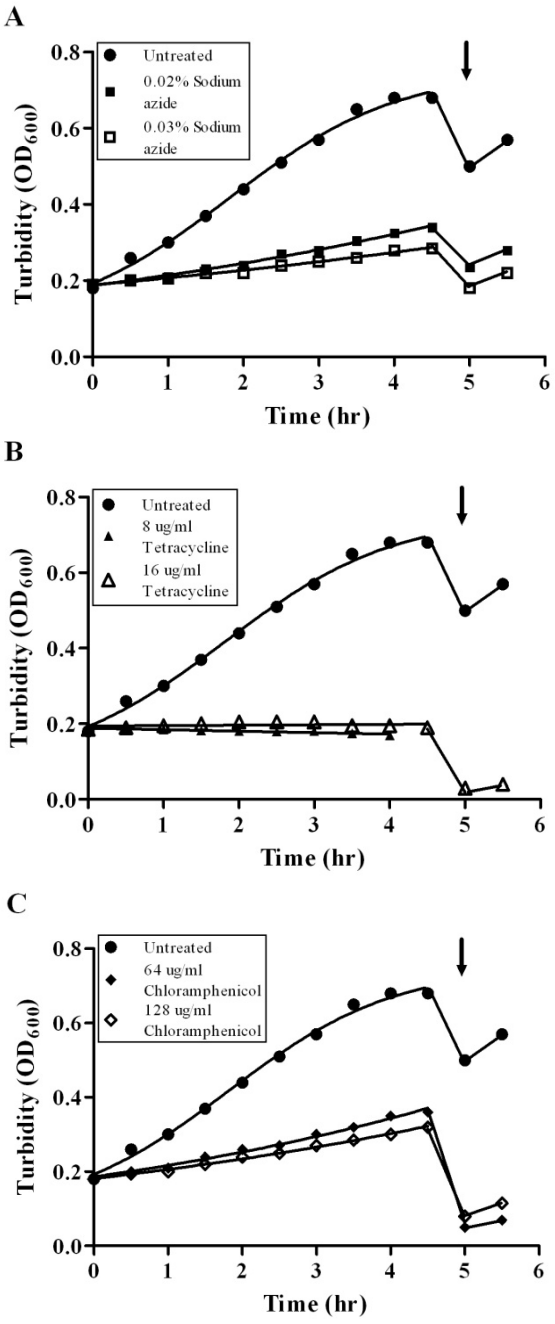

FIG. 1. The effect and reversibility of cell division inhibitors on the growth of planktonic *P. aeruginosa* PAO1. Cells were treated with (A) sodium azide, (B) tetracycline or (C) chloramphenicol for 4.5 hours and then inhibitors were removed by centrifugation (as indicated by the black arrow).

As seen in Figure 1B, the OD_{600} readings of samples treated with 8 ug/ml or 16 ug/ml of tetracycline were maintained within the range of 0.170 to 0.190, and 0.185 to 0.195, respectively, throughout the 4.5 hour incubation period. The 8 ug/ml tetracycline treated sample was lost after the 4 hour point due to a mechanical error when transferring samples. Due to the low OD_{600} readings (0.030-0.040) at the 5.0 hour time point, it was observed that the cells did not recover after the removal of the inhibitor (Fig. 1B). The use of tetracycline appeared to have caused cell death and was therefore not a suitable inhibitor for the purpose of this experiment.

Treatment of samples with 64 ug/ml or 128 ug/ml of chloramphenicol (Fig. 1C) resulted in slightly increased OD_{600} readings by 1.9-fold and 1.7-fold, respectively, throughout the 4.5 hour incubation time. Even though the inhibitor appeared to be effective in reducing cell growth, the growth recovery was not optimal. After the removal of the inhibitors at 4.5 hours, the OD_{600} readings remained below the value of 0.100. Due to the poor recovery of viable cells with the use of this inhibitor, it was not deemed suitable for this experiment.

Effect of alginate lyase on homogenized biofilm cultures as measured by plate counts. In order to replicate the observation made by Duncan *et al.* of increased CFU counts in alginate lyase-treated culture compared to untreated culture, plate counts of homogenized biofilm cultures treated through time with 20 U/ml of alginate lyase were analyzed (6).

In Figure 2A, the alginate lyase-treated and untreated cultures both exhibited an exponential growth phase lasting for about 2 hours. However, the alginate lyase-treated sample increased by 13.9-fold while the untreated sample increased by 10.5-fold throughout the exponential growth period. In another independent trial (data not shown), plate count data from alginate lyase-treated and untreated samples revealed a similar difference in growth trends.

When comparing the various growth phases with respect to time (Figure 2A), the untreated cells appeared to have begun in lag phase and then grew exponentially after 1.0 hour, and proceeded to stationary phase at 3.0 hours. In contrast, the alginate lyase-treated sample was initially at the lag phase and entered exponential growth at around 0.5 hours and began to plateau at the 2.5 hour time point. The alginate lyase-treated and untreated samples appeared to enter exponential growth phase at different time points of the incubation period, which may be indicative of the activity caused by alginate lyase.

A comparison of the calculated specific growth rate between samples was made. The specific growth rate of alginate lyase-treated culture was 1.32 hr^{-1} while untreated culture was 1.18 hr^{-1}. The use of alginate lyase appeared to increase the cells' specific growth rate which may also be indicative of the effect of alginate lyase on biofilm cultures.

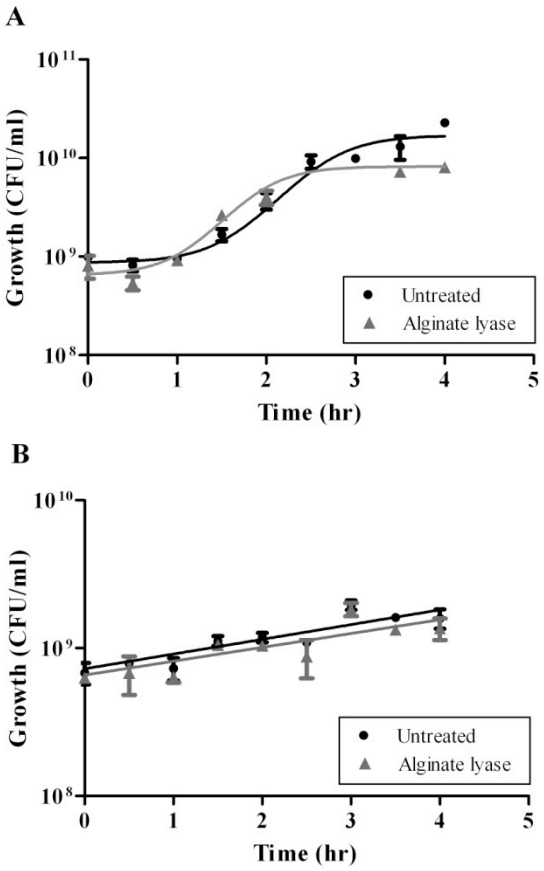

FIG. 2. Alginate lyase enhances the growth of physically homogenized *P. aeruginosa* PAO1 biofilms in the absence of sodium azide. Cells were treated with or without alginate lyase and grown in the absence (A) or presence (B) of 0.03% w/v sodium azide. Data points show the mean +/- SD of technical duplicates.

Effect of growth inhibitor on alginate lyase-treated and untreated, homogenized biofilms as measured by plate counts. Sodium azide as a cell division inhibitor was added to alginate lyase-treated and untreated biofilm cultures in order to elucidate whether the observed initial increase in plate counts was due to alginate lyase-enhanced cell division or alginate lyase-induced cell dispersal.

In Figure 2B, the CFU counts of the alginate lyase and sodium azide treated cells and the sodium azide treated cells did not increase significantly. The trendlines for both treatment conditions were similar and the cell concentrations at each time point were constant at approximately 10^9 CFU/ml. This data indicates the inhibitory effect of sodium azide on planktonic (Figure 1A) and biofilm cells (Figure 2B) was similar. Furthermore, an independent trial (data not shown) revealed a similar inhibitory effect of sodium azide on biofilm cells. Therefore, with the addition of a cell division inhibitor, the initial increase in plate counts in alginate lyase-treated samples compared to untreated samples was not observed.

DISCUSSION

The growth of untreated and alginate lyase-treated biofilms displayed a lag phase prior to exponential growth; however, the lag phase of the alginate lyase-treated biofilms was shorter than that of the untreated biofilms by approximately 0.5 hours. Furthermore, both the alginate lyase-treated and untreated biofilms grew exponentially for approximately 2 hours, but the cell concentration of the alginate lyase-treated biofilms increased 3.4-fold more than the untreated biofilms during the exponential phase. Thus, the alginate lyase-treated biofilms began to grow exponentially earlier than the untreated biofilms and exhibited a greater increase in cell concentration during the exponential phase. This suggests that the activity of alginate lyase initially contributed to the liberation of cells from the biofilm, which allowed cells to divide earlier and at a faster rate than cells within untreated biofilms. This result is in accordance with the fact that alginate lyase has been shown to increase dispersal of *P. aeruginosa* PAO1 from biofilms (2). Furthermore, this result is in agreement with the finding that cells detached from biofilms grew faster than cells present in biofilms due to limited nutrient and oxygen conditions present within biofilms (3, 12).

Moreover, the alginate lyase-treated biofilms entered stationary phase at approximately 2.5 hours post-treatment, whereas the untreated biofilms began to make the same transition at approximately 3 hours post-treatment. This difference may be due to the fact that the initial growth trends of the untreated and alginate lyase-treated biofilms were different. That is, the untreated biofilms may have entered stationary phase after the alginate-lyase treated biofilms as a result of their later entrance into exponential phase. Furthermore, the alginate lyase-treated biofilms did not enter stationary phase at the same cell concentration as the untreated biofilms possibly as a result of a difference in the initial cell concentrations of the two treatments. Although the initial cell concentrations were expected to be equal across treatments, due to the fact that the initial samples were split from a single culture of biofilms, the alginate lyase-treated biofilms displayed a slightly lower initial cell concentration. This may have been due to the fact that the single culture of biofilms was not completely homogeneous, and thus, there may have been variation between pipetted samples as a result of the absence or presence of conglomerations of cells.

To elucidate whether the shorter lag phase and greater increase in cell concentration during the exponential phase of growth in the alginate lyase-treated biofilms was a result of increased cell division or solely alginate lyase-induced cell dispersal, cell division was inhibited using 0.03 % w/v sodium azide, since it was found to reversibly inhibit cell division in planktonic *P. aeruginosa* PAO1 cells. As was similarly observed in planktonic cells, treatment of biofilms with 0.03 % w/v sodium azide resulted in a decreased growth rate in comparison to untreated biofilms. In addition, treatment of biofilms with 0.03 % w/v sodium azide and alginate lyase resulted in a decreased growth rate in comparison to alginate lyase-treated biofilms. Therefore, 0.03 % w/v sodium azide treatment of biofilms reduced cell division, as measured by CFU counts, similarly in the presence or absence of alginate lyase. This suggests that the alginate lyase-induced increase in growth rate was observable as a result of cell division, since the increase was not observed when cell division was prevented. Therefore, alginate lyase did not have an effect on CFU counts independent of cell division, and so, treatment of biofilms with 0.03 % w/v sodium azide and alginate lyase did not result in increased liberation of cells from the biofilm at a level that was observable by plate count enumeration. This result, in combination with the previously described ability of alginate lyase to liberate cells from the biofilm and subsequently allow cells to divide, indicates that an increase in cell concentration due to alginate lyase's ability to disperse cells was not discernable by plate count enumeration, but an increase in cell concentration due to dispersion of cells from biofilms by alginate lyase and the subsequent division of liberated biofilm cells was observable by plate count enumeration.

The results of this experiment are consistent with the finding by Duncan *et al*. that alginate lyase liberated cells from biofilms, resulting in increased cell growth in alginate lyase-treated biofilms (6). However, the findings of Duncan *et al*. were complicated as a result of uncertainty as to whether the observed increase in cell division in the presence of alginate lyase could have been due to increased cell dispersal by alginate lyase. This increased cell dispersal may have manifested as increased cell division due to the inability to distinguish between individual cells and clumps of cells by plate count enumeration.

Thus, the results of this experiment are important because they suggest that the increased CFU counts observed by Duncan *et al*. were due to alginate lyase-induced cell dispersal leading to cell division as opposed to alginate lyase-induced cell dispersal alone (6). Furthermore, the results of this experiment corroborate the suggestion made by Duncan *et al*. that alginate lyase treatment of *P. aeruginosa* PAO1 biofilms may increase antibiotic susceptibility, compared to physical disruption of the biofilm, as a result of the ability of alginate lyase to liberate cells from the biofilm into an environment in which they grow; this growth results in the susceptibility of biofilm cells to bacteriostatic antibiotics that target dividing cells (6).

However, the results of the current investigation should be interpreted with caution because although physical homogenization was performed prior to incubation and prior to serial dilutions, biofilm clumps were observed throughout the experiment. This suggests that physical homogenization did not fully disperse biofilms. In addition, biofilms could have been reforming throughout the incubation period, which could have resulted in conglomerations of cells being pipetted during the serial dilution procedure. This could have caused a misrepresentation of the number of cells present if physical homogenization did not produce homogenous samples, and thus, serial dilutions inconsistently resulted in the absence or presence of conglomerations of cells in the final sample that was plated. Furthermore, all plating experiments performed displayed some clumps and smears of colonies, which suggests that clumps of cells were present at the time of plating. Therefore, it is possible that the results were affected by clumps of biofilm cells and the inability to separate clumps and distinguish single cells.

The earlier exponential growth and increased growth rate of alginate lyase-treated biofilms in comparison to untreated biofilms demonstrates that alginate lyase increased biofilm growth by liberating cells into an environment conducive to growth, and that this growth was detectable by plate count enumeration. Furthermore, the abolishment of an alginate lyase-induced increase in growth rate when cell division was prevented by 0.03 % w/v sodium azide in alginate lyase-treated biofilms suggests that alginate lyase-induced cell dispersal alone was not detectable by plate count enumeration. Therefore, this study shows that plate count enumeration is a suitable method for determining the effect of alginate lyase on cell division in *P. aeruginosa* PAO1 biofilms.

FUTURE DIRECTIONS

As a result of the clumping of biofilm cells observed during the current study, future investigations could utilize a different method to validate the work completed. This could involve the use of a cell division tracker dye, such as carboxyfluorescein diacetate succinimidyl ester, to confirm the role of alginate lyase in *P. aeruginosa* PAO1 cell division as it can qualitatively by fluorescence microscopy and

densitometric programs, measure cell division over time. If alginate lyase increases cell division by increased cell dispersal, as proposed, the employed cell division tracker dye would decrease proportionally in intensity throughout the exponential phase of growth. In addition, fluorescence microscopy would reveal a greater proportion of individual cells as well as microcolonies, compared to untreated biofilms, due to increased dispersal by alginate lyase. On the other hand, if the results of the current study were a manifestation of errors resulting from clumping and alginate lyase does not truly increase cell division, the employed cell division tracker dye would neither decrease nor increase in intensity throughout the experiment; however, fluorescence microscopy may still reveal a greater proportion of individual cells and microcolonies due to dispersal. This experiment may also confirm the sufficiency of plate counting for measuring the effects of alginate lyase on cell division.

ACKNOWLEDGEMENTS

This work was generously funded by the Department of Microbiology and Immunology at the University of British Columbia. The authors would like to thank Dr. William Ramey and Matt Mayer for their support and suggestions in the execution of this experiment, as well as the Wesbrook Media room staff for their help

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