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Effects of Antibody Induced Localized Cell Crowding on Autoinducer-2 Levels in *Salmonella typhimurium* LT2

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Cell-cell communication in bacteria such as *Salmonella typhimurium* LT2 is mediated by quorum sensing. The mechanism involves the synthesis and subsequent increase in the extracellular concentration of molecules called autoinducers as bacterial population density increases. Effects on autoinducer levels caused by antibody induced localized cell crowding of *Salmonella typhimurium* in liquid culture were studied. The *Vibrio harveyi* bioluminescence assay system was used to measure the amount of autoinducer present in the cell-free supernatant of treated *Salmonella typhimurium* cultures. The data suggested that antibody mediated cell crowding caused a decrease in autoinducer level. This effect was consistent with artificial crowding experiments that did not utilize antibodies.

Approximately ten years ago, scientists studying *Salmonella typhimurium* gene expression discovered that genes expressed at high population density are different from the genes expressed when cell density is low (13). This observation led to the hypothesis that *S. typhimurium* employs a mechanism enabling the quantification of the number of cells in its immediate environment, similar to the quorum sensing phenomenon that was discovered over 25 years ago in *Vibrio fischeri* and *Vibrio harveyi* (6). Various species use this quorum sensing mechanism to regulate many of their physiological processes and gene expression (8), suggesting that a single bacterium behaves differently as an independent individual than as a member of a large population of the same species (23).

Quorum sensing involves the synthesis and secretion of small, unique, metabolically inexpensive signaling molecules called autoinducers (14, 15). Autoinducers (AIs) are generally classified into two categories: AI-1 or AI-2 (7). While AI-1 is theorized to be used for intraspecies communication (2), AI-2 is believed to be involved in interspecies signaling (1) since similar AI-2 molecules appear to be synthesized by a wide variety of organisms (17). *V. harveyi* expresses both the AI-1 and AI-2 quorum sensing systems (7). It has been suggested that there is a basal level of AI produced (16). As the population increases, the concentration of AI in the environment will increase proportionally (10). Once a critical level of AI is reached, *V. harveyi* cells upregulate a certain set of genes. One operon known to be regulated by quorum sensing is the *lux* operon, which encodes proteins involved in bioluminescence (3, 4). Because AI concentration is normally an indicator of cell density, higher AI concentration would lead to more luminescence (11).

Since AI-2 is involved in intercellular communication, *V. harveyi* can be utilized to indirectly measure AI-2 levels in other bacterial species. One well-studied organism that solely produces AI-2 is *S. typhimurium* (19). Mutant *V. harveyi* strains that are only capable of detecting AI-2, such as *V. harveyi* BB170, can be utilized as reporter strains to study the quorum sensing system in *S. typhimurium*. These reporter strains activate the *lux* genes in the presence of AI-2 produced by *S. typhimurium*, leading to increased luminescence (5). Utilizing this assay system, AI-2 levels of *S. typhimurium* have been shown to fluctuate in response to environmental conditions and culture growth phases (20). Increased AI-2 levels have been observed when *S. typhimurium* was grown in low pH, high osmolarity or preferred carbon sources (18). In this paper, induced crowding of *S. typhimurium* culture was studied to evaluate the effects of cell crowding on the level of AI-2 present in liquid culture. Based on the data, it seems that artificial crowding reduced the amount of AI-2 found in the culture supernatant.

MATERIALS AND METHODS

Bacterial Strains and Media. Strains of *S. typhimurium* LT2, *V. harveyi* BB170 and *V. harveyi* BB152 were obtained from Dr. William Ramey (University of British Columbia). *S. typhimurium* was grown in Luria-Bertani medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per litre) and where indicated, supplemented with 0.5% glucose. Luria-Marine media (10 g tryptone, 5 g yeast extract and 20 g NaCl per litre) was used to culture *V. harveyi*, while Autodinducer Bioassay (AB) medium (12) was used for the autoinducer assay.

Agglutination Test. A colony of *S. typhimurium* was mixed with a drop of water and a drop of antisera (Bacto Salmonella O Antiserum Poly A and B, Difco Laboratories) to determine which antisera contained antibodies specific for the LT2 strain.

Bacterial Growth Curve. An overnight culture of *S. typhimurium* LT2 was grown at 30°C with aeration (175 rpm). In the morning, the culture was diluted 1/50 in 3 mL of fresh LB media containing 0.5% glucose. At hourly intervals, this process was repeated over the course of 7 h.

Optical density (600 nm) readings were taken utilizing a spectrophotometer (Ultraspec 3000, Pharmacia Biotech). Antibody treated growth curves contained 0.1 mg of antisera A or B per mL of culture.

Antibody Treatments. As with the bacterial growth curve, *S. typhimurium* was inoculated overnight, and diluted 1/50 with supplemented LB media. To study the effects of varying antibody concentration, the diluted samples were incubated at 30°C with aeration for 4 h. At that point, the supernatant was removed, and fresh LB media with 0.5% glucose was added along with antisera A (0, 0.01, 0.1, 0.5 mg/mL of culture). To study the effects of antibody treatment on *S. typhimurium* at different growth phases, 3 mL aliquots of the diluted (1/50) sample were grown. Every hour, a sample was treated with 0.1 mg of antisera A or B per mL of culture, as described previously. For both experiments, the samples were incubated for 2 h after the addition of antisera. After incubation, cell-free supernatants were collected from the samples.

Cell Crowding. *Salmonella typhimurium* LT2 was grown overnight, as previously discussed for the bacterial growth curve. The cells were re-inoculated 1/50 in 3 mL LB media supplemented with 0.5% glucose, and incubated at 30°C with aeration for 4 h. The samples were centrifuged at 14,000 rpm, and the pellets were resuspended with 1, 2 or 3 mL of 0.4 M NaCl, and incubated at 30°C. At various times, the samples were removed and cell-free supernatants were generated.

Cell Free Supernatant. Optical density measurements at 600 nm were taken prior to collection. The remaining samples were centrifuged at 14,000 rpm for 5 min, with the supernatant filtered using 0.45 µm filters. The resulting cell-free supernatant was stored at –20°C until assayed.

Autoinducer Assay. *Vibrio harveyi* was cultured overnight at 30°C with aeration. The culture was diluted 1/5000 in the morning and used to assay the cell-free supernatant. Cell-free supernatant (150 µL) was added to 1.35 mL of diluted *V. harveyi* BB170 and incubated for 3.5 h. Similar procedures were followed for the positive control (cell free supernatant from *V. harveyi* BB152) and the negative control (cell free AB media). After incubation, the samples were analyzed using a luminometer (1250 Luminometer, LKB Wallac).

RESULTS

As reported earlier, *S. typhimurium* species have the ability to produce AI-2, which can be detected by *V. harveyi* (20). Figure 1A demonstrates autoinducer levels at various phases of the bacterial life cycle. There appeared to be little to no autoinducer present for the first 3 h of incubation, but autoinducer levels increased steadily for the duration of the experiment.

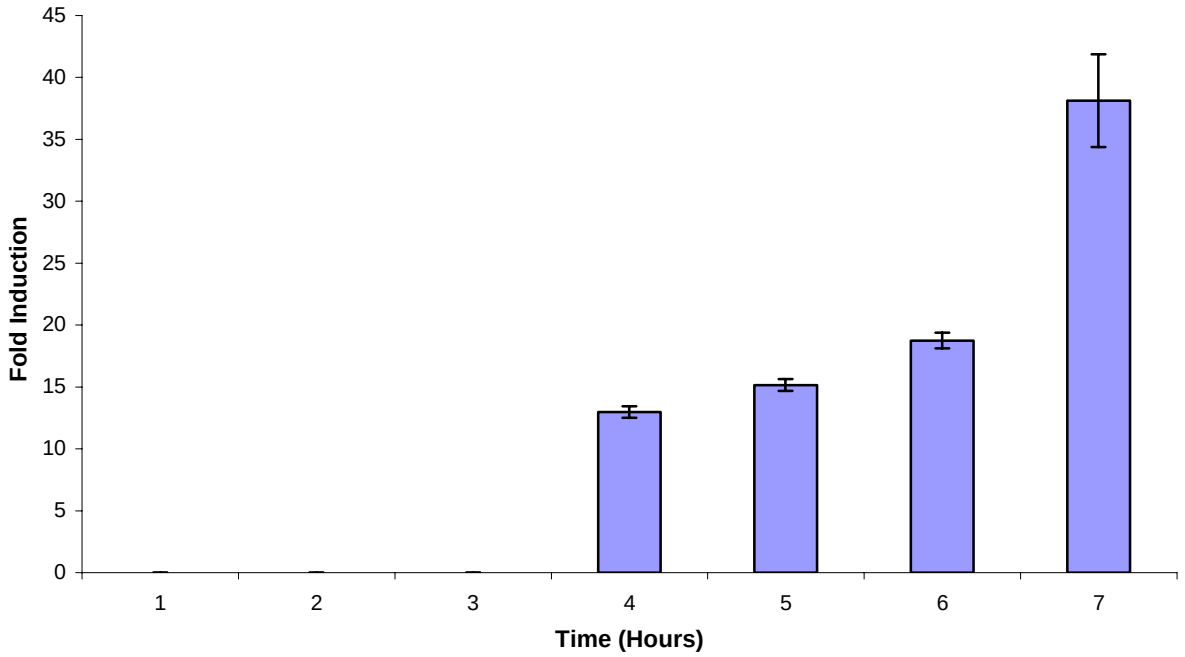

Figure 1A. Autoinducer levels of *S. typhimurium* LT2 at different phases of growth. Fold induction refers to the level of AI-2 present compared to the negative control (cell-free AB media). Error bars represent ±1 SD.

With the presence of AI-2 confirmed, the effects of antibodies specific to *S. typhimurium* LT2 were studied. Agglutination tests determined that antisera A was specific for strain LT2, as the cells visibly aggregated upon addition of the antisera (data not shown). Antisera B was also tested, but showed no agglutination properties.

By generating a bacterial growth curve over the course of 7 h, it appeared that antisera A treated *S. typhimurium* reached stationary phase at a cell density 1.5 times lower than the control (Fig. 1B). The exponential phase of these

cells also exhibited a shallower slope compared to the control. In contrast, the non-specific antisera (B) produced similar results as the control.

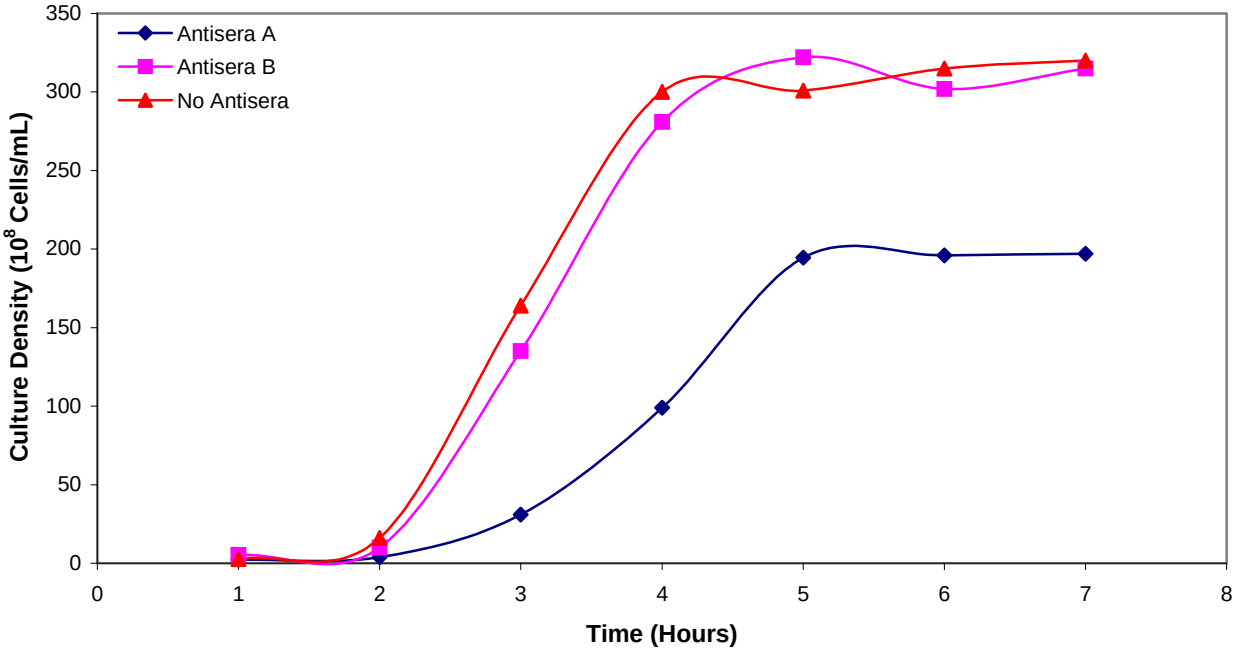

Figure 1B. Growth curve of *S. typhimurium* LT2 in the presence of specific antibody (antisera A) and non-specific antibody (antisera B)

The concentration of the specific antisera (A) was also tested to determine the consequence of altering the dosage of antisera A (Fig. 2). Antisera A was inversely proportional to AI-2, and AI-2 levels in the control sample (containing no antisera) were approximately 4 fold greater than the sample treated with 0.5 mg/mL of culture.

Experiments were also performed on *S. typhimurium* LT2 grown to different phases prior to the addition of 0.1 mg of antisera per mL of culture (Fig. 3). In this experiment, all samples were incubated for 2 h post antisera treatment. When treated with antisera A, the AI-2 levels at 4 h (mid-to-late exponential phase) was 3 and 5 times lower than the antisera B and the control, respectively. Due to uncertainty present in the results (as indicated by the error bars in Fig. 3), the difference between the two samples, control and antisera A, may not be as large as indicated. However, once the cells reached stationary phase (5 h), AI-2 levels of all three samples were relatively similar.

Specific antibodies can have various effects on a cell, including surface antigen binding and agglutination. To study the effects of cell crowding without antibody surface binding on AI-2, different volumes of 0.4 M NaCl were added to cells grown to mid-to-late exponential phase. Figure 4 depicts the samples suspended with 2 and 3 mL of NaCl undergoing an initial decrease in AI-2 levels. In each case, AI-2 levels surpassed the concentrations present with the sample suspended with 1 mL of NaCl. The 1 mL suspended sample remained unchanged and stable throughout the 2 h incubation. Other than the temporary decline in AI-2 exhibited at 40 and 80 min in the 3 and 2 mL conditions respectively, AI-2 levels in the 1 mL suspended sample were generally lower.

DISCUSSION

Prior to the antibody-based experiments, the *S. typhimurium* LT2 strain was tested to confirm the production of autoinducer-2 and to determine whether the *V. harveyi* bioluminescence assay functioned properly. Therefore, a growth curve experiment was performed to determine the autoinducer-2 levels of *S. typhimurium* at different phases of growth.

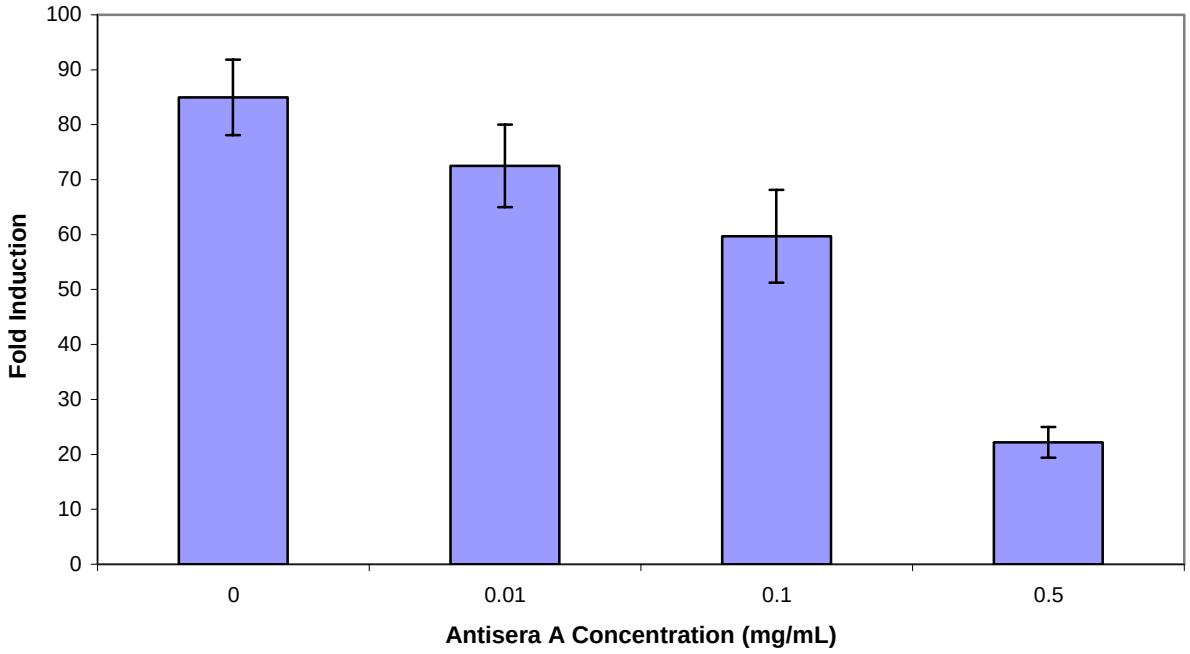

Figure 2. The effect of varying antibody concentration on AI-2 levels in *S. typhimurium* LT2 cultures. Error bars represent ±1 SD.

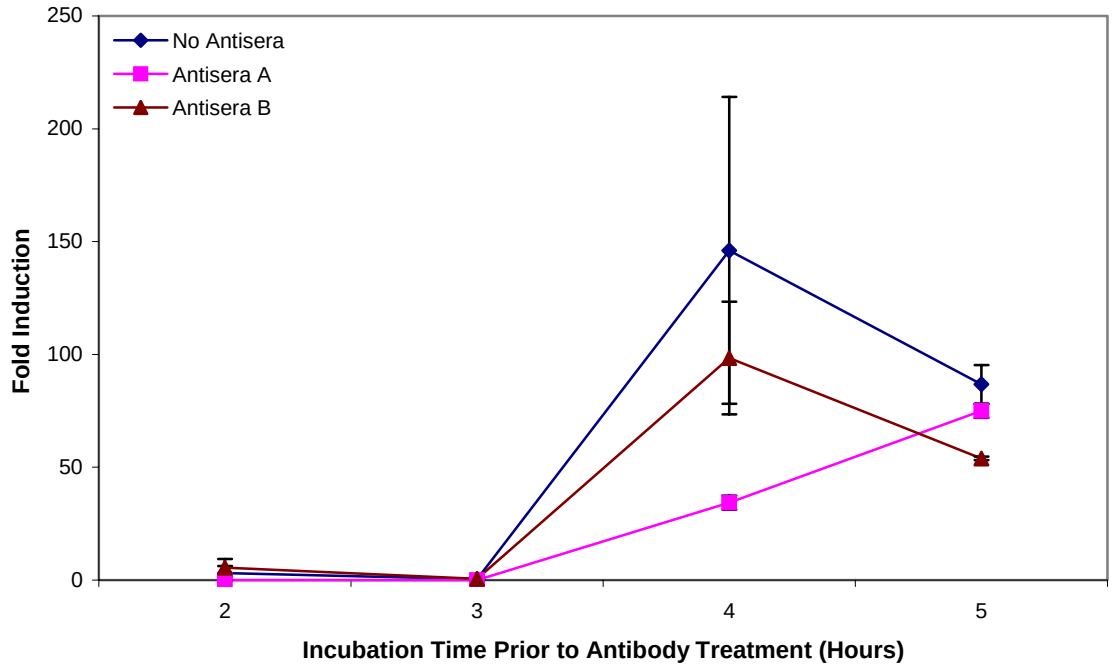

Figure 3. Effect on AI-2 levels caused by antibody treatment of *S. typhimurium* LT2 during different phases of growth. Error bars represent ±1 SD.

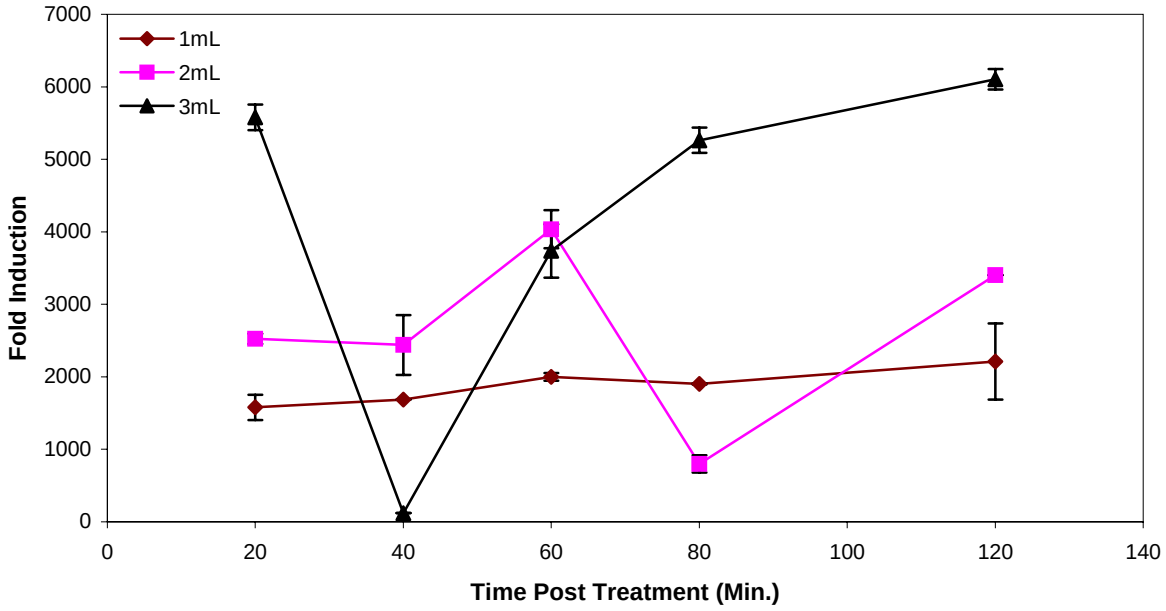

Figure 4. Autoinducer levels of *S. typhimurium* LT2 upon artificial crowding with 0.4 M NaCl. Error bars represent ±1 SD.

Salmonella typhimurium secrete and accumulate increasing amounts of autoinducer during different growth phases. As indicated in Fig. 1A, over a 7 h period, AI-2 levels increased to approximately 40 fold induction relative to the negative control (no AI-2). During the first 3 h after inoculation of *S. typhimurium*, the presence of AI-2 was not observed (Fig. 1A). The initial absence of autoinducer could be the result of the cells adapting to the fresh media. The inoculum was an overnight culture of cells that were in stationary phase. Once these cells were introduced to fresh LB broth, a lag phase ensued in the culture, as the cells adapted to the rich nutrient levels. The lack of AI-2 may have also been due to the fact that there was not enough time for the AI-2 to accumulate. *Salmonella typhimurium* may have been producing AI-2, but at levels which were initially too low to detect. In addition, while measuring luminescence, the luminometer settings for this experiment may not have been sensitive enough to detect the low AI-2 levels. The *V. harveyi* reporter strain has a minimal threshold level of AI-2 regulating the expression of the *lux* genes (3, 4). If *S. typhimurium* produced AI-2 at concentrations lower than the threshold, the AI-2 would not have been detected.

In Fig. 1A, a sudden increase in AI-2 levels was observed between the 3 and 4 h samples. At 3 h, the culture was entering exponential phase (Fig. 1B), and the increase in autoinducer may be attributable to the transition of the cells from lag phase to exponential phase. After 3 h, AI-2 levels steadily increased, doubling between the 6 and 7 h samples. This trend has been reported previously and is due to the fact that maximal levels of AI-2 occurs at mid-to-late exponential phase in the presence of glucose (19, 20). In this phase, the cell is metabolically most active. Additionally, compared to earlier time points, the cell density is higher, which naturally increases AI-2 levels. Although Fig. 1B indicates that stationary phase occurs at 6 h, recent reports have suggested that the exponential phase of *S. typhimurium* can extend up to 6 h post inoculation (19, 20). It has also been shown that AI-2 levels can remain relatively high in early stationary phase (19, 20). Thus, despite the seemingly contradictory data of Fig. 1A and 1B, it may be possible for AI-2 levels to remain high at 7 h post inoculation due to the variability of growth of *S. typhimurium*. However, AI-2 levels would likely decline at time intervals greater than 7 h (19, 20).

After determining the basal levels of AI-2 produced by *S. typhimurium*, the effects of specific and non-specific antibodies on growth were characterized. Antisera A, which binds to the O-antigen on the surface of *S. typhimurium*, is able to cause cell crowding, illustrated by the clumps observed in the agglutination test (data not shown). Antisera A appeared to inhibit the growth of *S. typhimurium*, while the non-specific antibody (antisera B) and control (no antisera) samples demonstrated similar growth throughout the 7 h incubation period. As discussed earlier, antisera A treated samples reached a lower cell density upon entering stationary phase and grew at a slower

rate (as depicted by the slope of the growth curve) in relation to the antisera B treated and control samples (Fig. 1B). This suggested that the presence of specific antibodies, which causes localized cell crowding and surface binding, negatively affected growth. However, the discrepancy between growth rates among the samples may have also been due to inaccurate OD_{600nm} readings and plate counts. From the agglutination tests, antisera A caused *S. typhimurium* cells to aggregate together. This aggregation may have underestimated cell density, particularly with respect to plate counts. For each colony counted, it may have represented an individual cell or several cells clumped together as a result of the antisera. Thus, although it appears as though antisera A inhibited growth, the magnitude of inhibition is unclear due to these potential sources of error.

From the introductory studies, it appeared that antisera A induced a negative effect on AI-2 levels. The effects of antisera A were further characterized by altering antisera concentrations. *Salmonella typhimurium* AI-2 levels were induced by incubating the cells for 4 h prior to the addition of antisera. As shown in Fig. 2, an increased concentration of antisera A resulted in a decrease in AI-2. It is possible that contaminants (eg. preservatives) in the unpurified antisera A stock solution may have caused an inhibitory effect on the growth of *S. typhimurium* or affected the levels of AI-2. At 0.5 mg of antisera A per mL of culture, 56.5 µL was added to the 3 mL culture (compared to 11.3 µL for 0.1 mg/mL of culture and 1.1 µL for 0.01 mg/mL of culture). The addition of 5 to 50 times more antisera may be a partial explanation for the decrease in AI-2.

Modification of the cell surface upon antigen binding by antibody and localized cell crowding are both possible causes for this decrease in AI-2. However, the discovery of an ATP binding cassette (ABC) transporter (Lsr transporter), which imports AI-2 into the cell, provides support for the latter. In the extracellular fluid, AI-2 is not degraded, as it has been shown to be stable in cell-free supernatant (18). Rather, AI-2 is eliminated from the supernatant through internalization by the Lsr ABC transporter (22). Recent reports determined that the Lsr transporter is upregulated in response to increased AI-2 (21). In this experiment, the decreasing levels of AI-2 with increasing antisera implied that a lower threshold AI-2 concentration was needed to activate the Lsr transporter in response to cell crowding. The concentrated antisera samples were able to express the Lsr transporter prematurely relative to those samples with less antisera (less cell agglutination). As a result, more AI-2 was eliminated from the extracellular fluid, resulting in the observed decrease shown in Fig. 2.

With the addition of increasing antisera, cell crowding was increased, forcing the cells to pack closely together. AI-2 secreted by one cell could be readily taken up by neighbouring cells. In contrast, without cell clumping, it may have required more time for AI-2 to encounter and be taken up by another cell. In essence, antisera A can increase the local concentration of existing AI-2. As a result, this higher local concentration of AI-2 induced the Lsr transporter prematurely, leading to a general decrease in AI-2 levels.

In addition to altering antisera A concentration, *S. typhimurium* was grown to various stages prior to a 2 h treatment with antisera. After the treatment, supernatant was collected and assayed utilizing *V. harveyi* BB170. As expected, antisera B and no antisera treatments exhibited maximal AI-2 levels at 4 h (Fig. 3), representing the mid-to-late exponential phase (Fig. 1B). As AI-2 levels increased, the Lsr transporter was upregulated (21), contributing to the decrease in AI-2 at 5 h.

Treatment with antisera A resulted in lower AI-2 levels (at 4 h), and a peak in AI-2 was not present in this sample. The absence of a peak suggested that there was no dramatic increase and subsequent decrease in AI-2 levels as observed for antisera B treatment and control (Fig. 3). This indicates that the premature induction of Lsr transporter upon antibody-mediated localized cell crowding, increased the uptake of AI-2, when the bacteria was at an earlier phase of growth. The subsequent increase in AI-2 levels for the antisera A treatment could be attributed to a general increase in the amount of AI-2 due to higher cell numbers and premature maximal induction of the Lsr transporter. In all likelihood, a decline in AI-2 would have been observed had more time points been collected due to changes in bacterial growth phase and metabolism.

Based on results from the previously discussed experiments, antisera A treatment affected AI-2 levels in *S. typhimurium* cultures. To determine whether the effect of antibodies on autoinducer levels is due to localized cell crowding, an experiment was performed to artificially induce cell agglutination. Similar to prior experiments, *S. typhimurium* was grown for 4 h to induce AI-2 expression. The supernatant was then removed and the cells were resuspended with varying volumes of 0.4 M NaCl. Resuspension with NaCl eliminated the nutrient variable, as the cells were prevented from growing in NaCl. Optical density readings of the samples over the course of the experiment confirmed the inhibition of growth (data not shown).

By utilizing 0.4 M NaCl instead of LB broth, cellular growth was no longer a factor that affected AI-2. An additional effect of high osmolarity treatment (using NaCl) was the amplification of AI-2 secreted by *S. typhimurium* (18). Under normal conditions, at such high AI-2 levels, the Lsr transporter would be expressed to compensate for the high extracellular concentration of AI-2. However, 0.4 M NaCl seems to interfere with the

effectiveness of this uptake apparatus, while simultaneously stimulating the AI-2 synthesis pathway (18). Despite this amplification effect, AI-2 levels still maintain a similar pattern, as AI-2 declines sharply after 120 min (18).

Although the concentration of NaCl was constant throughout the 1 mL, 2 mL and 3 mL trials, the absolute amount of NaCl differed. Also, each of the three trials had the same cell density (prior to NaCl treatment). As a result, the 3 mL samples had a higher ratio of salt to cells, relative to the 2 mL and 1 mL samples. For the 3 mL samples, the cells were exposed to higher osmotic pressure and as a result they had a sharp initial decline in AI-2 (Fig. 4). These results were similar to those published in recent reports, indicating that this was a reproducible phenomenon occurring as a result of osmotic shock (18). In the 2 mL samples, the effect of NaCl was delayed by 40 minutes, and the severity of the decrease was diminished. This may have been due to the lower ratio of salt to cells compared to the 3 mL samples. Despite the valleys in AI-2, both the 2 mL and 3 mL samples recovered and produced high levels of AI-2 by 120 min. In contrast to the 2 and 3 mL samples, the 1 mL sample had no visible decline in AI-2 levels. Since osmotic shock to the cell in the 1 mL sample was the lowest, an eventual decrease may have been observed had samples been taken from time points greater than 120 min.

In this experiment, artificial crowding was achieved by resuspending cells with similar density in less volume. In general, the 1 mL sample (representing the most crowded sample) maintained a consistently low level of AI-2, which was lower than either the 2 mL or 3 mL sample (Fig. 4). A comparison between Fig. 4 and Fig. 2 yielded similar results. By focusing on the 120 min samples in Fig. 4, it is apparent that increased crowding decreased AI-2. In Fig. 2, increased antisera A (which induces localized clumping) generated similar effects. Therefore, the results of the cell crowding experiment appear to lend support to the hypothesis that antisera A affects AI-2 levels by causing localized cell aggregation.

Throughout this study, the magnitude of autoinducer levels (expressed as folds of induction over the negative control) ranged widely from experiment to experiment. This inconsistency in autoinducer levels prevented comparisons of absolute amounts of AI-2 observed among the experiments. For instance, throughout our cell crowding experiment (Fig. 4), the 0.4 M NaCl solution contributed to the amplification of AI-2 in the three conditions by 1 to 2 orders of magnitude. It has previously been shown that high osmolarity leads to increased autoinducer levels by several orders (18). In addition, even among samples that were not treated with NaCl (Fig. 1A, 2 and 3), fold induction over AB media varied by 2 to 3 factors.

This difference in fold induction could be due to the inherent variability of the *V. harveyi* bioassay system (9). In particular, the presence of glucose in the cell-free supernatants can deter AI-2 detection. For *S. typhimurium* grown in 0.5% glucose for 6 h, half of the glucose remains in the sample. Traces of glucose are still present 20 h after inoculation (19). In this study, *S. typhimurium* was grown in 0.5% for a maximum of 7 h. At 7 h, slightly less than 50% of the glucose would be in the sample. Thus, it is likely that the detection of AI-2 had varying degrees of error depending on the amount of glucose remaining in the sample. This could explain the higher levels of fold induction observed in the artificial cell crowding experiment, where no glucose is present in the cell-free supernatant.

In conclusion, the presence of antibodies specific for *S. typhimurium* decreases the level of autoinducer-2, possibly through premature induction of the Lsr transporter. Both increasing antibody concentration and artificial crowding using 0.4 M NaCl treatments produced similar results. Thus, it appears as though cell crowding is the mechanism through which specific antibodies negatively affect AI-2 levels.

FUTURE EXPERIMENTS

One possible problem with antibody induced cell crowding would be that the effect could also be attributed to changes in cell membrane structure due to antibody binding. Therefore, a comparison of normal divalent antibody with cleaved monovalent antibody can be conducted to compare their effects on AI-2 levels. If autoinducer-2 levels do not decrease with the monovalent antibody treatment, then it will be further evidence to support the theory that antibodies regulate AI-2 levels via crowding. In addition, the use of heat-denatured antibody as a negative control can ensure that the effect observed is truly due to antibody cross-linking, rather than other components present in the antibody solution. More purified antibodies (eg. monoclonal antibodies specific for *S. typhimurium*) should also be utilized in an attempt to improve the consistency of the results.

Our data also suggests that localized crowding and the subsequent induction of the Lsr ABC transporter led to the decrease in autoinducer-2 (AI-2) levels in *S. typhimurium*. One future experiment that could be performed would be the use of a mutant *S. typhimurium* strain with a defect in the Lsr ABC transporter to evaluate whether antibody induced crowding leads to the same effect on AI-2 levels. If the Lsr ABC transporter is solely responsible for the reduction of AI-2 levels upon crowding, then this effect will not be seen in the Lsr mutant strain.

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