Mapping Stress-Induced Changes in Autoinducer AI-2 Production in Chemostat-Cultivated Escherichia coli K-12

MATTHEW P. DeLISA,1,2 JAMES J. VALDES,3 AND WILLIAM E. BENTLEY1,2*

Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute,1 and Department of Chemical Engineering,2 University of Maryland, College Park, Maryland 20742, and U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, Maryland 210103

Received 8 November 2000/Accepted 6 February 2001

Numerous gram-negative bacteria employ a cell-to-cell signaling mechanism, termed quorum sensing, for controlling gene expression in response to population density. Recently, this phenomenon has been discovered in Escherichia coli, and while pathogenic E. coli utilize quorum sensing to regulate pathogenesis (i.e., expression of virulence genes), the role of quorum sensing in nonpathogenic E. coli is less clear, and in particular, there is no information regarding the role of quorum sensing during the overexpression of recombinant proteins. The production of autoinducer AI-2, a signaling molecule employed by E. coli for intercellular communication, was first described in Vibrio harveyi (18, 44, 57), and similar autoregulatory mechanisms have since been reported in a wide range of bacteria, including Pseudomonas aeruginosa (34), Erwinia carotovora (5), and Agrobacterium tumefaciens (40), as well as E. coli (18, 44, 57). Although the existence of such a mechanism in E. coli has been uncovered, the genetic, physiological, and environmental factors that contribute to and regulate the quorum circuit remain poorly defined.

Evidence of intercellular communication in E. coli came with the discovery of a quorum-regulated transcriptional activator (SdiA) of the cell division genes in the ftsQAZ locus homologous to LuxR of V. fischeri (18, 44, 57) and a synthase protein (LuxS_{esp}) responsible for AI-2 signal molecule production (49). Further elucidation of native E. coli quorum circuit architecture resulted from the Vibrio harveyi cross-species activity assay of Surette and Bassler (47), which has greatly facilitated autoinduction research in facultative anaerobes (46, 47). This is particularly important as much of the information pertaining to the regulatory machinery controlling quorum-dependent gene expression in E. coli had previously been derived from studies of E. coli cloned with V. fischerilux genes or indirectly from cognate studies of V. fischeri (53). It has already been shown that AI-2-stimulated quorum sensing in E. coli is critical for regulating behavior during prestationary growth and that AI-2 communicates cell density, growth rate, glucose level, and metabolic potential of the environment (47, 48). Moreover, regulation of AI-2 in E. coli appears to be utilized for channeling conditions of stress and starvation into the quorum circuit, presumably through the GroESL chaperonin complex (12, 48, 53). In particular, a factor present in E. coli-conditioned medium (CM) stimulates expression of rpoS, sdiA, and the ftsQAZ cell division cluster (18, 44) and inhibits chromosomal replication (58), suggesting intimate involvement of the quorum circuit with stress- and starvation-mediated and cell cycle circuits in E. coli.

Still, autoinduction in E. coli remains enigmatic, as the signaling molecule, the precise mechanism of signaling, and the cellular and environmental stimuli involved in global AI-2 regulation are poorly defined. It is known that a variety of environmental cues play a role in regulating lux expression in E. coli, but a systematic study of the effect of these stimuli on the native quorum circuit in E. coli (i.e., AI-2 regulation) is lacking. Further, it is difficult to uncouple population density-dependent effects from growth phase effects in batch culture, making it unclear whether patterns of AI-2 regulation are population density or growth phase effects. In the present study, we assayed AI-2 production in continuous culture (41) to explore various transients commonly observed during fermentation processes, including physical and chemical insults, as well as the induction of heterologous protein. For example, growth of
E. coli is often regulated by transient substrate and nutrient limitations, oxygen transfer capabilities, formation of growth-inhibitory by-products, and limitations in heat dissipation (36). Also, the act of producing recombinant proteins elicits stress responses (2, 22, 24, 25, 33). Chemostat cultivation offered a reproducible, regulated growth environment so that regulation of AI-2 could be isolated from continuously changing variables, such as oxygen, glucose, and pH, which likely obscure AI-2 regulatory phenomena in batch cultures. Therefore, we monitored dynamic AI-2 production during the transitory period between steady states caused by either dilution rate changes or induced perturbations (i.e., heat shock and ethanol stress) to steady-state cultures of recombinant E. coli. Results presented here show that shifts in intracellular metabolism and stress resulted in significantly altered patterns of AI-2 accumulation, demonstrating that AI-2 production is a complex function of environmental and intracellular conditions.

Our ultimate objective is to better understand the communication between E. coli during fermentation processes in order to facilitate expression of heterologous genes. Importantly, little is known about the attenuated induction of heterologous proteins commonly observed at high cell densities (11, 19, 50), where factors such as decreased growth rate, increased cell death, increased lysis, decreased metabolic activity, and segregation into viable but nonculturable cells can negatively impact reactor productivity (2, 28, 43). We have shown dramatically altered transcript levels of several stress-related genes at cell densities near 80 g (dry weight) liter$^{-1}$ (20), and recent understanding of autoinduction suggests possible mechanisms that contribute to the altered metabolic and physiological state of cells grown to these cell densities. In this work, we have also explored the transcriptional response of 24 genes, including four known quorum-related genes as well as a known subset of genes up-regulated in response to human interleukin-2 (hIL-2) production, in order to begin mapping quorum-dependent transcriptional regulation with bacterial stress responses.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture media.** E. coli K-12 strain W3110, harboring the plasmid pH-II-2 for hIL-2 protein (9) or strain MDAI2 (W3110 ΔluxL A1-1::Tn5 sensor 1” sensor 2”) and BB152 (lux::Tn5 A1-1 ΔluxS”) were used for E. coli autoinducer activity assays and were kindly provided by B. L. Bassler. Luria-Bertani (LB) medium contained yeast extract (5 g liter$^{-1}$) and Tryptone (10 g liter$^{-1}$) and was adjusted to pH 7.0. Chemostat experiments. Temperature- and pH-regulated microfermentors (56) specifically designed for long-term continuous cultivation were inoculated with 1% (vol/vol) overnight LB cultures and maintained at pH 6.7 and 30°C with 225-rpm agitation. All chemostat experiments were either sparged with air (200 ml min$^{-1}$) or unsparged but vented as noted. For continuous culture, a steady state was defined by unchanged optical density at 600 nm (OD$_{600}$) for a period of 5 to 6 residence times. Dilution rate changes were implemented by altering the feed rate of fresh LB medium to the reactor. At steady state, the dilution rate is equivalent to the growth rate of the culture (41). Experimental data associated with a particular dilution rate (or transition between dilution rates) are the averages of three samples each assayed in triplicate using BB170 (n = 9), and all data are the averages of duplicate experiments.

**Intracellular and environmental stimuli.** Physical or chemical insults were applied to steady-state E. coli cells (μ = 0.75 h$^{-1}$) and 2-ml samples were drawn over a 4-h period (ca. 3 residence times), allowing dynamic measurement of the AI-2 response. Specifically, glucose, which has previously been implicated in AI-2 regulation (14, 38, 47), was pulse fed (ca. 50 mM) to steady-state cultures. The effect of iron, known to delay the onset of luminescence in V. fischeri (13), was investigated by pulse addition of ferric [Fe(III)] citrate (100 mg liter$^{-1}$). High osmolality has been shown to stimulate AI-2 production in Salmonella enterica serovar Typhimurium (48) and thus was investigated by addition of NaCl (35 g liter$^{-1}$). Increased aerobiosis was investigated by delivery of oxygen (200 ml min$^{-1}$) to the fermentor. Heat shock was induced by temperature elevation of the reactor jacket from 30 to 42°C, which resulted in culture temperature change over 5 min. Ethanol, which elicits a stress response similar to heat shock (55), was pulse added (4% [vol/vol]). Stringent stress was induced via serine hydrolaminate addition (32). Oxidative stress was generated by addition of hydrogen peroxide (54) or by addition of dithiothreitol (DTT) (a membrane-permeating reducing agent) to a final concentration of 1 g liter$^{-1}$ (21). The presence of acetate has been linked to low productivity in recombinant E. coli cultures (31) and was investigated by addition of 6 g of sodium acetate per liter. Finally, the expression of recombinant hIL-2 was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma).

**Analytical methods.** Cell growth was monitored by measuring OD$_{600}$ with a UV-visible light spectrophotometer (Beckman DU 640). To determine glucose concentration, 1-ml whole broth samples were centrifuged (10,000 × g at 4°C) and glucose concentration of the supernatant was measured using a glucose analyzer (YSI Model 2700). Green fluorescent protein (GFP) was assayed in whole cells as previously reported (8), and readings were made using a Perkin-Elmer LS-35 fluorescence spectrometer at an excitation wavelength of 495 nm and an emission wavelength of 509 nm. Specific fluorescence intensity was obtained by normalized relative fluorescence intensity of samples by OD$_{600}$ of the sample.

**Preparation of cell culture fluids and CM.** Cell culture fluids were prepared by centrifugation of 2-ml E. coli whole-broth samples for 5 min (10,000 × g at 4°C). Cleared supernatants were passed through 0.2-µm-pore-size HT Tuffyn filters (Pall Corp) and stored at −20°C. V. harveyi BB152 cell culture fluids were prepared likewise to obtain positive control samples as reported previously (47). CM was prepared by growing W3110 or MDAI2 in LB–50 mM glucose to an OD$_{600}$ of 2 to 3 (~6 to 8 h) followed by centrifugation (5 min, 10,000 × g at 4°C) and filtering of cleared supernatants as above.

**Autoinducer activity assay.** E. coli cell culture fluids were tested for the presence of AI-2 using the V. harveyi reporter strain BB170, which responds only to AI-2 (47). Luminescence assays were performed as outlined elsewhere (47) and luminescence was measured as a function of V. harveyi cell density by quantitating light production with a luminesimeter (EG&G Berthold). Data reported as fold activation were obtained by dividing the light produced by the reporter after addition of E. coli cell culture fluid by the light output of the reporter when growth medium alone was added. Importantly, for quantification of AI-2 activity level, a detailed standard curve of CM samples serially diluted in fresh AB medium (25-fold difference in CM volume) was utilized to demonstrate concentration dependence of the assay. The resulting standard curve had an r$^2$ of 0.9, and all CM samples outside the linear range were diluted appropriately in AB medium.

**RNA isolation and total RNA dot blotting.** Whole-cell samples (1 ml) were immediately flash frozen in liquid nitrogen and stored at −80°C. RNA purification was carried out using the RNAqueous total RNA isolation kit (Ambion). This kit typically purified 10 µg of total RNA per ml of E. coli culture at an OD$_{260}$ equivalent of 1.0.

Total RNA dot blots were performed by pipetting 1 to 5-µg total RNA samples (in 50% formamide, 6.5% formaldehyde, and 1X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) onto nylon membranes using a Schleicher and Schuell microsample filtration manifold. Fusing of nylon membranes was performed via UV-induced cross-linking. Membranes were prehybridized for 1 h in 15 ml of hybridization buffer (Boehringer Mannheim). Prehybridization solution was decanted, and 7.5 ml of fresh hybridization buffer containing 50 ng of digoxigenin (DIG)-labeled Northern probe ml$^{-1}$ was introduced. Northern probes were prepared using a PCR DIG Probe Synthesis kit (Boehringer Mannheim), 25-bp oligonucleotide primers (Gene Probe Technology), and E. coli K-12 template DNA isolated according to the method in reference 23, and resulting probes were approximately 400 bp in length. Hybridization was carried out overnight at 50°C followed by development utilizing anti-DIG alkaline phosphatase (Boehringer Mannheim) and CSPD chemiluminescent substrate (Boehringer Mannheim) according to the manufacturer protocol (Boehringer Mannheim). Developed membranes were incubated at 37°C for 30 min prior to X-ray film exposure for 2 h.

**Signal quantification and calculation of correlation coefficients.** For quantification of total RNA dot blots, a detailed standard curve of serially diluted
DIG-labeled λDNA was utilized. The dilution spanned a 50-fold difference in mass of DNA per dot, and the resulting standard curve had an $r^2$ of 0.9. Denitometric scanning was performed for signal analysis. Images of exposed films were acquired via an EAGLE EYE II (Stratagene) image acquisition system. Image quantification was performed using Scion Image software (Scion Corporation). Lastly, induction ratios (IR) based on the density of each dot were calculated by the following equation: 

$$IR = \frac{\text{density}}{\mu g \text{ of RNA}}_t / \frac{\text{density}}{\mu g \text{ of RNA}}_0$$

and induction ratios from repeated experiments were averaged, with the standard error calculated as the standard deviation ($s$) of the mean 

$$s = \frac{\sum (x - \bar{x})^2}{n - 1}$$

where $s_x$ and $s_y$ are the standard deviations within samples $x$ and $y$, respectively (51). Determination of the probability ($P$) that the $r$ value (correlation coefficient) obtained could have been calculated from uncorrelated samples is made such that the label "significant" was assigned to a $P$ of $<0.05$ and the label "highly significant" was assigned to a $P$ of $<0.01$.

RESULTS

Transient AI-2 response to increased growth rate and glucose perturbation. In this study, we used continuous cultures of *E. coli* to study AI-2 production in response to growth phase changes as well as to a number of environmental and intracellular factors. Results depicting the transient response in extracellular AI-2 levels following upshift in culture growth rate (from 0.10 to 1.25 h$^{-1}$) of W3110/phIL-2 chemostat cultures. The AI-2 production rate is the activity times the dilution rate, D (D = reactor volume/flow rate). Steady state was achieved in ca. 3 to 5 residence times. Increased growth rate was implemented by increasing the feed rate of fresh LB medium–50 mM glucose. Replicate samples agreed within 15%.

As the growth rate of the culture was incrementally increased from a growth rate of 0.10 to 1.25 h$^{-1}$ (in steps of ca. 0.15 to 0.20 h$^{-1}$), a concomitant increase in the fold activation of AI-2 was observed. The changes in glucose concentration and cell density (OD$_{600}$) over this range were less than 17 and 14% respectively (not shown). The increase in AI-2 activity from one steady state to the next was accompanied by a sharp overshoot during a transitory period prior to settling at a new steady-state AI-2 level. This observed overshoot was seen to increase in conjunction with increasing growth rate, with the largest overshoot occurring during the transition to the highest growth rate evaluated (1.10 to 1.25 h$^{-1}$). In addition, the rate of AI-2 production was seen to increase with increasing growth rate (Fig. 1b). To help distinguish between the effect of glucose levels and growth rate, we performed chemostat experiments for which W3110/phIL-2 cells were grown in the absence of glucose. Importantly, results for growth of W3110/phIL-2 cells in LB medium without glucose demonstrated similarly that the increase of AI-2 with increasing culture growth rate occurred, but at a lower overall level for each growth rate compared to cells grown in 50 mM glucose (not shown). Therefore, glucose affected only the relative magnitude of AI-2 but was not required for AI-2 production under the conditions studied.

![FIG. 1. AI-2 activity (a) and rate of AI-2 production (b) during steady-state transitions achieved by incremental upshift in culture growth rate (from 0.10 to 1.25 h$^{-1}$) of W3110/phIL-2 chemostat cultures. The AI-2 production rate is the activity times the dilution rate, D (D = reactor volume/flow rate). Steady state was achieved in ca. 3 to 5 residence times. Increased growth rate was implemented by increasing the feed rate of fresh LB medium–50 mM glucose. Replicate samples agreed within 15%.

![FIG. 2. AI-2 activity during transition between low growth rate (region I and III) (0.10 h$^{-1}$) and high growth rate (region II) (1.25 h$^{-1}$) of W3110/phIL-2 cultures grown in the presence of 5 (a) and 50 (b) mM glucose. Replicate samples agreed within 15%.

state cultures (maintained at $\mu = 0.75$ h$^{-1}$) exhibited a relatively rapid upswing followed by decline back to the preperturbed level. However, unlike other perturbations studied, a second peak in AI-2 activity was observed as the glucose concentration fell below ca. 10 mM due to consumption and washout. This response was unlike other perturbations studied, a second peak in AI-2 activity produced by increasing the growth rate in 0.10 h$^{-1}$ steps (Fig. 1) was performed and exhibited by the open triangles. Replicate samples agreed within 15%.

Glucose-induced perturbations from steady state stimulate AI-2 production. We next perturbed steady-state W3110/phIL-2 cultures grown in LB medium–5 mM glucose with a glucose spike (50 mM pulse fed to reactor). Both cases ($\mu = 0.75$ and 0.10 h$^{-1}$) exhibited a relatively rapid upswing followed by decline back to the preperturbed level. However, unlike other perturbations studied, a second peak in AI-2 activity was observed as the glucose concentration fell below ca. 10 mM due to consumption and washout. This response was likely triggered by a decline of glucose concentration below a threshold value, which was a fixed point common to both chemostat experiments (47, 48) that show a spike in AI-2 activity concomitant with glucose depletion. As expected based on prevailing glucose consumption rates, the time required for the entire glucose response was much shorter for the higher growth rate case (not shown), but normalizing the perturbation time demonstrates that the AI-2 responses were similar (Fig. 3). That is, the elapsed time was normalized by dividing the time postperturbation by the time at which the glucose had reached 8% of its peak (ca. 40 to 45 mM) value, a fixed point common to both chemostat experiments. Notably, comparing Fig. 3a and b reveals that the fold activation of AI-2 signal activity in response to pulsed glucose was almost 2 times greater at the higher growth rate versus the fold AI-2 activation at the lower growth rate.

Intracellular and environmental stimuli perturb AI-2 signal. Since several environmental cues are known to affect lux gene expression in V. fischeri or in E. coli (via plasmid-borne lux genes), we investigated the effect of these stimuli on AI-2 activity in steady-state chemostat cultures of W3110/phIL-2 grown in LB medium–5 mM glucose. Specifically, pulse addition of several stimuli (see Materials and Methods) to steady-state cultures (maintained at $\mu = 0.75$ h$^{-1}$) was performed and resulted in ca. 2.5- and 6.3-fold increase in AI-2 activity in the presence of 5 and 50 mM glucose, respectively. Interestingly, in the presence of a higher glucose concentration (50 mM) and a one-step upshift (Fig. 2b), increasing the growth rate led to 2.5 times more AI-2 activity compared to that observed for cells grown in the presence of a lower glucose concentration (5 mM) (Fig. 2a) but was ca. 1.6 times lower than AI-2 activity produced by increasing the growth rate in 0.10 h$^{-1}$ steps (Fig. 1 versus Fig. 2b). In all cases, transition to a higher growth rate resulted in overshoot of the AI-2 activity during the 2.5-h transitory period (region II) prior to reaching the new steady-state level. Decreasing the culture growth rate back to 0.10 h$^{-1}$ (region III) resulted in AI-2 activity equal to the original steady-state levels. The time required to reach steady state during this downshift was ca. 4.5 h (compared to 2.5 h during upshift).

AI-2 activity was monitored at high frequency for the subsequent 4 h (i.e., 3 residence times) (Fig. 4). Superimposed onto these responses (Fig. 4a [iron pulse] and [hIL-2 expression]) is the theoretical concentration (normalized) of a pulse-added, nonmetabolized tracer, illustrating in some cases the transient intensity of the stimulus.

Responses to increased iron [100 mg of Fe(III) citrate liter$^{-1}$], increased osmolarity (35 g liter$^{-1}$), and decreased culture redox potential (1 g of DTT liter$^{-1}$) exhibited AI-2 accumulation within the first 60 min that remained elevated for 3 h before returning to approximately the original steady-state level (Fig. 4a). Notably, exposure to iron resulted in the largest (ca. 3.5-fold) and most rapid (ca. 30 min) transient AI-2 increase. Additionally, a feed of LB–50 mM glucose–100 mg of Fe(III) citrate liter$^{-1}$ resulted in -2.5-fold increase in AI-2 activity (compared to cultures fed LB–50 mM glucose only [not shown]) over 5 residence times, confirming that the pulse-induced response was dependent upon iron concentration experienced by cells. Exposure to sodium acetate (6 g of sodium acetate liter$^{-1}$) resulted in an immediate decrease of AI-2 for 60 min followed by a sharp and reproducible 1.5-fold increase at 80 min, remaining elevated for 80 min before finally falling below the original steady-state level. Finally, exposure to increased oxygen (pulse input of pure O$_2$ [200 ml min$^{-1}$]) resulted in immediate decrease of AI-2 activity that persisted for 120 min. At this point, the oxygen supply to the reactor was ceased, and an immediate increase in AI-2 activity to ca. 1.5-
fold ensued before settling back towards the steady-state level. An identical experiment was performed where oxygen was supplied over the entire 4-h period and the AI-2 activity remained low over the entire period (not shown).

The rpoH-mediated stresses heat shock (42°C; change in temperature, 12°C; change in time, 4 h) and ethanol (4% [vol/vol]) both resulted in decreased AI-2 production for ca. 60 min followed by an oscillatory response before reaching a steady state (Fig. 4b). In the case of heat shock, the AI-2 activity remained well below the prestress level as the reactor temperature was maintained at 42°C for the entire 4-h period. The final AI-2 level in response to ethanol (which was transiently depleted) returned to the prestress level. Interestingly, pulse addition of serine hydroxamate (100 mg liter\(^{-1}\)), known to stimulate a stringent response (42), resulted in down-regulation of AI-2 that persisted over the entire 4-h period, even after the serine hydroxamate was washed out of the reactor. Exposure to hydrogen peroxide (2 mM), known to induce oxidative stress (54), was observed to negatively regulate AI-2 production over the first 30 min; however, an increase in AI-2 production occurred over the next 100 min, reaching a peak level of 1.5-fold before settling back to the prestress steady-state level. Lastly, the effect of recombinant hIL-2 formation was investigated by addition of 1 mM IPTG. AI-2 production decreased initially but returned to the prestress level in a damped oscillatory manner. Also interesting is the observation of increased AI-2 degradation (or inhibition) poststress. That is, because AI-2 activity decreased faster than the predicted washout of AI-2 in the absence of continued synthesis, the AI-2 was either degraded or inhibited by a secreted factor, as in the cases depicted in Fig. 4b. Importantly, negative controls were performed by addition of each perturbation agent directly to CM following removal of \textit{E. coli} cells and screened using BB170. In all cases, no measurable change in luminescence was
we grew the changes in cell density, glucose consumption, and growth rate, Valdes, and W. E. Bentley, submitted for publication). To cell division gene cluster in batch culture (M. P. DeLisa, J. J. conferring the inability to synthesize AI-2, but pGFPuv-ftsQ2p activity when LB–50 mM glucose–1% Casamino Acids was fed state (Fig. 5), coincident with almost no measurable AI-2 ac-
of ability to express GFP in response to SdiA-mediated activation (chemostat culture of MDAI2/pGFPuv-ftsQ2p
FIG. 5. SdiA-mediated activation of ftsQ4 via the p2 promoter in chemostat culture of MDAI2/pGFPuv-ftsQ2p (μ = 0.75 h−1). Region I feed, LB–50 mM glucose–1% Casamino Acids; region II feed, W3110 CM (AI-2 activity = 500-fold)–50 mM glucose–1% Casamino Acids; region III feed, W3110 CM (AI-2 activity = 830-fold)–50 mM glucose–1% Casamino Acids; and region IV feed. MDAI2 CM (AI-2 activity = 5-fold)–50 mM glucose–1% Casamino Acids. The condition of steady state between feed regions was achieved after ~5 residence times. Specific activity was determined as the relative fluorescence intensity of GFP-expressing whole cells normalized by the OD600 of the sample.
observed in the reporter strain. Additional negative controls were run by adding each agent to fresh AB medium at a concentration comparable to that in steady-state samples. Results confirmed that the presence of each agent alone did not stimulate light production in the reporter strain.
AI-2 signals through SdiA in steady-state culture. We previously observed that AI-2 signals through SdiA, a LuxR-type transcriptional activator, through the p2 promoter of the ftsQ4 cell division gene cluster in batch culture (M. P. DeLisa, J. J. Valdes, and W. E. Bentley, submitted for publication). To confirm that this observation was independent of dynamic changes in cell density, glucose consumption, and growth rate, we grew the luxS mutant strain MDAI2 harboring plasmid pGFPuv-ftsQ2p in a chemostat culture. Strain MDAI2 was unable to synthesize AI-2, but pGFPuv-ftsQ2p conferred the ability to express GFP in response to SdiA-mediated activation of ftsQ4. Only a basal level of GFP was expressed at steady state (Fig. 5), coincident with almost no measurable AI-2 activity when LB–50 mM glucose–1% Casamino Acids was fed continuously to the reactor (μ = 0.75 h−1 [region I]). However, introduction of CM (generated from W3110 wild-type cells grown to an OD600 of 2.0 in LB–50 mM glucose; AI-2 activity = 560-fold [region II]) supplemented with 50 mM glucose–1% Casamino Acids resulted in an ~3-fold increase in GFP expression coincident with a 400-fold steady-state level of AI-2 activity in the extracellular chemostat medium. Note that we previously demonstrated the sensitivity and linearity of GFP as a transcriptional promotor probe (8), and a threefold increase in GFP fluorescence corresponded to a threefold increase in transcription. Feeding of CM generated identically from W3110 wild-type cells grown to an OD600 of 3.0 (AI-2 activity = 830-fold [region III]) and supplemented as described above resulted in a higher steady-state level of AI-2 in the reactor and a corresponding ~4-fold induction of GFP over the basal (region I) level. Finally, introduction of CM generated from the luxS mutant MDAI2 grown to an OD600 of 3.0 in LB–50 mM glucose (AI-2 activity = 5-fold [region IV]) supplemented as described above resulted in no measurable AI-2 activity in the reactor with a simultaneous return of GFP expression to the basal level observed in region I. Overall, these results confirm that AI-2 signals to SdiA in chemostat culture independent of cell density, glucose concentration, and growth rate changes.
Quorum-regulated genes respond to induced hIL-2 expression. Using a chemostat culture, it was possible to perform a biological perturbation analysis in that steady-state cells could be physically or chemically perturbed and the characteristic footprint response, either AI-2 signaling or mRNA transcription, could subsequently be mapped. An hIL-2 induction experiment was performed exactly as described in Fig. 4b, with the addition of a second 1 mM IPTG pulse at 60 min in order to prolong the expression of hIL-2 during the 4-h response period. The transcription of 24 genes, including four known quorum-related genes (luxS, ftsQ, ftsA, and groEL) and 20 genes previously shown to be upregulated in batch cultures following hIL-2 expression in E. coli, was monitored (Table 1 and Fig. 6) (20, 22). Quantified transcript levels were converted to Z values ([(sampleIR − averageIR)/standard deviation]) to allow magnitude-independent analysis of the dynamic response (16). Converted data were organized via a one-di-

dimensional self-organizing map (SOM) to guide the flipping of the nodes in subsequent hierarchical trees obtained from clustering (10). Correspondingly, the output of the SOM was analyzed using the hierarchical clustering method as described in Eisen et al. (15). The most interesting cluster borne from this analysis contained 10 genes—namely, recA, rpoS, ftsI, groEL, dnaK, grpE, luxS, ftsQ, and ftsA (correlation coefficient of 0.74; P = 0.008)—which were highly significantly correlated (see Materials and Methods). Three of these genes (luxS, ftsQ, and ftsA) are directly involved in AI-2-stimulated quorum sensing (49), while rpoS has been implicated in E. coli quorum sensing (30, 35, 44) and recA (LexA controlled) and groEL and groES (both rpoS controlled) are known to regulate the V. fischeri lux genes in recombinant E. coli (53). Inclusion of rpoH and is5 formed a 12-gene cluster which resulted in a statistically significant correlation coefficient (r = 0.56; P = 0.037). Finally, addition of sdiA (whose gene product is a LuxR-type homologue) and ompT still resulted in a statistically significant correlation (r = 0.52; P = 0.048). To strengthen our claim that quorum sensing overlaps stress circuits, we grew several heat shock protein mutants (groEL140, groES30, dnaK756, dnaJ259, and gpeE280) in chemostat cultures (LB–0.8% glucose; μ = 0.75 h−1) and determined AI-2 production (Table 2). We observed that groES and groEL mutants produced a steady-state level of AI-2 approximately twofold higher than their nonmu-
tuated parent while strains mutated in dnaK, dnaJ, and gpeE all produced a steady-state level of AI-2 lower than their nonmu-
tuated parent. In all cases, strains were grown under identical conditions (μ = 0.75 h−1) and attained nearly identical cell densities (OD600 = 1.2 ± 0.1), confirming that observed differences in AI-2 production between mutant and parental strains was a consequence of an altered σ20-regulatory pathway.
The AI-2 response and Z values of the 10 most tightly
TABLE 1. Identified stress- and quorum-related genes analyzed by total RNA dot blotting

<table>
<thead>
<tr>
<th>Stress- or quorum-related gene</th>
<th>Stress or quorum characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>aceA</td>
<td>Stationary, utilization of acetate</td>
</tr>
<tr>
<td>clpA</td>
<td>Heat shock, ATP-binding subunit</td>
</tr>
<tr>
<td>clpP</td>
<td>Heat shock, SOS, ATP-dependent proteolytic subunit</td>
</tr>
<tr>
<td>degF</td>
<td>Heat shock, serine protease</td>
</tr>
<tr>
<td>dnaK</td>
<td>Heat shock, chaperone</td>
</tr>
<tr>
<td>dnaQ</td>
<td>Heat shock, stationary, chaperone</td>
</tr>
<tr>
<td>ftsA</td>
<td>Quorum-regulated cell division</td>
</tr>
<tr>
<td>ftsH (ftsB)</td>
<td>Heat shock, bacteriophage, cell growth control</td>
</tr>
<tr>
<td>ftsI</td>
<td>Heat shock, cell division and growth</td>
</tr>
<tr>
<td>ftsQ</td>
<td>Quorum-regulated cell division</td>
</tr>
<tr>
<td>groEL</td>
<td>Heat shock, bacteriophage, quorum-related chaperone</td>
</tr>
<tr>
<td>groES</td>
<td>Heat shock, bacteriophage, quorum-related chaperone</td>
</tr>
<tr>
<td>ppE</td>
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<tr>
<td>rpoA</td>
<td>Heat shock, inclusion body protein, chaperone</td>
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<tr>
<td>s75 transposase</td>
<td>Transposition</td>
</tr>
<tr>
<td>lon</td>
<td>Heat shock, SOS, ATP-dependent protease</td>
</tr>
<tr>
<td>luxS</td>
<td>Quorum-related, autoinducer synthase</td>
</tr>
<tr>
<td>ompT</td>
<td>Heat shock, outer membrane protease</td>
</tr>
<tr>
<td>ppdA</td>
<td>Heat shock, bacteriophage, regulation of PSP operon</td>
</tr>
<tr>
<td>recA</td>
<td>SOS, bacteriophage, possible quorum regulator</td>
</tr>
<tr>
<td>rpoH</td>
<td>Heat shock, RNA polymerase σ^54 subunit</td>
</tr>
<tr>
<td>rpoS</td>
<td>Stationary, RNA polymerase σ^70 subunit</td>
</tr>
<tr>
<td>sdiA</td>
<td>Quorum-regulated, regulation of cell division ftsQA4Z cluster</td>
</tr>
<tr>
<td>urlb</td>
<td>SOS, UV resistance</td>
</tr>
</tbody>
</table>

clustered genes (Fig. 7a and b) and the time-dependent average of these 10 genes (Fig. 7c) show that, initially, transcript levels increased coincident with a drop in AI-2 activity (thus, they are inversely related). Between 60 and 120 min, the initial pulse of IPTG was being washed out of the reactor and the dynamic response of AI-2 and mRNA transcripts were both presumably migrating back to the approximately steady-state levels (t = 0 min). At 120 min, the second pulse of IPTG resulted in a more attenuated AI-2 response, in that a lag of ~40 min was observed before AI-2 declined and the magnitude of the decline was less severe than that observed following the first pulse. The transcriptional response during this period was similar, in that it was also much more attenuated, maintaining a relatively constant level for ~80 min before declining at the end of the experiment.

DISCUSSION

To date, all regulatory studies of quorum sensing have been performed in shake flask cultures, where biomass, dissolved oxygen concentration, pH, and glucose all change continuously, which can obscure quorum-dependent regulatory phenomena that are otherwise independent of these variables. Therefore, we have used a V. harveyi cross-species bioassay (47) and continuous culture (37, 41) to track E. coli quorum signaling in response to intracellular stress and/or environmental deficiencies. Specifically, we monitored transient AI-2 production by steady-state chemostat cultures of W3110/phIL2 perturbed by either changes in dilution rate (and therefore in growth rate) or imposition of an intracellular or environmental stimulus. As noted previously by Freeman and Bassler (17), the autoinducer assay for measuring light emission is extremely simple, enabling precise quantification of light production over a wide range of magnitudes such that the effects of quite subtle perturbations of the system can be measured reliably. Chemostat culture enabled decoupling of population density and growth rate effects, enabling the demonstration that AI-2 production not only is growth rate dependent but also exhibits interesting dynamic behavior during upshifts (overshoot) and downshifts (sluggish lag) in dilution rate. Further, it was found that production of AI-2 in chemostat culture did not require glucose metabolism. More frequent sampling of identically performed batch cultures grown in the absence of glucose confirmed that infrequent sampling may have excluded discovery of this subtle feature previously (45).

Further, we confirmed that AI-2 signals to SdiA in chemostat culture independent of cell density, glucose concentration, and growth rate dynamics, suggesting a role for AI-2 signaling in ftsQA-dependent control of cell division. Previous reports regarding quorum-activated ftsQA expression from the upstream p2 promoter have been somewhat ambiguous. Two early reports suggested that SdiA modestly regulated the expression of the cell division locus ftsQAZ in response to an unidentified extracellular factor (18, 44). Surette and Bassler recently reported that CM from E. coli 0157 and DH5α (which does not produce AI-2) had no measurable effect on β-galactosidase produced by a reporter strain (E. coli MC4100) harboring a ftsQ1p2p-lacZ fusion (48). However, fusion of both the p1 and p2 promoters of ftsQA to the lacZ gene may be problematic, as the p1 promoter is rpoS-mediated and the promoters together demonstrate a bimodal control mechanism (44). This prevents study of the effect of AI-2 directly on the p2 promoter as the p1 and p2 promoters are differentially regulated within the cell under transient growth conditions. Further support of this comes from Sitnikov and colleagues, who observed that CM induced the p2 promoter in E. coli W3110 and W3110::Tcr (approximately fourfold maximum induction). That is, they found increased SdiA activity in response to CM through just p2 at a level (approximately fivefold) comparable to that which we observed (approximately fourfold maximum induction).

Finally, as we performed our experiments using CM from the same species (W3110 wild type and W3110 luxS::Tc') and in chemostat mode, where dynamic contributions were precluded, we were able to show that SdiA-mediated p2 activation involved the extracellular AI-2 signal. We do not, however, demonstrate that AI-2 acts directly on SdiA or any other regulatory gene, yet there is existing evidence that suggests the effect is indirect. For example, detection of AI-2 in V. harveyi occurs via the cognate sensor LuxPQ, where LuxP is homologous to the ribose binding protein of E. coli. Freeman and Bassler (17) have proposed that LuxP is the primary sensor for AI-2 and that the LuxP–AI-2 complex interacts with LuxQ for transduction of the signal to an intermediary protein (LuxU) prior to activation of the response regulator (LuxO). Interestingly, Kamamuru and colleagues found that culture supernatants of E. coli 0157:H7 bound the N-terminal portion of SdiA, and while the factor(s) was not AI-2, they proposed a dual quorum-sensing system involving AI-2 and perhaps AI-like homoserine lactone analogues might bimodally regulate the activity of SdiA (32).
The broad number of gram-negative bacteria which employ quorum sensing, coupled with the genetic and mechanistic similarities of their quorum systems, indicate that autoinduction is an evolutionarily conserved process used to orchestrate gene expression with population density. However, an evolving body of evidence supports the presumption that quorum sensing, coupled with the genetic and mechanistic overlap responses, for example induction of the psp operon (7).

In general, however, the perturbations observed in Fig. 4 may not be specific to each individual stress, but more likely are indicative of a shift in the metabolic activity or state of the cells caused by the individual stress. We hypothesize that this change in metabolic state (physiologically seen as a change in growth rate but which may include other phenomena) was then responsible for changes in AI-2 signaling. In support of this notion, cells cultured entirely at a temperature of 42°C showed a constant higher level of AI-2 signal compared to cells grown entirely at 30°C. Importantly, this suggests cells cultured at a higher metabolic rate (i.e., higher growth rate) produce a higher level of AI-2 activity more than threefold within the first 30 min. Contrarily, several stimuli (heat shock, ethanol, H$_2$O$_2$, serine hydroxamate, sodium acetate, hIL-2 overexpression, and oxygen) not only elicited a decrease in AI-2 activity but also increased degradation (or possibly inhibition). Of note, both heat and ethanol shock resulted in immediate down-regulation followed by highly reproducible oscillatory AI-2 responses, which, to our knowledge have not been previously reported. The observation that these two stimuli elicited similar AI-2 responses is not entirely surprising, as heat shock and ethanol shock exhibit overlapping responses, for example induction of the psp operon (7).

TABLE 2. Steady-state (μ = 0.75 h$^{-1}$) AI-2 produced by mutant strains of E. coli at 30°C

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic or genotype</th>
<th>Specific fold AI-2 activity (s.s.$^*$)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B178</td>
<td>W3110 galE relA</td>
<td>518</td>
<td>F. Baneyx</td>
</tr>
<tr>
<td>CG712</td>
<td>B178 zjd::Tn10 groES30</td>
<td>972</td>
<td>52</td>
</tr>
<tr>
<td>CG714</td>
<td>B178 groEL140</td>
<td>1,014</td>
<td>52</td>
</tr>
<tr>
<td>JZ12</td>
<td>B178 zjd::Tn10 groES519 zjc::Kan$^*$</td>
<td>1,137</td>
<td>60</td>
</tr>
<tr>
<td>C600</td>
<td>F$^-$/thi thi1 leuB6 lacY1 tonA21 supE44 λ$^-$/</td>
<td>578</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>CG804</td>
<td>C600 pheA::Tn10 gypE280</td>
<td>442</td>
<td>3</td>
</tr>
<tr>
<td>MF746</td>
<td>C600 dnaK756</td>
<td>335</td>
<td>59</td>
</tr>
<tr>
<td>MF634</td>
<td>C600 dnaJ259</td>
<td>479</td>
<td>59</td>
</tr>
</tbody>
</table>

$^*$ Steady-state (s.s.) AI-2 activity level divided by OD$_{600}$. Fold activation represents the induction of luminescence (relative light units) in V. harveyi reporter strain BB170 (sensor 1 'sensor 2') from steady-state E. coli cultures grown in LB-50 mM glucose in relation to luminescence (relative light units) with fresh medium alone. Values are reported as the average of three independent activity measurements from three independent steady-state samples. OD$_{600}$ of all cultures and replicate samples agreed within 10%.
and/or capacity of stressors have been observed to decrease the metabolic activity of hIL-2 overexpression, in that all three support of this idea is seen in the responses to sodium acetate, magnitude-independent gene expression by steady-state W3110/phIL-2 cultures. Note, magnitude of hIL-2 expression with pulse addition of 1 mM IPTG at t = 120 min (black arrow). Seven genes clustered (correlation coefficient of 0.74) following induction of steady state-culture (b) and average Z value (mRNA response) of seven clustered genes (c). Z values were calculated as (sample − average)/standard deviation to allow examination of the dynamic changes in a variable independent of magnitude.

![Graph of relative AI-2 expression](image)

**FIG. 7.** Dynamic profile of highest correlated gene cluster during hIL-2 expression by steady-state W3110/phIL-2 cultures. Note, magnitude-independent Z values are reported. (a) AI-2 response to induction of hIL-2 expression with pulse addition of 1 mM IPTG at t = 0 and t = 120 min (black arrow). Seven genes clustered (correlation coefficient of 0.74) following induction of steady state-culture (b) and average Z value (mRNA response) of seven clustered genes (c). Z values were calculated as (sample − average)/standard deviation to allow examination of the dynamic changes in a variable independent of magnitude.

support of this idea is seen in the responses to sodium acetate, serine hydroxamate, and hIL-2 overexpression, in that all three stressors have been observed to decrease the metabolic activity and/or capacity of *E. coli* (11, 31, 42). Therefore, it was not entirely surprising that the production of AI-2 was observed to decrease relative to the steady-state level following each of these perturbations. Overall, these results demonstrate the utility of transient AI-2 profiling for comparative stimulus analysis, thereby elucidating future studies involving overlapping regulatory mechanisms.

Lastly, we attempted to correlate quorum-dependent transcriptional regulation with a subset of genes known to be up-regulated in response to recombinant protein overexpression in batch culture (22). The transient transcriptional response of 24 genes, including four known quorum-related genes (*sdiA*, *luxS* _E.c._, *ftsQ*, and *ftsA*), to hIL-2 production, following an SOM-clustering reduction algorithm (15), resulted in 10 genes (*recA*, *rpoS*, *ftsI*, *groEL*, *dnaK*, *grpE*, *clpP*, *luxS*, *ftsQ*, and *ftsA*) having highly significant statistical correlation (r = 0.74). The inclusion of *rpoS* in this cluster was not entirely surprising, as there is a growing body of evidence, including the presence of quorum and *rpoS*-regulated promoters upstream of *ftsQA* (44) and the induction of *rpoS* and *ftsQA* by homoserine lactone (29, 44), that *E. coli* may use quorum sensing as a signal to prepare for entry into stationary phase (reviewed in reference 35). Also, it is well-known that *dnaK*, *grpE*, *groEL*, and *recA* are all intimately involved in the folding of overexpressed heterologous proteins as well as the clearance of misfolded aggregates and premature polypeptides. Therefore, the similarity of responses among these genes, which includes four genes involved in the *E. coli* quorum circuit (*luxS* _E.c._, *sdiA*, *ftsQ*, and *ftsA*), suggests that autoinduction is intimately intertwined with the stress response to abnormal protein formation as well as other related stress responses (25). Consistent with these findings, both *recA* and *groEL*, which are similarly upregulated following hIL-2 expression, have been implicated in regulating lux expression in bioluminescent *E. coli* (53). Lastly, chemostat cultures of *groEL140*, *groES30*, and *groES619* and *dnaK756*, *dnaJ259*, and *grpE280* further demonstrated the link between these stress-induced genes and AI-2 quorum signaling, as all produced altered levels (ca. 2-fold difference) of AI-2 quorum signal relative to those of their parent strain at 30°C. These results provide a direct link between AI-2 signaling and O23-mediated proteins, suggesting that a chaperone-mediated folding pathway exists that directly affects the accumulation of extracellular AI-2 under nominal growth conditions, similar to that proposed for bioluminescent *E. coli* (52).

Finally, it is well-known that environmental adaptation in *E. coli* involves increased rates of spontaneous mutation, shuffling or rearrangement of genomic content, and DNA methylation. We previously hypothesized that an adaptation of this nature was likely occurring during the transition to high cell density and during recombinant protein overexpression (20), and while this hypothesis was not specifically tested here, the dissimilar responses observed following the initial and subsequent pulse addition of IPTG is likely indicative of culture adaptation to heterologous protein formation. Similar adaptation, consistent with this result, has been demonstrated for glucose uptake and protease up-regulation in response to two consecutive pulses inducing recombinant protein expression (27).

Importantly, quorum sensing in gram-negative bacteria is an excellent example of how individual prokaryotic cells transduce physiologically based signals into coordinated, multigenic responses. An emerging blueprint holds that the *E. coli* quorum circuit is regulated by a complex, overlapping network built upon a distinct autoinducer molecule (AI-2), synthesized by LuxS (homologous to *V. harveyi* LuxS), and used to channel environmental information to a response regulator protein, SdiA (homologous to *V. fischeri* LuxR), for density-dependent control of gene expression. We have demonstrated that studying the transients in chemostat culture has improved our knowledge of global regulation of AI-2 in the prevailing bioreactor environment, where the effect of transients, such as physical and/or chemical gradients, on overall cell and/or heterologous protein productivity are poorly defined. Understanding the role of cell-to-cell signaling in complex environ-
ments (e.g., high density, low oxygen solubility, substrate and nutrient gradients or limitations, evolution of excess metabolic heat, and overflow metabolism) common among numerous manufacturing bioprocesses may begin to explain attenuated productivity as well as many other physiological changes (i.e., cell morphology, increased lysis rates, reduced metabolic activity rates, and decreased reproductive viability) that occur as cells progress to higher densities rarely seen in the natural environment.

ACKNOWLEDGMENTS

We thank B. Bassler, P. Dunlap, and F. Baneyx for generously providing strains used in this study.

This research was supported by the U.S. Army Engineering Research and Development Center, Edgewood, Md. (grant DAAH01-96-0037).

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