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Growth Inhibitory Levels of Salicylic Acid Decrease *Pseudomonas aeruginosa fliC* Flagellin Gene Expression

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Pseudomonas aeruginosa **is an opportunistic pathogen that forms recalcitrant biofilms. It has previously been observed that biofilm formation in *P. aeruginosa* can be attenuated by the addition of salicylic acid (SA). As cellular motility is involved in biofilm formation, this study investigated whether salicylic acid could affect the expression of *fliC* which produces flagellin, an important component of the flagella required for motility. Turbidity measurements and luminescence assay conducted on parent strain *P. aeruginosa* PAO1 and the derivative H1001 strain containing a *fliC*::*luxCDABE* reporter construct showed stasis in turbidity and a significant decrease in substrate-dependent luciferase activity when treated with 50 mM salicylic acid. Phase contrast microscopy observation also correlated the loss of motility in the *P. aeruginosa* PAO1 and H1001 strain with SA treatment at 50 mM. These results suggest that the salicylic acid may inhibit motility and cell growth by decreasing *fliC* expression.**

The aggregation of bacteria into coordinated biofilms offers protection against external stresses such as lack of nutrients or antimicrobial agents (9). For *Pseudomonas aeruginosa*, growth as persistent and recalcitrant biofilm on both biotic and abiotic surfaces is of great concern to modern health care in terms of nosocomial infections (5, 2, 7). Thus, elucidating the biological mechanisms regulating *P. aeruginosa* biofilm formation would prove to be invaluable as it could potentially allow for the design of novel anti-biofilm products, reducing persistent infections. Salicylic acid (SA) is an anti-inflammatory drug that has previously been demonstrated to have the ability to reduce the formation of biofilms by *P. aeruginosa* yet the exact mechanism remains to be elucidated (4). In previous studies, microarray analysis has suggested that the sub-inhibitory concentrations of SA induced the downregulation of multiple virulence factors in *P. aeruginosa* (10). Likewise, culturing *P. aeruginosa* 6294 in sub-inhibitory levels of SA reduced the production of virulence factors associated with microbial keratitis as well as affecting the twitching and swimming motility (1). In another study, SA showed a similar effect in modulating the swimming, twitching, and swarming abilities of *P. aeruginosa* to decrease biofilm production, possibly through the impairment of the flagellum (3). Although the components of flagellum are not usually considered as classical biofilm forming matrix molecules, they are involved in the formation of the mushroom-shaped multicellular structure that is indicative of mature biofilms (8).

A potential explanation for the inhibitory effect of SA in motility and biofilm formation on solid surfaces is that it may downregulate the expression of flagella genes by affecting their transcriptional activity. Since in *P. aeruginosa,* flagellin produced from *fliC* expression forms the filament on the single polar flagellum of the bacterium, it is possible that *fliC* transcription may be reduced by the presence of sub-inhibitory concentrations of SA described in previous studies that were conducted with overnight culture on solid medium (3). In order to investigate whether salicylicylic acid affects the expression of flagellin gene *fliC* in *Pseudomonas aeruginosa*, a *P. aerugionsa* H1001 strain containing a *luxCDABE* gene cassette under the control of the *fliC* promoter was used to test the expression of *fliC* (6) by measuring relative luminescence. By subjecting *P. aeruginosa* to treatments of SA at varying concentrations, SA negatively affected the kinetics of *P. aeruginosa* cell turbidity and the relative luminescence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 BW25113 (Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lambda-, rph-1, Δ(rhaD-rhaB)568, hsdR514) was obtained from MICB 421 culture collection from the Microbiology and Immunology Department at University of British Columbia. Strains of *P. aeruginosa* PAO1 and *P. aeruginosa* H1001, a PAO1 derivative containing a construct of *Tn5-luxCDABE* transposon insertion into *fliC* gene was provided by R.E.W Hancock Laboratory (6).

Growth conditions. Cultures were grown on Luria-Bertani (LB) agar (1 % tryptone (w/v), 0.5 % yeast extract (w/v), 1 % sodium chloride (w/v) and 1.5 % agar) plates at 37º C, mildly aerated and stored at 4º C for subculture. For all experimental conditions, each

overnight culture was grown in LB media at 37º C in Excella E24 air shaker (New Brunswick Scientific), aerated and shaken at 200 rpm for 20 hours. Overnight PAO1 cultures were seeded into 50 mls of fresh LB media in a 125 ml Erlenmeyer flask at 0.2 OD_{600}. Overnight H1001 cultures were diluted to 300 ml fresh LB media at 0.2 OD_{600} and then split into four subcultures of 50 ml in 125 ml Erlenmeyer flasks. The seeded morning cultures were incubated in a shaking Gyrotory Water Bath Shaker (New Brunswick) at 200 rpm and 37° C.

Chemicals. Salicylic acid stock solution (SA) was prepared 1 hour prior to use by dissolving sodium salicylate (Sigma, Cat. No. S2679-100G) in distilled water at 1 M.

Growth curve determination and luminescence assay. OD_{600} reading (measured by DU Series 500 Beckman Spectrophotometer) was measured at t = 0 minutes for each subculture to standardize starting experiment turbidity to 0.2 OD_{600} units. Measurements were taken at an interval of 10 minutes until t = 60 minutes. SA solution was then added to *P. aeruginosa* H1001 cultures to obtain final concentrations of 10 mM, 25 mM and 50 mM. After the addition of SA, turbidity was measured every 10 minutes until t = 120 minutes, and then every 30 minutes until t = 210 minutes.

Luminescence of each culture was measured concurrently with turbidity readings using the TD-20/20 Luminometer (Turner Biosystems) at an integration time of 5 seconds. The linear range of the machine was tested using a serially diluted H1001 culture and was determined to be linear in the 10-1000 luminescence unit (LU) range. Luminescence of undiluted PAO1 culture was measured and cultures of H1001 in the presence or absence of SA were measured at 1/100 dilution in LB. Relative Luminescence Unit (RLU) was calculated using measured LU normalized to cell mass in turbidity.

Phase contrast microscopy. At t = 0 minutes, PAO1 and H1001 cells were cultured in the presence or absence of SA at various concentrations (Table 1) in LB media shaken at 200 rpm (Gyrotory Water Bath Shaker, New Brunswick) at 37° C. Samples were then taken at t = 0 minutes and t = 180 minutes to be observed on wet mount slides using the phase contrast microscope at 400 x magnification (Zeiss Axiostar plus).

Statistical analysis. Student's t-test was used to analyze statistical differences between *P. aeruginosa* H1001 subjected to various concentrations of SA treatments.

RESULTS

SA exhibited a dose-dependent inhibition on *P. aeruginosa* growth and at 50 mM decreased relative luminescence of *P. aeruginosa* H1001 strain (Fig. 1). *P. aeruginosa* cell mass turbidity was measured as a control to determine whether SA had any effect on luminescence due to any growth modulating effect it might have had. As shown in Fig. 1, during the first 60 minutes prior to the addition of SA, the *P. aeruginosa* growth showed a similar trend in all cultures. After the addition of SA, a decrease in both growth (Fig. 1A) and luminescence (Fig. 1B) was observed in a dose dependent fashion that became apparent at both 50 mM and 25mM. When SA was added to H1001 cultures during the exponential phase, a differential response was observed for the different treatment conditions. At 10 mM SA, there was no effect on turbidity or luminescence; however, there was a slower increase when 25 mM or 50 mM SA were added. The decrease in luminescence observed in 25 mM treatment correlated to a decrease in turbidity of similar amplitude. At 50 mM, a greater decrease in luminescence was observed when compared to changes in turbidity (Fig. 1A and B). This effect is better demonstrated in Fig. 1C,

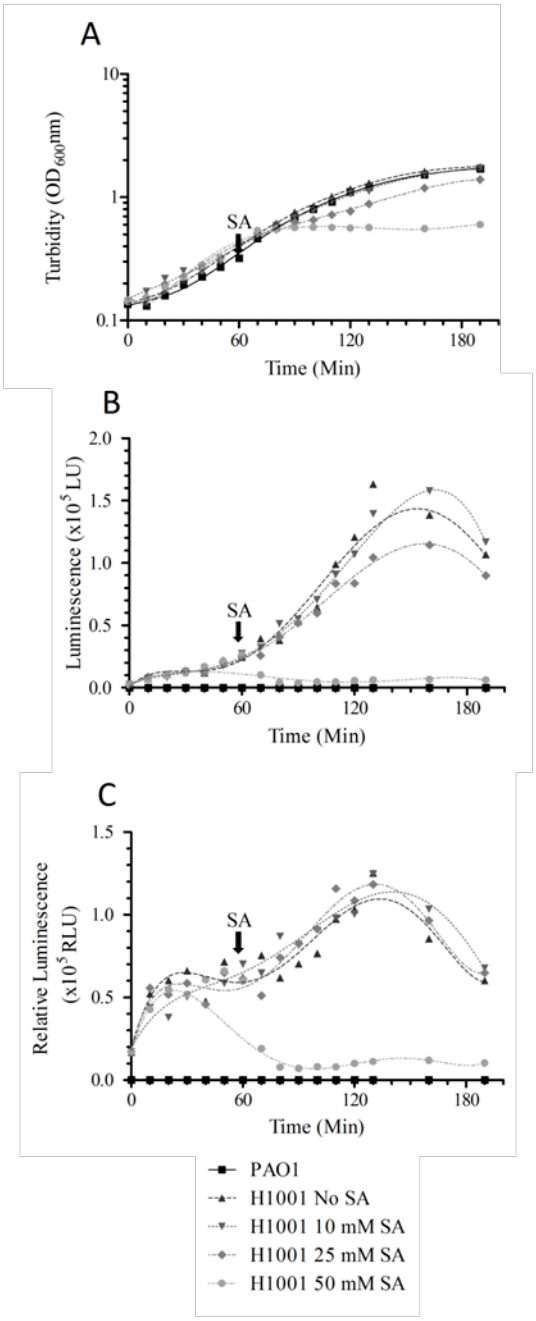

FIG. 1. Comparison of growth and luciferase activity from SA treatments in *P. aeruginosa*. The trendlines represent in (A) change in cell mass, (B) change in luminescence, and (C) change in relative luminescence. Data is representative of four independent experiments.

TABLE 1. Effect of SA treatments on *P. aeruginosa* swimming motility.

Sample	Motility (Swimming)	
	T = 0 minutes	T = 180 minutes
PAO1 no SA	Yes	Yes
PAO1 + 50 mM SA	n/a	No
H1001 no SA	Yes	Yes
H1001 + 10 mM SA	n/a	Yes
H1001 + 25 mM SA	n/a	No
H1001 + 50 mM SA	n/a	No

in which the relative luminescence curves were similar for all treatment conditions before the addition of SA, but a decrease of relative luminescence was observed only at 50 mM of SA. A peak in both luminescence and relative luminescence at t = 150 minutes were observed (Fig. 1B and C).

The collective results from four independent experiments are shown in Fig. 2. Fold changes of turbidity, luminescence and relative luminescence (RLU) were examined as total fold changes when RLU was at its peak values (t = 120 minutes) compared to when SA was added (t = 60 minutes). The RLU increases of H1001 cultures treated with SA, compared with the untreated culture, was half-fold higher at 10 mM and half-fold lower at 25 mM. However, these changes were not statistically significant. In contrast, at 50 mM, SA significantly decreased RLU ($p = 0.02$) and showed an average of 0.59 fold decrease whereas the control RLU increased by 0.60 fold (Fig. 3C). This dramatic difference was in part due to the fold change in turbidity in the 50 mM SA treated condition, which was 14% as much as the untreated control, and the luminescence fold change in which the treated culture showed a decrease (0.26 fold, $p = 0.43$) while the control experienced an increase (2.55 fold, $p = 0.21$). Together, these results show that there was a significant collective difference in RLU at 50 mM SA treatment only when compared to the untreated controls.

SA inhibited the swimming ability of *P. aeruginosa.* To investigate whether the reduction in accumulated luminescence translated into functional differences related to *fliC* expression, the swimming motility of *P. aeruginosa* PAO1 and H1001 cultures after SA treatment were examined. Although the flagellum itself could not be seen with phase contrast microscope at 400X magnification, the loss of swimming motility of cells was observed when the cultures were treated with SA at 25 mM and 50 mM

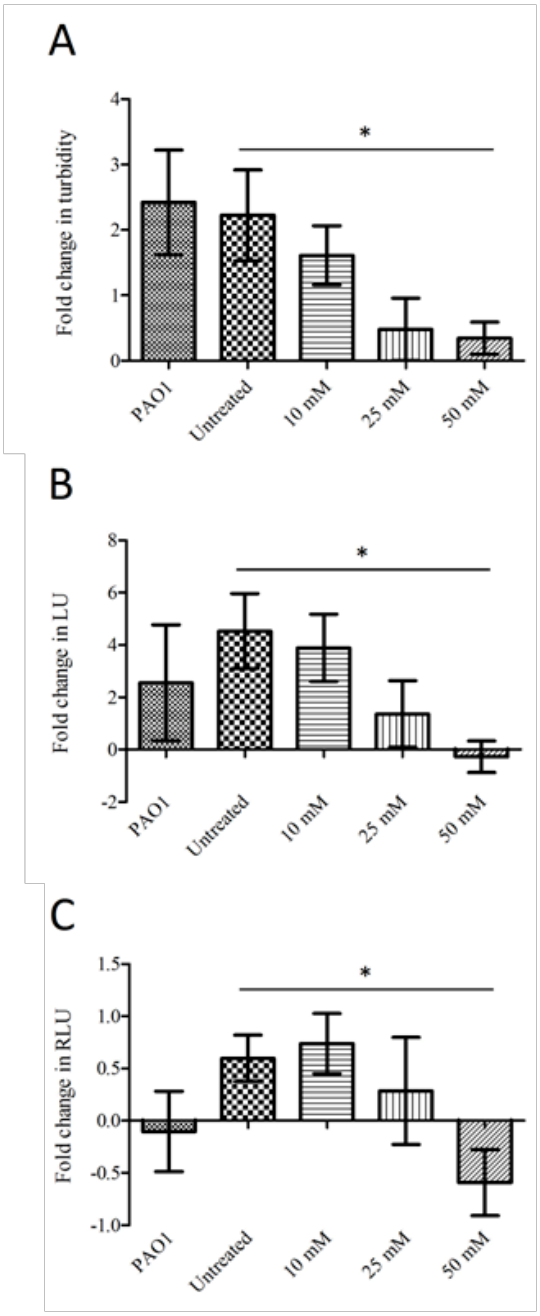

FIG. 2. Effect of SA treatments on *P. aeruginosa* growth and maximum luciferase activity. Average fold changes of (A) cell mass, (B) luminescence, and (C) relative luminescence, immediately before and one hour after SA addition. Mean and SEM were calculated from four independent experiments. Significance determined by unpaired t test, $p < 0.05$.

after 180 minutes of incubation (Table 1). This effect was observed in both PAO1 wild type strain and the H1001 reporter strain. These observations indicate the SA had a functional repercussion on cell motility at both 25 mM and 50 mM while a visible difference in RLU was only seen at 50 mM (Fig. 1 and 2) .

DISCUSSION

Previous studies have shown that SA disrupted biofilm formation; the purpose of this study was to investigate the effect of SA on the transcription of *fliC* gene and flagellin protein as a possible mechanism that contributes to this observation. As flagellar structure requires the flagellin protein, inhibition of motility observed in *P. aeruginosa* culture on solid medium in the presence of previously suggested sub-inhibitory concentrations of SA (3) may be caused by changing the expression in *fliC*. Our initial hypothesis is that SA at 25 mM and 50mM concentrations inhibit *fliC* expression by affecting gene transcription. *P. aeruginosa* H1001 strain containing the *luxCDABE* transposon insertion into the *fliC* gene was used as a model to test the effect of SA at different concentrations on the change in *fliC* expression. The *luxCDE* genes encodes for the fatty acid reductase complex that generates the substrates used by *luxAB* genes that encodes the luciferase complex to produce luminescence.

A trend involving a dose-dependent inhibition of growth on *P. aeruginosa* grown in liquid media as measured by cell mass in turbidity upon addition of SA was observed at 25 mM and 50 mM concentrations (Fig. 1), while a significant inhibition of growth was only observed when the culture was challenged with 50 mM SA (Fig. 1A). This observation may be due to the small number of replicas that were done (n = 4), or the existence of a threshold concentration of SA that has detrimental effects of *P. aeruginosa* growth. This partially supports previously reported results that SA is sub-inhibitory at less than 50 mM concentrations (3). However, our results that significant growth inhibition was observed at 50 mM SA during the first two hours of treatment in liquid condition conflicts with a previous study (3) in which 50 mM showed mild sub-inhibitory effect on growth on solid media. This indicates that the growth of *P. aeruginosa* is more sensitive to SA treatment in our experiment condition. Since *P. aeruginosa* form cell aggregates in liquid culture at 10-400 μm (11), the aggregation are not as resistant to higher concentrations of SA compared to cultures in solid media as dissolved SA may freely infiltrate more efficiently in liquid culture.

The RLU value was designed to reflect *fliC* transcription activity independent of growth. As shown in Fig. 1C, a significant decrease in RLU was only observed when *P. aeruginosa* was treated with 50 mM SA when compared with other treatments. This coincided with the growth inhibition also observed at 50 mM, indicating that SA may have more than one effect on *P. aeruginosa* when grown in liquid media. It should be noted that due to the limitations of the assay employed in this study, it was not conclusive whether the SA truly affected *fliC* expression, as the *luxCDABE* genes that were encoded in the *fliC* gene does not report instantaneous activation of the promoter. Instead, the *lux* genes encode proteins that produce the substrate for the luciferase complex and the luciferase proteins. As the proteins are made when cells are actively expressing *fliC,* even when transcription is turned off, the proteins made previously before the SA treatments that are stable in the cell can continue to produce light as long as the metabolic precursors to make the substrates are available. Therefore, the observed values in luminescence reflect the total accumulated *fliC* promoter activity.

When comparing the change in turbidity with change in RLU at 50 mM SA (Fig. 1A and C), it reveals that SA possesses a unique role in the context of *P. aeruginosa* growth and metabolism. Overnight stationary phase cells were diluted and cultured for 60 minutes to exponential phase before adding SA treatment, therefore the *lux* proteins previously made prior to the treatment should not be affected if SA inhibited growth or *fliC* expression and should remain active for the duration of our experiment. Given the nature of the reporter system, if SA inhibited *fliC* expression and thus the expression of reporter proteins, it was expected that the level of luminescence to remain at a constant level in the effective treatments of SA whereas the luminescence would increase in the control and at sub-inhibitory concentrations of SA. However, at 50 mM, the observed RLU decreased in parallel with the inhibition in growth measured by turbidity (Fig. 1). This unexpected observation suggests that SA may have an inhibitory effect on either the activity of the luciferase enzymes or the enzymes involved in the pathway that produces the substrates for the luciferase complex. Because the precursors used by *luxCDE* proteins to generate the luciferase substrate in *P. aeruginosa* are reduced flavin-mononucleotide ($FMNH_2$) and molecular oxygen (8), it is possible that SA affects certain metabolic pathways that involve $FMNH_2$ in the culture conditions. This explains why a sharp decrease was observed in luminescence in conjunction with inhibition of growth. A previous study

has showed that when metabolically limited, *P. aeruginosa* in biofilms disperse into single cells (11). If SA indeed inhibits metabolism, this could explain the mechanism in which biofilm formation was limited. The fact that luminescence reading is affected by the $FMNH_2$ and molecular oxygen level also explains why a decrease of luminescence and RLU at t = 150 minutes were observed in the experiment. This effect is possibly due to the consumption of nutrient and oxygen in the media during the growth in the first 150 minutes and the decrease reflects the depletion of nutrient and oxygen level as well as the FMNH2 in the cell.

Likewise, a conclusion cannot be made whether the effects of SA treatment at 10 mM or 25 mM on *fliC* expression are accurately represented by the luminescence observed, due to the limited sensitivity and consistencies in our replicates and measurements. The luminometer requires a hundred fold dilution of the sample for the values to fall within a linear range of the assay, and thus increases the range of error of the measurements. In addition, by adding the samples to fresh LB for dilution, the luminescence reading will change as the luminescence is dependent on available $FMNH_2$, which might be produced at a higher level by the cells in fresh media (6). This may have brought in errors to the measurement and potentially explain the insignificant dose-dependent trend observed in 10 mM and 25 mM treatments. However, it can be concluded that there is a correlation between *fliC* expression and SA treatment as the fold change at 50 mM treatment displayed a slight increase in turbidity and a slight decrease in RLU at 60 minutes after the addition of SA where RLU was maximum (Fig. 2A and C). This suggests that the effect of SA on the expression of *fliC* may be masked by its growth inhibitory effects. If protein synthesis is inhibited to allow complete turnover in the *lux* proteins in culture, followed by the removal of the protein synthesis inhibitor and addition of fresh media for renewed growth, the effect of SA on *fliC* expression versus growth may be determined by comparing the accumulation of luminescence and change in turbidity at different concentrations of SA.

The results from phase contrast microscopy show that at 25 mM and 50 mM SA, the swimming motility of the cells was abrogated compared to the untreated and 10 mM SA treatment. It is interesting that at 25 mM SA treatment, there was a similar blocking in swimming when compared to the 50 mM SA treatment but significant effects of SA on turbidity and RLU were not observed at 25 mM (Table 1, Figure 2). This is partially in agreement with previous study in that the inhibition effect on mobility of SA was observed even when growth is not significantly inhibited (3) and that the *luxCDABE* transposon insertion did not block *fliC* function. This observation suggests that SA may have a role in one or multiple aspects of the *P. aeruginosa* flagella such as transcription, translation, assembly, and/or function, yet the exact role of SA remains unclear. It may be possible that SA at threshold concentrations of 50 mM does inhibit *fliC* expression in addition to inhibiting metabolic activities of *P. aeruginosa* and reduces the total available energy in the cells. If the latter is true, then *fliC* expression may be turned off not due to a direct effect of SA inhibition on transcription, but rather due to a change in the transcriptome of the cell due to different energy demand and needs results of different environmental stress. Contrary to our findings, a previous report showed significant inhibition of motility at 25 mM of SA (3).This difference may be due to the slight differences in culture method and the preparation of SA solution in two studies or the inaccuracy in results caused by the small number of replicates in each study.

In this study, we observed that the growth inhibitory effect SA correlated with RLU at varying concentrations, with the effect being most significant at 50 mM treatment. Although the study could not differentiate the growth inhibitory effect of SA to effects it may have on *fliC* expression, the reduction in swimming motility using phase contrast microscopy suggests flagella impairment. Inhibition of *fliC* transcription by SA cannot be concluded nor excluded by the results of this study.

FUTURE DIRECTIONS

Due to the observation of SA induced growth inhibition, it is possible that SA may inhibit *P. aeruginosa* through metabolic means that is independent from biofilm formation at concentrations equal or greater to 50 mM. This could be confirmed by examining the total energetic activity such as the ATP level of *P. aeruginosa* cultures. If such is the case, it would be in agreement with the observation that SA inhibited growth and also swimming motility, both of which requires ATP. If reduced metabolic activities could be observed in the presence of SA, it would support the conclusion that SA may inhibit motility and biofilm formation through its inhibition effect on metabolism.

In addition, as our results suggest that SA may affect the activities of proteins involved in the luciferase pathway, it would be helpful if another reporter system can be used to study the transcription of *fliC* to avoid this problem. Instead of using *luxCDABE* reporter which produces its own substrate using cellular

metabolic product, *luxAB* reporter alone for example requiring exogenous substrate may be used to avoid the energetics variable of the assay. This could allow a measurement of gene transcription level independent of cellular metabolism.

Finally, the assay used in this study was limited for the purposes of examining the transcriptional activities of *fliC* when *P. aeruginosa* was treated with different concentrations of SA. Western blot experiments were attempted using a commercially available flagellin antibody against *Escherichia coli* flagellin to investigate whether there was a difference in flagellin protein levels in *P. aeruginosa* cultures treated with different SA concentrations. However, since the antibody was not specifically designed for *P. aeruginosa* flagellin, and the experimental conditions were not optimized, a specific band corresponding to *fliC* product was not successfully obtained in the study. If the experimental protocol and conditions were improved enough, it will be interesting to examine whether *fliC* expression changes translated into protein levels. As RLU results could not be interpreted independently from growth due to the accumulation of *lux* proteins, flagellin protein levels will be an independent measurement that navigates around this problem. If a decrease of flagellin protein level could be observed in the western blot in presence of SA, it would suggest a possible inhibition effect on *fliC* expression.

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