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The Effects of Altering Autoinducer-2 Concentration on Transfer Efficiencies of the F and RPI plasmids to the Quorum Sensing Recipient *Escherichia coli* Strain AB1157

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Many strains of bacteria constitutively produce and secrete autoinducers that allow populations to coordinate their behavior based on cell density. Previous research has shown that autoinducers are capable of up-regulating the expression of certain genes, outer membrane receptors, which are known to function in bacterial conjugation. *Escherichia coli* strain AB1157 is known to produce and respond to autoinducer-2 (AI-2). The purpose of this investigation was to examine the relationship between conjugation efficiency and increasing concentrations of AI-2. In this study we report a positive correlation between the conjugation efficiency of RP1 to AB1157 and increasing concentrations of AI-2, from 0% to 20% (v/v). This evidence supports the hypothesis that AI-2 provides a positive regulatory role in bacterial conjugation.

Bacteria use quorum sensing to regulate gene expression in response to cell density. This enables members of a population to coordinate their gene expression, and consequently the behavior of their entire community. Many physiological processes are subject to regulation by quorum sensing, including competence, conjugation, biofilm formation and virulence factor expression. Quorum sensing is widespread among bacteria, and many species rely on it to regulate multiple activities (7).

A key component of all quorum sensing systems is the autoinducer. The structures of autoinducers differ, but they are all small, water-soluble signaling molecules that are secreted into the extracellular space. Gram positive species generally use processed oligo-peptides, where gram negative bacteria rely on N-acyl-homoserine lactones. A third class of autoinducer was first discovered in the marine bioluminescent bacteria *Vibrio harveyi*. It is called autoinducer-2 (AI-2), and its structure remains unknown (7).

A previous study by Surette and Bassler showed that a signaling substance acts on *Escherichia coli* AB1157 to induce *lux* expression in *V. harveyi* through "Signaling System 2" (9). A second research group expanded on the findings of Surette and Bassler by examining the role of autoinducer-2 in *E. coli*. Using the techniques of Surette and Bassler, DeLisa and colleagues attempted to induce expression of AI-2 regulated genes in an F^- *E. coli* strain (2). These differences were detected using cDNA microarrays. Their experiments indicated that 5.6% of the *E. coli* genome exhibited significant transcriptional changes in response to increasing AI-2 concentration. Included in the upregulated genes was *ompA*, which encodes an outer membrane protein. It has been shown that the OmpA protein is required for the stabilization of F-mediated mating pairs in liquid culture. (1) The authors also demonstrated that the F-plasmid (IncF1) encodes the TraN protein in the donor, which interacts with OmpA on the surface of the recipient. Disruption of the *traN* gene drastically decreases the efficiency of conjugation suggesting that quorum sensing may affect the receptivity of recipients to F plasmid conjugation (6).

E. coli is also host to other conjugative plasmids, including the IncPα plasmid, RP1 (also known as RK2 and RP4). The probability of encountering possible mating partners increases with cell density, and up-regulation of conjugation machinery serves to increase the chance of successful transfer. Given recent findings suggesting a connection between F plasmid transfer and quorum sensing, it is reasonable to infer that the conjugative systems of plasmids, such as RP1, may also be quorum sensing-regulated. The exact nature of the mating pair connection in RP1 transfer is unknown, but it differs substantially from the F plasmid system in several respects. First, there is no homolog to TraN in the RP1 system (8). Therefore OmpA is not believed to be involved in mating pair stabilization in this system. In addition, RP1 pili are thin and rigid, and are easily disrupted in liquid matings. In fact, RP1-mediated conjugation is increased by 4-fold when matings are performed on solid media (8). These characteristics contrast the F plasmid system, which includes long, flexible pili, and high frequency conjugation in liquid mating, but considerably lower frequency on solid media (5).

The object of this study is to further the characterization of autoinducer-2 by studying the effect of increasing AI-2 concentrations in the recipient culture on conjugation efficiency in
E. coli. The F plasmid and RP1 plasmid will be used as model conjugative systems. Both systems are under extensive investigation and as such the relation between AI-2 and conjugation could be used in the future to study AI-2 molecule structure and function. The F plasmid system is especially well characterized, with the complete sequence of the F plasmid transfer region available in literature (4).

Based on the results of the previously mentioned studies, we hypothesize that increasing the concentration of AI-2 will have the effect of increasing conjugation efficiency in *E. coli*. Conjugation efficiency will be measured by the extent of successful transfer of a marker from a donor *E. coli* strain to the quorum sensing *E. coli* AB1157. The transfer of the ability to metabolize lactose from an F'lac strain to AB1157 will be used to assess F plasmid conjugation success. Assaying for the RPI plasmid will be performed via an ampicillin-resistance gene that can be selected for in successfully conjugated recipients.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Conjugation experiments were carried out using *Escherichia coli* AB1157 (F-, thr-1, leu-6, thi-1, lacY1, galK2, ara-14, xyl-5, mtl-1, proA2, his-4, argE3, str-31, tsx-33, sup-37) (supplied by Dr. Ramey, UBC) as the recipient strain. *E. coli* RP1 (genotype unavailable) (supplied by Dr. Jullian Davis, UBC) and *E. coli* JCFLOxK1200 (F'Lac, Nal resistant, lacΔ, U124Δ, nadA, aroG, gal, attL, bio, gyrA) (supplied by Dr. Laura Frost, U of A) were used as donor strains. Unless otherwise specified, cultures were grown at 30°C with aeration in Luria-Bertani broth containing 0.5% glucose. Overnight cultures of AB1157 contained streptomycin at 100 ug/mL. For growth curves and conjugation trials, AB1157 was grown in media containing 0, 10 and 20% (v/v) AI-2.

Preparation of AI-2 media. AI-2-containing culture supernatant was prepared as previously described (9). Briefly, an overnight culture of *E. coli* AB1157 was diluted 1:100 into fresh LB media. This new culture was incubated at 30°C for 8 h with aeration. Following this incubation, cell-free media was prepared by pelleting the cells for 10 min at 3500 rpm in a GH-3.8 rotor in a Beckman Coulter Allegra 6 Centrifuge. The supernatant was then passed through a 0.2 um HT Tuffryn filter (Pall Gelman Laboratory) into sterile falcon tubes and stored at –20°C. In making the 0, 10 and 20% (v/v) AI-2 media, constant volume was maintained by the addition of LB, filtered in the same manner as the AI-2 supernatant.

Growth curves. Growth curves were plotted for each of the three strains to be used in conjugation. To prepare the curves, overnight cultures were diluted 1:50 into fresh LB broth. Starting at time zero, turbidity readings were taken every 30 min until cultures reached stationary phase. All readings were taken using a Spectronic-20D spectrophotometer set at
660 nm. For the recipient strain, growth curves were plotted for cells grown in media containing 0, 10 and 20% AI-2.

Conjugation. The donor and recipient strains were cultured overnight in 50 ml LB broth. The strains were then diluted 1:50 into 50 ml media containing 0, 10 or 20% AI-2 (v/ v) for the recipient and LB broth for the donor. When the cultures reached a turbidity of 0.1, conjugation was initiated by mixing the donor strain with each of the recipient strains at a ratio of 1:9. Each mixture was placed in a 37°C water bath and agitated gently by hand every few minutes for a total of 30 min. Prior to mixing, samples of donor and recipient cultures were plated on LB to determine viable donor to recipient ratios. After 30 min, conjugation was disrupted by vortexing for 15 sec.

Screening for transconjugants. Immediately after vortexing, the cells were diluted in sterile LB and spread on selective plates. For the conjugation between AB1157 and RP1, dilutions were spread on LB plates containing streptomycin (100 ug/ml) to determine the total number of recipients, as well as plates containing streptomycin (100 ug/ml) and ampicillin (100 ug/ml) for assaying transconjugants. For the conjugation between JCFLO and AB1157, dilutions were spread on LB plates containing X-gal and IPTG at 80 ug/plate and 800 ug/plate respectively. All plates were incubated at 37°C for approximately 24 h. Conjugation efficiencies were calculated by comparing ratios of successful transconjugants to total recipients.

RESULTS

In order to measure the effect of increasing AI-2 concentration on conjugation efficiency, we isolated AB1157 cells in early log phase. It was thought that at early log phase, the effects of constitutive AI-2 production by the AB1157 cells in culture would cause minimal interference with any affects observed from the addition of supplemental AI-2. To determine the time of early log phase, growth curves were constructed in order to determine the growth profiles of the various strains in the culture conditions of this experiment.

TABLE 1: Cell concentration for pre-conjugation cultures grown in increasing AI-2 concentrations

Percent of Supplemental AI-2	Viable donor cells (cells/ml)(x10^8)	Viable recipient cells (cells/ml)(x10^8)	Donor : recipient ratio
0	0.67	19.1	28
10	0.67	4.74	7
20	0.67	1.61	2

Total viable cell concentrations were determined by counting cfu on LB plates incubated at 37°C for 24 hours.

TABLE 2: Conjugation efficiencies for RP1 and AB1157 grown in increasing AI-2 concentrations

Percent of supplemental AI-2	Total concentration of viable recipients (cells/ml) ($x10^8$)	concentration of transconjugants (cells/ml)	% transconjugants
0	1.24 +/- 0.06	2105 +/- 103	0.002 +/- 0.0001
10	0.92 +/- 0.06	3600 +/- 693	0.004 +/- 0.0008
20	1.17 +/- 0.06	10500 +/- 778	0.009 +/- 0.0008

Following conjugation, total viable recipients were selected for by plating on LB plates containing streptomycin. Transconjugants were selected for on LB plates containing both streptomycin and ampicillin.

Early log phase growth as determined by turbidity: Mixing of AB1157 and RP1 cultures for conjugation was performed when cultures were in approximately equal stages of their growth curve. Figure 1 shows the time of early log phase for AB1157 in 0%, 10% and 20% AI-2 to be approximately 90 minutes. The growth curves of donor strains RP1 and JCFLO also reached early log phase by 90 minutes (Fig 2). Dilutions of 1:50 were used for initial growth curve culture conditions to match the conjugation protocol. Due to time constraints, repetition of growth curves was not possible, hence standard deviations of these curves was not constructed. This may explain the discrepancy between growth profiles as determined by turbidity measurements in the growth curve (Fig. 2) and total viable concentration of recipients (Table 2).

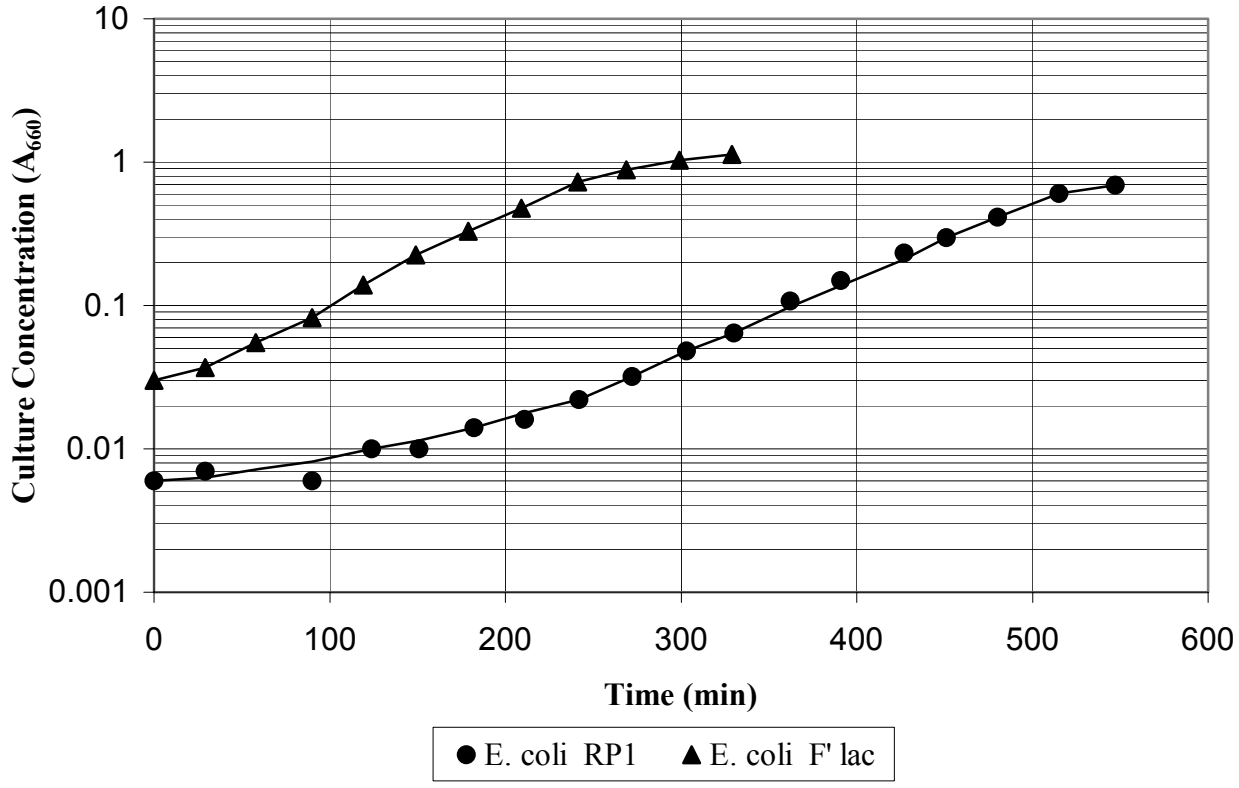

FIGURE 1: Growth Curves for Donor *E.coli* RP1 and *E.coli* F'Lac grown in LB at 30°C with Aeration

Conjugation results as determined by colony forming units: Total viable bacteria of pre-conjugants were determined through spread plating on LB plates with or without selective agents. To measure the ratio of donor RP1 to recipient AB1157 prior to conjugation, cultures were plated on Luria Broth and Luria Broth with streptomycin, respectively. Table 1 shows a deviation from a desired constant ratio. The ratio of donor to recipients was 1:28.5,

1:7.1 and 1:2.4 for the 0%, 10% and 20% AI-2 treatments, respectively. The relative conjugation efficiency used to test our hypothesis appears in Table 2. To determine the number of total viable recipients, cultures were plated on LB with streptomycin while transconjugants were measured by growth on LB in the presence of streptomycin and ampicillin. Conjugation efficiency was found to increase from 0.002 +/- 0.0001% to 0.009+/- 0.0008% as AI-2 concentration increased from 0% to 20%. This trend can be observed in Figure 3 and suggests that increased AI-2 concentration positively affects conjugation efficiency. A parallel experiment was performed to test the affects of AI-2 on conjugation efficiency of F'Lac into AB1157. The results from this trial indicated that the F'Lac was unable to transfer into the recipient strain under all conditions tested.

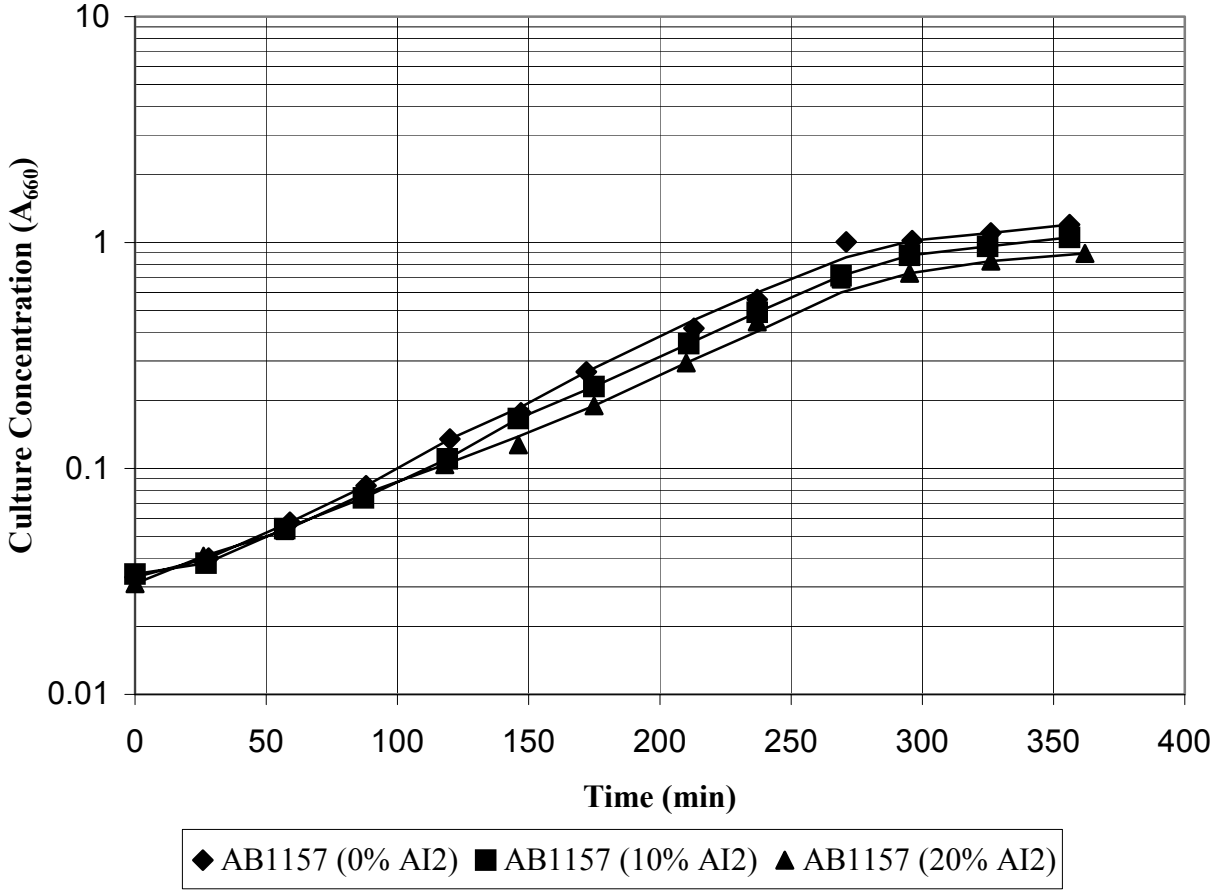

FIGURE 2: Growth Curves for *E.coli* AB1157 Grown in the Presence of 0%, 10%, and 20% AI2 Media in Luria Broth at 30C with Aeration.

DISCUSSION

When AB1157 was used as the recipient and RP1 as the donor, AI-2 concentration was positively correlated with conjugation efficiency. When cultures were grown in the presence of 20% AI-2, there were significantly more transconjugants detected than any other AI-2 condition. The donor to recipient ratio was determined by plating samples of the respective cultures before conjugation. The smallest donor to recipient ratio occurred with no AI-2 treatment, while the largest donor to recipient ratio occurred at the 20% AI-2 treatment. It is thought that as donor to recipient ratio decreases, conjugation efficiency would increase due to the increased probability of mating-pair establishment. In contrast, our results showed that transformation efficiency was greatest at the largest donor to recipient ratio suggesting that increasing AI-2 concentration was responsible for increased conjugation efficiency. It

is proposed that supplementing cultures with AI-2 exposes the cells to an environment that is suggestive of a higher cell density, stimulating the expression of genes that are normally not expressed until later in culture. A number of these genes could encode for an outer membrane receptor that would bind to pilli and allow the cell to engage in conjugation. Although a positive correlation between AI-2 concentration and conjugation efficiency is evident, this efficiency is lower than the typical value of 1% (personal communication, Dr. George Spiegelman, UBC). A possible explanation for this low absolute efficiency may be the low cell density of the initial cultures prior to mixing.

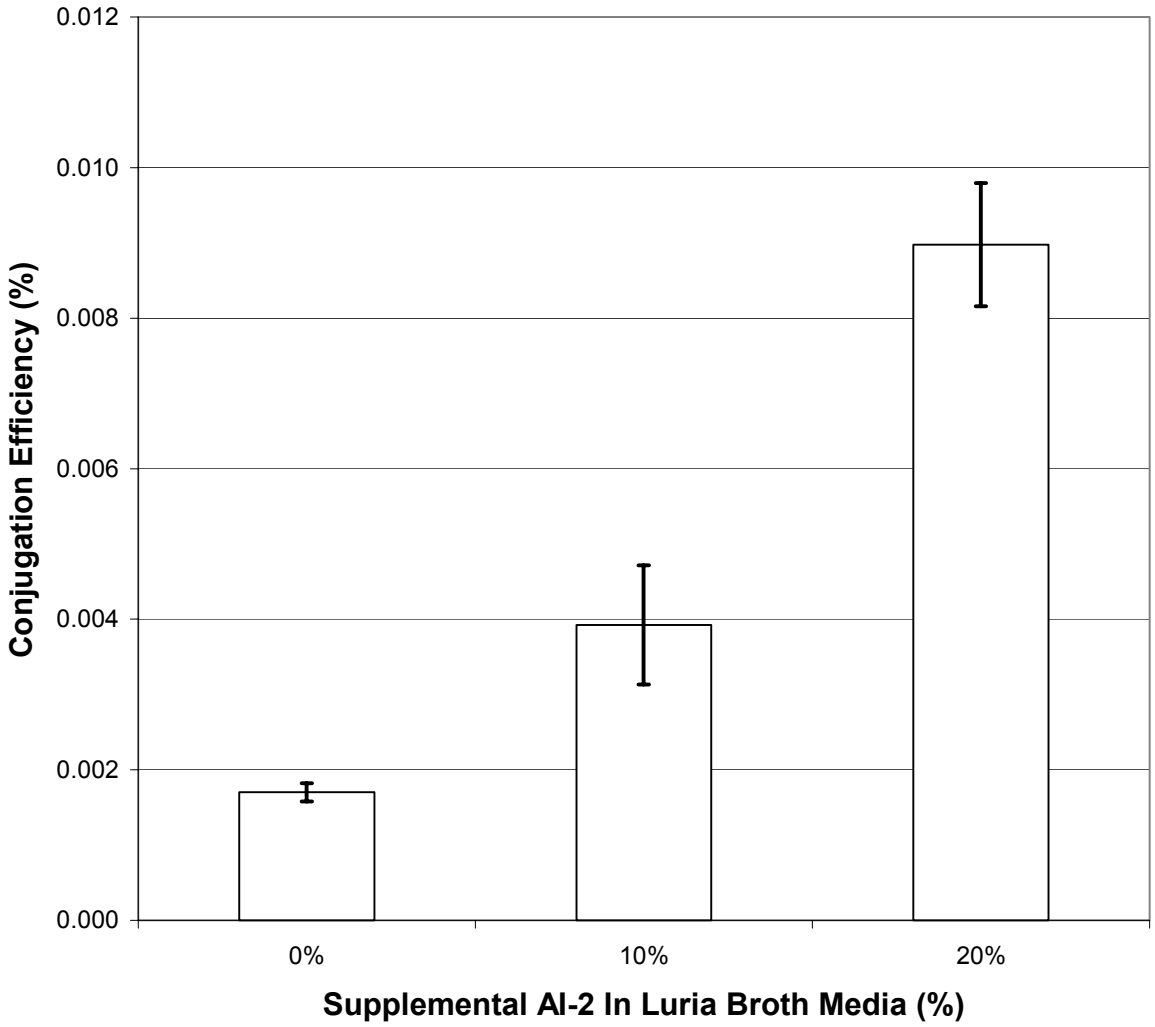

FIGURE 3: Relative Conjugation Efficiency between *E.coli* RP1 and *E.coli* AB1157 with Increasing Concentrations of AI-2

A second factor that could have effected growth rates is culture pH. The media preparation protocol used in this experiment involved the use of spent media, which would have greater acidity, and thus increasing concentrations of AI-2 would have been accompanied by decreasing pH. In preparing our AI-2 media, we did not equalize the pH of the various AI-2 concentrations. Therefore, it is possible that slower growth was a result of increased acidity at higher AI-2 concentrations. We could address this problem by using filtered media from a non-quorum sensing strain, such as DH5α, instead of filtered LB media (9).

When the JCFLO strain was used as the donor and AB1157 was used as the recipient, there were no transconjugants detected, even though the F' plasmid is known to conjugate at an extremely high efficiency (3). A second trial was performed in which the conjugation temperature was altered, and initial cell density was increased from a turbidity reading of 0.1 to 0.3 in an attempt to optimize conjugation conditions. These changes did not yield any detectable transconjugants.

There are several possible reasons for the lack of successful transconjugants in the F plasmid system. One possibility for our lack of successful transconjugants is the chance that the F'Lac plasmid was unstable, resulting in the integration of the *lac* gene into the chromosomal DNA, thus eliminating the conjugative abilities of the strain, while preserving the Lac+ phenotype (Dr. W. Ramey, personal communication). The presence of a functioning *lac* gene in the donor is not in dispute because ß-galactosidase activity was demonstrated through blue colonies observed on X-gal plates. Alternatively, it has been suggested that the lack of Lac+ transconjugants may have been due to the phenotype of the recipient, and not the donor. The presence of an F plasmid in the recipient would prevent the transfer of the F'Lac due to the incompatibility of the plasmid origins of replication. (Dr. J. Davies, personal communication) Transfer of RP1 would not be inhibited in this situation because it is a member of a different incompatibility group. This explanation is unlikely, however, since AB1157 is an F^- strain historically used for conjugation studies.

The results of this study support our hypothesis that RP1 conjugation is quorum sensing-regulated. A positive correlation between the conjugation efficiency of *Escherichia coli* RP1 to AB1157 and increasing concentrations of AI-2 was demonstrated. This model could provide a convenient means for further AI-2 study. The results obtained for the conjugative transfer of F'Lac are inconclusive. Therefore, the affects of AI-2 concentration in this system could not be determined.

FUTURE EXPERIMENTS

The results of this experiment are promising, however extensive work is required before any conclusive connections can be drawn between the presence of autoinducer in the environment and the efficiency of DNA transfer in the F plasmid and RP1 conjugation systems.

There are several issues of immediate concern. If research is to continue with the current F'Lac strain, it is imperative to confirm the presence of the conjugative plasmid. This can be accomplished through a combination of molecular and genetic screening. In addition, the AB1157 strain must be screened for the presence of plasmids that may interfere with its ability to accept the F'Lac. The conjugation protocol can also be altered in order to increase conjugation rates in both of the conjugative systems studied. Such alterations include delaying the matings until a later growth phase (eg. mid to late exponential phase), or using a filter mating protocol rather than the liquid mating protocol employed in this study. In addition, the duration of the conjugation could be increased. Alternatively, other recipients such as *Escherichia coli* χ^{478} could be tested for the change in conjugal efficiency and quorum sensing. In addition, maintaining constant volume in increasing AI-2 concentrations through the use of spent medium from a non-quorum sensing strain rather than filtered LB should provide comparable pH.

In the long-term, several experiments could be conducted to further the results found in this study. It would be interesting to establish whether the contribution of AI-2 to conjugation efficiency reaches a threshold at some concentration, over which conjugation rates are unaffected by the presence of the molecule. This study did not examine the contribution of quorum sensing to the regulation of gene expression in the donor cells. It is advisable for future experiments to examine of the affects of AI-2 concentration on isolated donor cultures to gain a better understanding of the full contribution of quorum sensing to the conjugation system.

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