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Escherichia coli genes regulated by cell-to-cell signaling

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ABSTRACT **Utilizing the bicistronic reporter transposon mini-Tn*5 lacZ-tet/1*, we have identified *lacZ* fusions to four *Escherichia coli* genes/operons that are strongly activated by the accumulation of self-produced extracellular signals. These fusions were designated *cma9, cma48, cma113,* and *cma114* for conditioned medium activated. Each of the *cma* fusions was expressed in a growth phase-dependent manner, and the presence of conditioned medium from a stationary phase *E. coli* culture resulted in the premature activation of these fusions in cells at early to mid-logarithmic phase. The *cma48* and *cma114* fusions were dependent on RpoS for growth phase expression and response to extracellular factors. The extracellular factors that activated the *cma9, cma48,* and *cma114* fusions were produced in both rich complex and defined minimal media. The *cma* fusions were shown to be within the *cysK* (*cma9*), *astD* (*cma48*), *tnaB* (*cma113*), and *gabT* (*cma114*) genes. These genes function in the uptake, synthesis, or degradation of amino acids that yield pyruvate and succinate.**

Bacteria are capable of regulating gene expression in response to a variety of extracellular signals. When the signal is produced by the bacterium itself, this type of regulation is termed autoinduction or quorum sensing (1–4). The composition of signaling molecules can include mixtures of amino acids, peptides, fatty acids, and acyl derivatives of homoserine lactone (1–4, 5–7). These signaling molecules can regulate gene expression by a number of mechanisms, including modulating the activity of members of the LuxR family, interacting with two-component systems, and inhibiting phosphatases (1, 3, 8–10). The cellular processes regulated by quorum sensing are diverse, and some examples include spore formation, activation of luminescence, competence, conjugal transfer of plasmid DNA, regulation of virulence genes, regulation of peptidoglycan O-acetylation, and biofilm maturation (11–20).

In *Escherichia coli,* the regulation of gene expression by extracellular signals has also been established. Expression of the *rpoS* gene, encoding the alternate sigma factor σ^S (σ^{38}) involved in stationary-phase and osmoregulated gene expression is stimulated by the presence of a factor in conditioned medium (21, 22). Studies by Huisman and Kolter (23) indicate that *rpoS* expression is decreased in a *thrA, metL, lysC* triple mutant that is defective in production of homoserine, and expression could be restored by exogenous homoserine lactone. However, the role of homoserine lactone or an acylated derivative in RpoS expression is unclear. Additional *E. coli* genes subject to regulation by extracellular factors include *sdiA* and the *ftsQAZ* cell division gene cluster (22, 24). Recent studies by Surette and Bassler (25) have revealed that *E. coli* produces a signal that can substitute for AI-2, one of two *Vibrio harveyi* signals that control luminescence gene expression. The *E. coli* signal was heat labile, produced at mid-exponential phase of growth, and degraded at stationary phase. In addition, *E. coli* DH5α did not produce the signal (25).

Given the extensive studies in *E. coli,* it is surprising that only three genes have been identified as quorum sensing regulated. The role of quorum sensing in the stationary-phase physiology of *E. coli* and other nondifferentiating bacteria is largely unknown, although a recent study has shown a role in the inhibition of chromosomal replication (26). To expand on these areas, a search was initiated for *E. coli* genes that are activated by the accumulation of extracellular factors. A subset of these genes appear to have a role in amino acid metabolism.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* strains MC1061, MG1655, and GS015 *rpoS*::Tn*10* (27–29) were used in this study. To prevent lysogenization by λ *pir* released from donor strains, a bacteriophage λ and rifampicin-resistant derivative of MC1061, designated MC1061.LR, was used as a host strain for transposon mutagenesis. *E. coli* MCS1061 *rpoS*::Tn*10* was constructed by bacteriophage P1 transduction of MC1061 using a lysate prepared from GS015 (29). Derivatives of MC1061 (MT9, MT48, MT113, and MT114) and MCS1061 *rpoS*::Tn*10* (MTS9, MTS48, MTS113, and MTS114) containing the *cma* fusions were constructed by P1 transduction using lysates prepared from the MC1061.LR mini-Tn*5 lacZ-tet/1* derivatives. Plasmids pBR325 and pACYC184 were used as cloning vectors. Growth medium consisted of LB broth containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. M9 medium was modified to contain lower concentrations of salts and consisted of 3 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 0.25 g of NaCl, and 0.5 g of NH_4Cl per liter. After autoclaving, the following were added per liter: 10 ml of 0.01 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, and glycerol to a final concentration of 0.4%. Antibiotics were used at the following concentrations: kanamycin 20 μg/ml, rifampicin 30 μg/ml, tetracycline 1 and 3 μg/ml, ampicillin 100 μg/ml, and chloramphenicol 25 μg/ml.

Transposon Mutagenesis. A library of mini-Tn*5 lacZ-tet/1* insertions in MC1061.LR was constructed by a plate mating. Briefly, 100-μl portions of overnight cultures of MC1061.LR and S17.1λ *pir*/pVD24 (30) were mixed together and spotted on an LB agar plate. After growth for 18 hr at 37°C, the mating mixture was resuspended in 10 ml of LB broth and dilutions were plated on LB agar plates containing kanamycin (20 μg/ml), rifampicin (30 μg/ml), and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) at 100 μg/ml and incubated for 48 hr at 37°C. To isolate mini-Tn*5 lacZ-tet/1* insertions within genes expressed at high density, individual blue colonies were streaked for single colonies, using toothpicks, at a density of 30 restreaks per plate on each of the following three plates: LB + tetracycline (1 μg/ml), LB + tetracycline (3 μg/ml), and

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Abbreviation: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.
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a master plate of LB + kanamycin (20 μg/ml), rifampicin (30 μg/ml), and X-Gal. Tetracycline sensitivity was scored after 18 hr of growth as an obvious defect in single colony formation.

Preparation of Cell-Free Supernatants. Conditioned medium was prepared by inoculating 30 ml of LB broth with 5 μl of a 10^{-3} dilution of an overnight culture of *E. coli* MC1061 followed by shaking (250 rpm) at 37°C. Cells were typically harvested 3–4 hr after entering stationary phase (OD_{600} of 1.6–1.7), and cells were pelleted at 4300 × *g* for 10 min. The resulting supernatants were supplemented with the addition of 20× TY (tryptone, yeast extract) to a final concentration of 0.5×, and the pH was brought to 7.5. Conditioned M9 medium was prepared in a similar manner, except for harvesting at an OD_{600} of 1.5. Conditioned medium was then filter-sterilized through a 0.2-μm-pore filter and stored at −80°C. Freezing had no apparent effect on activity. Growth-phase expression of the *cma* fusions was done using 30 ml of supplemented conditioned medium or 0.5× LB as a control in 250-ml flasks. Each type of medium was inoculated at a final dilution of 1/1,000 from a low-density culture of the appropriate strain, and flasks were shaken at 300 rpm and 37°C.

Factor Purification in Minimal Medium. Conditioned medium was prepared from early stationary-phase cultures of MG1655 cells grown in 100 ml of M9 minimal medium. The medium was adjusted to pH 7.5 with NaOH and filter sterilized. The resulting supernatant was concentrated by lyophilization and resuspended at a 20× concentration in sterile water. Five milliliters of material was applied to a Sephadex G-10 column (Pharmacia), and 5-ml fractions were collected by elution with 0.04 M NaCl, pH 7.5. Under these conditions, fraction 12 typically represented the void volume, and the M9 salts eluted at fraction 22. Fractions 1–11 displayed no activity. Factor activity was found in the included volume, and recovery of factor activity after fractionation was 55–60%. Fractions were stored at −80°C. Upon thawing, a white precipitate was occasionally observed and was removed by centrifugation. Fractions were tested for activity by mixing 1 ml of each fraction with 2 ml of 0.5× LB, pH 7.5, and using the appropriate *cma* fusion as a biosensor. The activating factors eluted approximately one-third into the included volume, typically fraction 15 or 16. Fractions with activity were further purified by passing through a solid-phase C_{18} matrix, which was preconditioned and equilibrated as instructed (Sep-Pak, Waters). For the *cma9*, *cma48,* and *cma114* fusions, the activating factor did not bind to the C_{18} matrix, and activity was present in the flow-through. Purified material was stored at −80°C.

Identification of the *cma* Fusions. To clone the *cma* fusions, we used the restriction enzyme *Pst*I, which cuts once in mini-Tn*5 lacZ-tet/1* immediately after the *lacZ* gene. Southern blot analysis of four *cma* fusions was performed, using a *lacZ* probe to determine the size of the *Pst*I fragments containing the *lacZ* gene and adjacent chromosomal DNA. On the basis of the Southern blot analysis, chromosomal DNA from MT9, MT48, and MT113 was digested with *Pst*I, and DNA in the appropriate size range was purified by using a Gene-clean kit (Bio-101) and ligated to *Pst*I-digested pBR325. Ligations were electroporated into the XL1 Blue strain, and transformants were selected on medium containing chloramphenicol (25 μg/ml) and X-Gal. Blue colonies were subjected to restriction analysis to confirm the presence of the proper fragment. To clone the *cma114* fusion, a *Sau*3A partial digest was performed and ligated to *Bam*HI-digested pACYC184. Ligation mixtures were then plated on LB agar containing kanamycin (20 μg/ml) and X-Gal. Blue and kanamycin-resistant colonies contained the Km^R and *lacZ* genes from mini-Tn*5 lacZ-tet/1* and in some cases a portion of flanking chromosomal DNA. For all plasmids, the DNA sequence of chromosomal DNA at the insertion junction was determined by using the Cy5-labeled primer 5′-GGTTTTCCCAGTCACGACGTTG-3′, which reads outward from the 5′ end of *lacZ.* Sequencing reactions were done using a cycle sequencing kit (Amersham) and reactions were run on an ALF sequencer (Pharmacia).

RESULTS

Use of Mini-Tn5 *lacZ-tet/1* to Isolate *E. coli* Genes Regulated by Cell–Cell Signaling. A characteristic of gene regulation by quorum sensing is activation or repression at high cell density. To isolate genes activated by quorum sensing, we used a strategy similar to the strategies previously described for the isolation of stationary-phase and sporulation-specific genes (30, 31), except that an additional step was added to discriminate between stationary-phase expression and quorum sensing controlled loci. Utilizing the transposon mini-Tn*5 lacZ-tet/1* (30), we constructed a library of random mini-Tn*5 lacZ-tet/1* insertions in *E. coli* MC1061.LR as described in *Materials and Methods*. This transposon contains promoterless *lacZ* and tetracycline-resistance genes in tandem as transcriptional reporters and a kanamycin-resistance gene for selection of transposition events. Resulting colonies were allowed to fully develop for 48 hr to identify fusions expressed at all phases of growth. Fusions of *lacZ-tet/1* within a gene expressed during the early stages of growth will result in a blue colony on X-Gal plates that is tetracycline resistant. However, an insertion in a gene expressed only at high cell density (late-logarithmic or stationary phase) will display a tetracycline-sensitive phenotype when restreaked for single colonies. Using this strategy, we screened approximately 900 mini-Tn*5 lacZ-tet/1* insertions with a blue colony phenotype, and 166 of these grew poorly or not at all when restreaked on tetracycline plates. These colonies presumably contained insertions in genes expressed during late-logarithmic or stationary phase. To determine whether the mini-Tn*5 lacZ-tet/1* insertions were within a transcription unit activated by the accumulation of an extracellular signal, we screened individual insertions at low cell density (OD_{600} = 0.3–0.4) for stimulation of β-galactosidase expression in the presence of conditioned medium prepared from a stationary-phase culture of MC1061 (see *Materials and Methods*). This allowed for the identification of 16 fusions which displayed at least a 2.5-fold increase in β-galactosidase accumulation when grown in the presence of conditioned medium. These fusions were designated *cma* (conditioned medium activated) The vast majority of fusions displayed similar levels of β-galactosidase accumulation in the presence or absence of the conditioned medium, indicating that conditioned medium itself did not affect β-galactosidase activity. Four mini-Tn*5 lacZ-tet/1* fusions, activated between 8- and 15-fold by conditioned medium, were chosen for further study, and P1 transduction was used to move the mini-Tn*5 lacZ-tet/1* insertion into a fresh MC1061 background.

Activation of the *cma* Fusions by Cell-to-Cell Signaling. The expression of each *cma* fusion in MC1061 was monitored by β-galactosidase accumulation at various stages of growth in LB or in conditioned medium (Fig. 1). In these experiments, nutrients in the conditioned medium were replenished with concentrated tryptone and yeast extract to a final concentration of 0.5×, and the pH of both the conditioned medium and the LB was adjusted to 7.5. Cells exhibited similar doubling times in LB or in the supplemented conditioned medium up to mid-logarithmic phase at an OD_{600} of 0.4–0.6 (data not shown). After this stage in growth, cells in the conditioned medium grew more slowly.

In cells grown in LB broth only, the expression of β-galactosidase from each fusion was increased in a density-dependent manner (Fig. 1). The induction ratio for each fusion between cells at early-logarithmic phase vs. stationary phase was 11-fold (*cma9*), 82-fold (*cma48*), 9-fold (*cma113*), and 74-fold (*cma114*). A control fusion, *zxx3,* did not exhibit significant changes in expression at different phase of growth (Fig. 1). The presence of conditioned medium from early

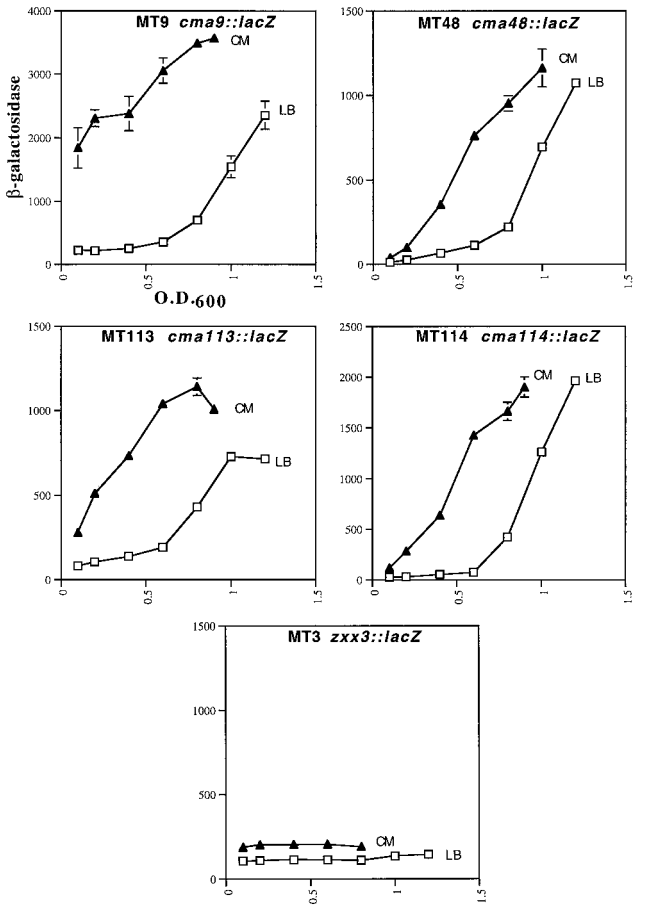

FIG. 1. Effect of conditioned medium on expression of the *cma* fusions. The accumulation of β-galactosidase from the *cma* fusions at various stages of growth is shown. Cells were grown in 0.5× LB (LB) or in conditioned medium from early stationary-phase cells (CM). Each point in growth represents an OD_{600} of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2. The β-galactosidase values are in Miller units and represent the average of duplicate samples from a typical experiment. Repeated experiments gave similar values.

stationary phase cultures resulted in the premature activation of each *cma* fusion in cells at early to mid-logarithmic phase (Fig. 1). At mid-logarithmic phase, the induction of each fusion in the presence of conditioned medium vs. LB was 11-fold for *cma9,* 7-fold for *cma48,* 5-fold for *cma113,* and 19-fold for *cma114.* The induction of each fusion by conditioned medium became less pronounced as cells reached high density.

Control experiments ruled out the possibility that oxygen depletion was responsible for activation of the *cma* fusions at high density or by conditioned medium. Concentrated conditioned medium was able to activate the *cma* fusions when it represented only 15% of the culture volume. In addition, conditioned medium prepared from *Mycobacterium smegmatis* at the same OD as *E. coli* had no significant effect on expression of the *cma* fusions (data not shown). These data and additional controls indicate that activation of the *cma* fusions by *E. coli* conditioned medium did not result from (*i*) a reduced growth rate, (*ii*) O_2 depletion, (*iii*) nutrient depletion, or (*iv*) changes in medium pH. We conclude that activation of the *cma* fusions occurs in response to the production of one or more extracellular factor(s) produced by *E. coli*.

Role of RpoS in *cma* Expression. The expression of each *cma* fusion was examined in isogenic MC1061 and MCS1061 (*rpoS*::Tn*10*) backgrounds in cells at mid-logarithmic and early-stationary phase. As shown in Table 1, the accumulation of β-galactosidase from the *cma9* and *cma113* fusions in mid-logarithmic and early-stationary phase cells was not significantly altered by the *rpoS*::Tn*10* allele. In contrast, the accumulation of β-galactosidase from the *cma48* fusion in stationary-phase cells was reduced 4.2-fold in the *rpoS*::Tn*10* background, relative to wild-type. The *cma114* fusion exhibited an even greater dependence on *rpoS* in stationary phase, where an 18-fold reduction in β-galactosidase accumulation was seen in the *rpoS*::Tn*10* background.

Table 1. Role of *rpoS* in expression of the *cma* fusions

Strain	Fusion	β-Galactosidase accumulation* Mid-log†	Stationary phase‡
MT9	*cma9*	248 ± 9	1,847 ± 202
MTS9 *rpoS*::Tn*10*	*cma9*	214 ± 38	1,796 ± 27
MT48	*cma48*	23 ± 1	460 ± 7
MTS48 *rpoS*::Tn*10*	*cma48*	18 ± 1	110 ± 2
MT113	*cma113*	83 ± 7	1,182 ± 109
MTS113 *rpoS*::Tn*10*	*cma113*	96 ± 6	1,959 ± 122
MT114	*cma114*	36 ± 5	992 ± 47
MTS114*rpoS*::Tn*10*	*cma114*	26 ± 1	55 ± 8

*Determined in Miller units; mean ± SD.
†Determined in cells at OD_{600} of 0.6.
‡Determined in cells at OD_{600} of 1.4.

The role of *rpoS* on the activation of each fusion by the conditioned medium was then examined in cells at early-logarithmic phase. Wild-type MC1061 or MCS1061 *rpoS*::Tn*10* cells containing each *cma* fusion were grown in LB or in LB + conditioned medium and harvested at early-logarithmic phase. The activation of the *cma9* and *cma113* fusions by conditioned medium was similar in the wild-type and *rpoS*::Tn*10* backgrounds (data not shown). For the *cma48* fusion, the activation by conditioned medium was slightly lower (2.8-fold) in the *rpoS*::Tn*10* background relative to wild-type (4.0-fold). In contrast, *cma114* was strongly dependent on *rpoS* for activation by conditioned medium, where a 1.7-fold induction was observed in the *rpoS*::Tn*10* background, relative to the 8.3-fold induction observed in wild-type cells.

Characterization of the Extracellular Factor(s). The extracellular factors activating each *cma* fusion were resistant to pH 12 for 20 min and heating to 100°C for 10 min. Acid treatment of the conditioned medium (pH 2 for 20 min) resulted in a 20–35% decrease in activation of the *cma* fusions, relative to untreated conditioned medium. Production of the factor(s) by *E. coli* was observed in LB broth without glucose, with optimal production at early stationary phase. Using each *cma* fusion as a biosensor, we produced the factors in a variety of *E. coli* strains, including MG1655, DH5α, MCS1061 *rpoS*::Tn*10*, and CF1693 Δ*relA*/Δ*spoT.* The ability of various metabolites to activate the *cma* fusions was examined. The addition of pyruvate (2 mM), α-ketoglutarate (2 mM), homoserine lactone (5 mM), homocysteine thiolactone (5 mM), acetate (2 mM), Casamino acids (0.5%), or EDTA (5 μM) had no significant effect on expression of the *cma* fusions in cells at low density (data not shown). Furthermore, the factor(s) activating the *cma* fusions were produced at equivalent amounts in the presence of excess iron (200 μM) and are unlikely to be siderophores.

Partial Purification of the Activating Factors. Conditioned medium prepared from a defined M9 salts medium + 0.4% glycerol was tested for activation of the *cma* fusions. Weak activation was observed, ranging from 1.5- to 2-fold. However, when conditioned M9 glycerol medium was concentrated 20-fold by lyophilization and fractionated by gel filtration on a Sephadex G-10 column (*Materials and Methods*), fractions exhibiting significant activation of the *cma* fusions were identified. The relative activation of each fusion by the fractions is shown in Fig. 2. Peak activation values were 4-fold for *cma9* (fraction 16), 3-fold for *cma48* (fraction 15), 2-fold for *cma113*

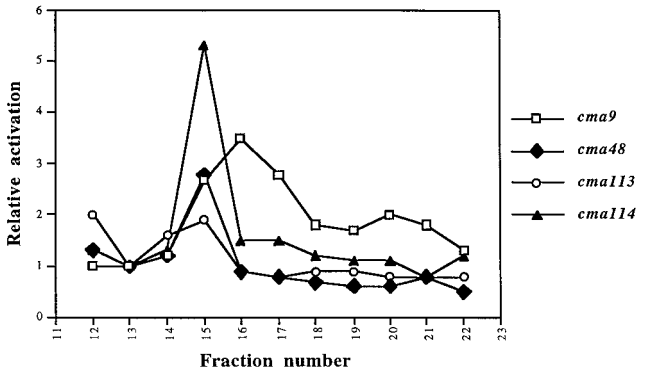

FIG. 2. Fractionation of the factors involved in *cma* activation. Conditioned medium prepared from M9 minimal medium + glycerol was fractionated on a Sephadex G-10 column as described in the text. Column fractions 11 and 12 represented the void volume, and the M9 salts eluted at fraction 22. For fractions 12–22, the same column fraction was used to assay each of the four *cma* fusions by mixing 1 ml of fraction with 2 ml of 0.5× LB. The activation of each fusion was determined by β-galactosidase expression in cells at early-logarithmic phase and was normalized to the levels of expression in the absence of factor.

(fraction 15), and 5-fold for *cma114* (fraction 15). Conditioned medium was also prepared from cells grown in M9 salts + 1% Casamino acids. This conditioned medium supported a stronger activation of the *cma9, cma48,* and *cma114* fusions, ranging from 5- to 11-fold (data not shown). The *cma113* fusion was activated 3-fold under these conditions. Fractionation of the conditioned medium prepared from M9 + Casamino acids resulted in fractions with similar levels of activation for the *cma9, cma48,* and *cma113* fusions as seen in Fig. 2. However, for the *cma114* fusion, the peak fraction resulted in a 10-fold activation.

Preliminary experiments indicated that the factors activating the *cma9, cma48,* and *cma114* fusions were polar and did not bind efficiently to a reversed-phase C_{18} column under several different conditions. Conditioned medium prepared from cells grown in M9 glycerol medium was fractionated by gel filtration, and fractions containing activity were identified by using each *cma* fusion as a biosensor. Active fractions were then passed through a solid-phase C_{18} matrix. This provided a convenient step for additional purification of active fractions off the G-10 column, and the activity for the *cma9, cma48,* and *cma114* fusions was present in the flow-through. Purified factors were then used to examine the dose dependence for activation of the *cma* fusions. On the basis of the results in Fig. 1, in which optimal activation of the *cma* fusions occurred at mid-logarithmic phase, this condition was used for the dose-dependence assays. As shown in Table 2, activation of the *cma9*, *cma48,* and *cma114* fusions was dose dependent with little or no activation occurring at a level below 0.15× purified material.

Chromosomal Location of the *cma* Fusions. Each of the mini-Tn*5 lacZ-tet/1* fusions and flanking upstream chromosomal DNA were cloned as described in *Materials and Methods*. The chromosomal location of each *cma* fusion was determined by DNA sequence analysis and homology searches were conducted using the BLAST server.

cma9. The *cma9* fusion contains mini-Tn*5 lacZ-tet/1* inserted within the *cysK* gene, which encodes *O*-acetylserine lyase A. This enzyme catalyzes the formation of L-cysteine from *O*-acetylserine and sulfide (32).

cma48. The *cma48* fusion contains mini-Tn*5 lacZ-tet/1* inserted in the *astD* gene at 39.4 minutes. The *astD* gene is the third gene of a putative five-gene operon (*astCADBE*), which functions in the degradation of arginine and ornithine to generate succinate and glutamate (33). The AstD protein is a probable succinylglutamate-semialdehyde dehydrogenase.

Table 2. Dose response of the *cma* fusions to purified factors

Growth condition*	Fusion	β-Galactosidase†
LB	*cma9*	430 ± 25
CM 0.3×	*cma9*	1644 ± 95
CM 0.15×	*cma9*	950 ± 35
CM 0.075×	*cma9*	645 ± 12
LB	*cma48*	87 ± 5
CM 0.3×	*cma48*	327 ± 34
CM 0.15×	*cma48*	192 ± 11
CM 0.075×	*cma48*	81 ± 1
LB	*cma114*	82 ± 2
CM 0.3×	*cma114*	706 ± 79
CM 0.15×	*cma114*	219 ± 11
CM 0.075×	*cma114*	108 ± 4

*The conditioned medium (CM) concentration represents the amount of active fraction used.

†Determined in Miller units at an OD_{600} of 0.35. Values represent the mean ± SD of quadruplicate samples from two independent experiments.

cma113. The *cma113* fusion contains mini-Tn*5 lacZ-tet/1* inserted in the *tnaB* gene, which encodes a low-affinity tryptophan permease and is the distal gene within the *tnaAB* operon (34, 35). The tryptophanase enzyme encoded by *tnaA* is capable of degrading tryptophan, serine, and cysteine to pyruvate and NH_3, and in the case of tryptophan, indole is also produced (36).

cma114. The *cma114* fusion contains mini-Tn*5 lacZ-tet/1* inserted in the *gabT* gene, encoding a glutamate:succinate semialdehyde aminotransferase. The *gabT* gene is located within the *gabDTP* operon, in which *gabD* encodes an NADP-dependent succinate-semialdehyde dehydrogenase and *gabP* encodes a permease for γ-aminobutyrate (37, 38). This operon functions in the generation of succinate from γ-aminobutyrate.

DISCUSSION

We have developed a screen for the identification of quorum sensing regulated genes in *E. coli*. By using this screen, the *cysK, astD, tnaB,* and *gabT* genes have been identified as activated by the accumulation of self-produced extracellular factors. In the course of screening approximately 900 random mini-Tn*5 lacZ-tet/1* fusions, a total of 16 quorum sensing activated fusions were identified. Given the 4,300 genes present in *E. coli* (27), these 16 fusions potentially represent only 20–25% of the total number of quorum sensing regulated genes. Therefore, quorum sensing may have a global role in the regulation of gene expression in *E. coli.* A subset of the cellular functions controlled by quorum sensing should be revealed by analysis of the remaining *cma* genes. The quorum sensing activated *cysK*, *astCADBE*, *tnaAB,* and *gabDTP* loci identified in this study function in the uptake, synthesis, and catabolism of amino acids to generate pyruvate or succinate. This may be important for energy production in stationary phase.

As shown in Fig. 1, the presence of these factors resulted in the premature activation of the *cma* genes in early to mid-logarithmic phase. Interestingly, the activation profiles were different for each gene. For example, the *cma9* (*cysK*) and *cma113* (*tnaAB*) fusions were activated at very early-logarithmic phase (OD_{600} = 0.1) in the presence of conditioned media. In contrast, the *cma48* (*astD*) and *cma114* (*gabT*) fusions were not fully activated by conditioned medium until early to mid-logarithmic phase. This finding may indicate a lower concentration threshold for activation of the *cma9* and *cma113* fusions. Alternatively, since both the *cma48* and *cma114* fusions are RpoS dependent (Table 1), there may be

insufficient levels of RpoS at early-logarithmic phase for optimal activation of these fusions.

The extracellular factors that activated the *cysK, astD,* and *gabT* genes were polar and were produced in both complex and defined medium. The factor activating the *tnaAB* operon was preferentially produced in rich LB medium and displayed hydrophobic properties. This information suggests the involvement of at least two distinct factors for activation of the different *cma* genes. Results from gel-filtration chromatography also support the existence of more than one factor. Peak activity for the *cma48* and *cma114* fusions was observed in a different fraction from the peak fraction for *cma9*.

A number of characteristics suggest that the factors that activate the *cma* genes are distinct from the *E. coli* factor that activates the signaling system-2 pathway of *Vibrio harveyi* (25). These characteristics include stability to base and 100°C, optimal production at stationary phase, production by *E. coli* DH5α, and production in medium without glucose. Thus, it appears that *E. coli* has at least two quorum sensing systems, one that operates at intermediate density described by Surette and Bassler (25), and the system described in this study, which operates at stationary phase. The *cma* fusions can now be used as biosensors to isolate and determine the structure of the chemical signal(s) that activate these genes. In addition, pathway(s) involved in response to the extracellular signals are being identified by the isolation of extragenic mutations that alter the expression of these fusions.

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