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Mechanisms of Iron Regulation of Luminescence in *Vibrio fischeri*

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Synthesis of luciferase (an autoinducible enzyme) is repressed by iron in the symbiotic bioluminescent bacterium *Vibrio fischeri*. Possible mechanisms of iron regulation of luciferase synthesis were tested with *V. fischeri* and with *Escherichia coli* clones containing plasmids carrying *V. fischeri* luminescence genes. Experiments were conducted in complete medium with and without the synthetic iron chelator ethylenediamine-di(*o*-hydroxyphenyl acetic acid). Comparison of the effect of ethylenediamine-di(*o*-hydroxyphenyl acetic acid) and another growth inhibitor, (2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide), showed that iron repression is not due to inhibition of growth. A quantitative bioassay for autoinducer was developed with *E. coli* HB101 containing pJE411, a plasmid carrying *V. fischeri* luminescence genes with a transcriptional fusion between *luxI* and *E. coli lacZ*. Bioassay experiments showed no effect of iron on either autoinducer activity or production (before induction) or transcription of the *lux* operon. Ethylenediamine-di(*o*-hydroxyphenyl acetic acid) did not affect luciferase induction in *E. coli* strains with wild-type iron assimilation (ED8654) or impaired iron assimilation (RW193) bearing pJE202 (a plasmid with functional *V. fischeri lux* genes), suggesting that the genes responsible for the iron effect are missing or substituted in these clones. Two models are consistent with the data: (i) iron represses autoinducer transport, and (ii) iron acts through an autoinduction-independent regulatory system (e.g., an iron repressor).

Luminescence in *Vibrio fischeri*, a marine bioluminescent bacterium, is regulated by a small molecule [autoinducer; *N*-(β-ketocaproyl) homoserine lactone] excreted by the bacterium that, when it reaches a critical concentration, induces synthesis of the components of the luminescence system (14). This phenomenon is known as autoinduction. Factors affecting autoinduction include oxygen availability (18) and the composition of the growth medium (10). The addition of iron to low-iron minimal medium has been shown to repress induction of luciferase, whereas iron chelation of complete medium with ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDA), a specific iron chelator, causes induction at lower cell densities than control cultures (10).

Luminescence, unlike other iron-regulated systems such as iron transport, has no obvious function under conditions of limiting iron, and it is not clear why it should be subject to iron regulation. Understanding the mechanism of iron regulation of luminescence may thus help to clarify the relationship between iron metabolism and bioluminescence. Accordingly, in this paper, we examine the possible mechanisms whereby iron might regulate the synthesis and activity of luciferase in *V. fischeri*. Responses of marine bacteria to low-iron conditions have rarely been studied, yet are potentially important since marine environments are especially low in iron relative to terrestrial and freshwater environments (Haygood, Ph.D. thesis, University of California, San Diego, 1984). Iron regulation of luminescence may also play a role in the symbiotic associations of *V. fischeri* (10).

We have considered here two mechanisms that could be responsible for iron repression of luminescence: (i) interactions between iron repression and autoinduction, and (ii) mechanisms in which iron repression and autoinduction are independent. In the first category four mechanisms can be postulated: (i) low iron might slow cell growth while autoinducer production continues, so that the critical concentration is reached at a lower cell density; (ii) the autoinducer might be inactivated by the binding of iron, resulting in effectively lower concentrations of autoinducer under high-iron conditions; (iii) iron might repress the production of autoinducer; (iv) low iron might increase sensitivity to the autoinducer, perhaps by increasing its transport in analogy to the low-iron effect on amino acid transport in *Escherichia coli* (2).

At least two mechanisms can be postulated in which autoinduction and iron repression are separate phenomena, although they may interact. First, iron might act directly on transcription of the *lux* operon, independently of autoinducer. Second, iron might act indirectly on transcription via either mechanisms such as the undermodified tRNAs in *E. coli* under low-iron stress (2) or a regulatory protein activated or inactivated by iron such as that postulated to occur in *E. coli* (7). The experiments described here suggest either that iron reduces autoinducer transport or that an iron-regulatory system separate from autoinduction exists.

MATERIALS AND METHODS

Media. Artificial seawater-glycerol (ASG) contains (per liter) 15.5 g of NaCl, 0.75 g of KCl, 12.35 g of $MgSO_4 \cdot 7H_2O$, 2.9 g of $CaCl_2 \cdot 2H_2O$, 1 g of NH_4Cl, 0.1 g of glycerophosphate, 3 ml of glycerol, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 2 mM $NaHCO_3$, and 16 mg of ferric ammonium citrate (FAC) unless otherwise noted; FAC (filter sterilized, 0.2-μm Gelman Acrodiscs) HEPES, and $NaHCO_3$ were added as 100× stock solutions after autoclaving. FAC must be made up fresh to prevent precipitation. Seawater complete (SWC) contains 5 g of peptone, 3 g of yeast extract, and 3 ml of glycerol per liter of 75% seawater and is buffered with 20 mM HEPES (pH 7.4). The iron contributed to SWC by yeast extract and peptone is calculated to be approximately 18 μM (4). Luria (L) broth contains 15 g of yeast extract, 10 g of tryptone, and 10 g of NaCl per liter of distilled water. Nutrient broth was obtained from Difco Laboratories. Solid media for plates contained 15 g of agar per liter. EDDA

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(Sigma Chemical Co.) was deferrated by the method of Rogers (17), washed with cold distilled water, and dried at 50°C. The stock solution (1.4 mM) was made up in 1 M NaOH, and the pH was adjusted to approximately 9. Other iron-binding reagents (deferriferrichrome A, dipyridyl, and KCN) gave results similar to those with EDDA (data not shown). The stock solution of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO; Sigma; 190 μM) was made up in 10 mM NaOH.

Maintenance of bacteria. *V. fischeri* strain MJ1 was isolated from *Monocentris japonicus* (18). *E. coli* K-12 AN193 (F^- B_1^- *tonA ent A proC leu− trp− lacY rpsL galK ara mtl syl azi tsx supE44*), a mutant defective in enterobactin synthesis and ferrichrome uptake; RW193 (same as AN193 but *tonA+*), a mutant defective in enterobactin synthesis; RWB18 (same as RW193, but $ColB^r$ and fep^-), lacking the enterobactin receptor; and RWB7 (same as RW193, but *tonB*), a *tonB* mutant deficient in all high-affinity iron uptake, were provided by J. B. Neilands (16, 19). The *ompR* mutant used is derived from LE392 (F^- *hsdR supE supF lacY galK galT metB trpR* $lambda^-$) (11). Plasmid pJE202 (5) was transformed in RW193 and in ED8654, a strain of *E. coli* with wild-type enterobactin synthesis and transport, by J. Engebrecht. pJE411 is pJE202 with a mini-Mu insertion in *luxI* in the proper orientation for transcriptional fusion between *luxI* and *lacZ* (which codes for β-galactosidase) carried on the mini-Mu. pJE411 in *E. coli* strain HB101 was provided by J. Engebrecht (5, 6). MJ1 cultures were maintained on ASG and SWC plates for preparation of overnight cultures used to inoculate experiments. All *E. coli* strains were maintained on L agar with appropriate antibiotics. It was necessary to transfer ASG plate cultures at 1- or 2-day intervals to ensure good growth when inoculated into liquid ASG; cultures on L and SWC plates were transferred weekly. Broth culture experiments were inoculated from liquid overnight cultures grown in the same medium. Experiments with *V. fischeri* were at 20°C, and those with *E. coli* were at 29°C.

Complete medium plate experiments. SWC soft agar (2.5 ml, 7.5 g of agar per liter) at 45°C with the indicated level of EDDA was mixed with 3 drops of SWC overnight culture and poured on SWC plates. Sterile concentration disks containing 0.3% FAC or pure synthetic autoinducer (100 μg/ml in ethyl acetate; 10 μl added and then evaporated) were placed on the plates. The plates were examined for growth and luminescence periodically for 48 h.

Growth and light measurements in liquid media. Cell density was measured as optical density (OD) at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. Light was measured with an Electro Megadyne Inc. type 9781A phototube and Pacific Photometrics model 110 amplifier. One light unit equals 5.5×10^5 quanta per s according to the "J" standard of Hastings and Weber (9). Cells were grown in new, HCl-washed, 18- by 150-mm glass tubes containing 7 ml of medium and capped with plastic Morton closures on a shaker at 80 to 100 rpm.

Luciferase experiments. The luciferase experiments were performed with 100 ml of culture in 300-ml nephelo flasks. The OD was measured; 1-ml samples were periodically removed and placed in scintillation vials, and light was measured. These samples were centrifuged for 2 min in an Eppendorf microcentrifuge, and the pellets were frozen for the luciferase assay (8). FAC added at 32 μg/ml to in vitro assays has no effect on luciferase activity. This concentration of FAC is more than sufficient to repress induction in minimal medium (see Fig. 10). MJ1 was lysed by the method of Hastings et al. (8); ED8654(pJE202) and RW193(pJE202) were lysed for 10 min at room temperature in 1 mM EDTA–10 mM Tris (pH 7.4)–1% glucose with 2 mg of lysozyme per ml. Extracts were chilled for 10 min in an ice-water bath, sonicated for 5 s in an ice-water bath, and assayed for luciferase.

Autoinducer bioassay. The autoinducer was assayed by measuring production of β-galactosidase by HB101(pJE411) in response to either synthetic autoinducer (supplied by A. Eberhard) or MJ1 conditioned medium. This strain was used to develop a bioassay because, unlike *V. fischeri* or *E. coli* strains containing the intact plasmid pJE202, HB101(pJE411) does not produce the autoinducer (because of the mini-Mu insertion in *luxI*), yet it induces β-galactosidase synthesis in response to autoinducer (5). Standard curves are linear from between 0 and 25 ng of autoinducer per ml to between 100 and 150 ng of autoinducer per ml in different experiments.

Effect of iron and EDDA on response to pure autoinducer. HB101(pJE411) cells were grown to the midlog phase in L broth at 29°C. Pure synthetic autoinducer in ethyl acetate was added to 13- by 100-mm glass tubes to give a standard curve (0 to 150 ng of autoinducer per ml, final concentration, with ethyl acetate control) and evaporated at 50°C. FAC (0 to 200 μg/ml, final concentration) was added to tubes containing 100 ng of autoinducer per ml. A 1-ml sample of culture was then added to all tubes. EDDA (2 to 10 μM) was added to some tubes after the addition of culture. A parallel experiment to test the effect of Fe(II) was performed in a Coy anaerobic chamber; 0 to 200 μM $FeSO_4$ (prepared in oxygen-free distilled water) was added. Cultures were incubated for 2 h at 29°C and then lysed and assayed for β-galactosidase activity (13).

Preparation of MJ1 conditioned medium. MJ1 was grown in 100 ml of SWC in 300-ml nephelo flasks with 0 or 5 μM EDDA as described above. The OD was measured, and subsamples were periodically removed for light and luciferase measurements. A 6-ml sample of culture was also removed at each time point and filtered through a glass fiber filter (Whatman) and then through a 0.2-μm Nuclepore filter, and the filtrate (conditioned medium) was frozen at −20°C.

Assay of MJ1 conditioned medium. HB101(pJE411) was grown to an OD of 0.3 in SWC containing 80 μg of ampicillin per ml and 100 μg of kanamycin per ml at 29°C. Autoinducer standards were made up as described above in Eppendorf microcentrifuge tubes. After evaporation of the ethyl acetate, 0.9 ml of either SWC (two replicates) or SWC containing 5 μM EDDA was added, followed by 0.1 ml of culture. For assay of the conditioned medium, 0.1 ml of culture was added to 0.9 ml of conditioned medium or 0.9 ml of 10-fold-diluted conditioned medium in SWC (three replicates). All samples were incubated for 90 min at 29°C, placed on ice, and centrifuged for 2 min in an Eppendorf microcentrifuge, and the supernatant was gently aspirated. The pellets were then assayed for β-galactosidase (13). It was necessary to remove the SWC supernatant before assaying β-galactosidase to prevent precipitation with the phosphate buffer used in the assay.

RESULTS AND DISCUSSION

Experimental approach. Three experimental approaches, which used both *V. fischeri* and *E. coli* clones with plasmids containing *V. fischeri* luminescence genes, were employed to examine the mechanisms outlined in the introduction. First, the hypothesis that premature induction of luciferase in the presence of EDDA is a secondary effect of reduced growth rate in the presence of EDDA was tested by exam-

ining the effect on luciferase induction of other substances that affect growth like EDDA. Second, we developed a quantitative bioassay for autoinducer to examine mechanisms involving alteration by iron of activity or production of autoinducer. Measurement of production of luciferase by *V. fischeri* in response to autoinducer is not an adequate assay, since the addition of autoinducer results in the production of more autoinducer in a positive feedback cycle (5). Therefore, to develop the bioassay, we chose a clone of *E. coli* containing a transposon mutant of a plasmid with *V. fischeri* luminescence genes [HB101(pJE411)], which responds to autoinducer by producing β-galactosidase. Because the mechanism by which autoinducer causes increased production of β-galactosidase in HB101(pJE411) is the same as that which occurs in autoinduction of luciferase in *V. fischeri*, the bioassay could be used to test the hypothesis that iron reduces the biological activity of autoinducer. These experiments showed that iron and EDDA had no effect on the activity of autoinducer, and therefore the bioassay could be used to measure the concentration of autoinducer in media, without regard to the presence of EDDA or iron. The effect of EDDA on autoinducer production by *V. fischeri* was thus tested by assaying the concentration of autoinducer in media in which *V. fischeri* was grown in the presence and absence of EDDA. Third, to determine whether any of the known *V. fischeri* luminescence genes could account for the iron effect, the effect of EDDA on luminescence and luciferase content of *E. coli* clones with a plasmid containing seven intact *V. fischeri* luminescence genes of known function (pJE202, autoinducer production and response and luciferase and aldehyde production) was examined. Since growth of wild-type *E. coli* is not affected by levels of EDDA that affect both growth and luminescence in *V. fischeri* (presumably because *E. coli* has a more effective means of iron assimilation than does *V. fischeri*), it was necessary to test both a strain of *E. coli* with wild-type iron assimilation (ED8654) and one with impaired iron assimilation (RW193) to ensure that the plasmid was submitted to iron stress. The absence of an iron effect on induction of luciferase in these clones would imply that the iron effect is mediated in *V. fischeri* by genes which are entirely absent or substituted in the *E. coli* clones. Specifically, the lack of an iron effect in luminous *E. coli* would support either (i) the hypothesis that iron reduces autoinducer transport, since genes for autoinducer transport are absent in these clones, or (ii) the hypothesis that a separate iron regulatory system (e.g., repressor) exists in *V. fischeri*.

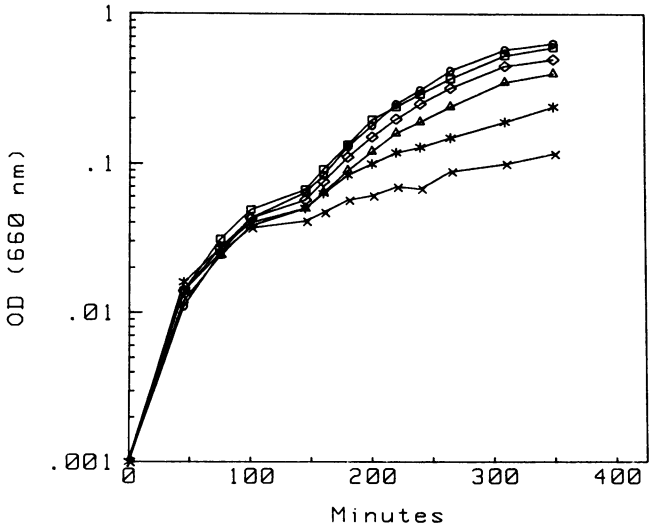

FIG. 1. Effect of EDDA on growth of MJ1 in SCW with (○) 0, (□) 3, (◇) 5, (△) 7.5, (*) 10, and (×) 20 μM EDDA. EDDA slows the growth of MJ1 in SWC. Final optical densities (after overnight growth) are approximately equal.

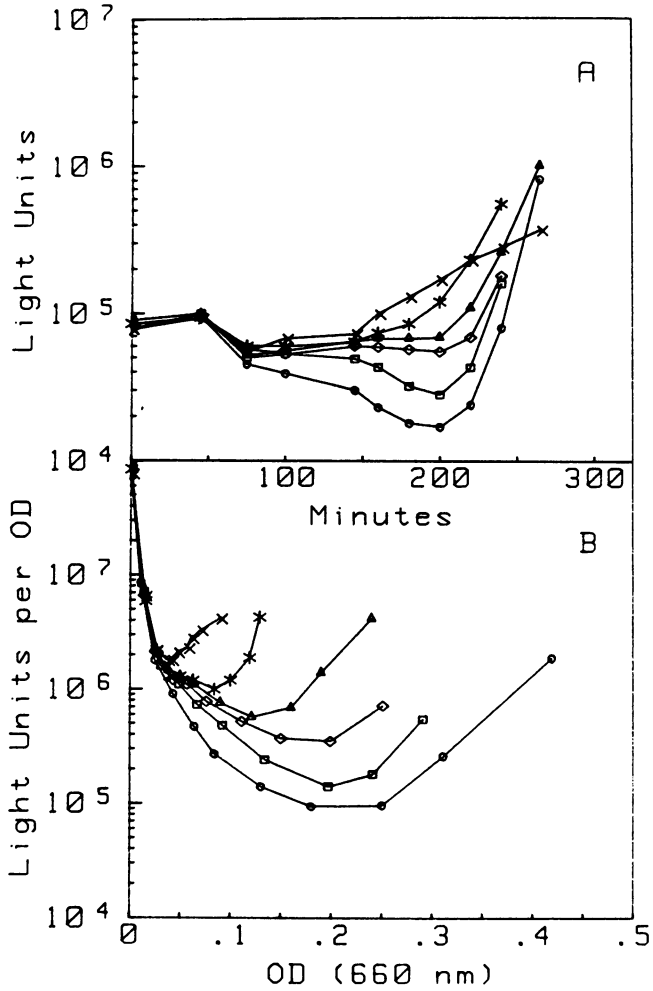

FIG. 2. Effect of EDDA on induction of luminescence on MJ1 in SWC with (○) 0, (□) 3, (◇) 5, (△) 7.5, (*) 10, and (×) 20 μM EDDA. (A) Luminescence per OD unit as function of time. (B) Luminescence per OD unit as a function of growth (OD).

Cell growth. The iron chelator EDDA, when added to MJI in SWC, causes a reduction in growth rate (Fig. 1) and induction of luciferase at a lower cell density (Fig. 2). The initial growth rates are similar, presumably because the inocula are iron replete and EDDA does not affect the cells until a few cell divisions have occurred. The effect on induction takes place between OD 0.05 and 0.3, after the growth rates have diverged. It could be argued that the premature induction is simply a consequence of slowed growth; i.e., if autoinducer is produced at the same rate per cell, but cell division is slower, the critical concentration of autoinducer might be reached at a lower cell density. If this were the case, the time of induction should be later in cultures containing EDDA, since there would be fewer cells producing autoinducer at any given time after the start of the

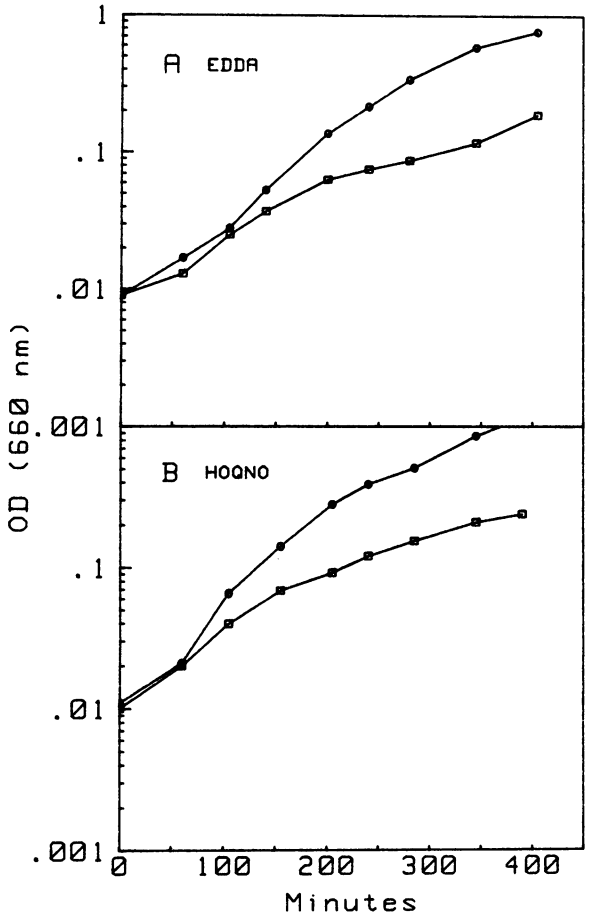

FIG. 3. Effect of EDDA and HOQNO on growth of MJ1 in SWC. (A) SWC contained (○) 0 and (□) 5 μM EDDA. (B) SWC contained (○) 0 and (□) 10 μM HOQNO.

experiment. However, the time of induction is the same (or even earlier) in the presence of EDDA (Fig. 2). Further, if the EDDA effect is a growth effect, other substances that slow growth to an equivalent extent might also be expected to cause induction at a lower cell density. HOQNO, an electron transport inhibitor acting at the level of cytochrome *b* (3), at a concentration of 10 μM had an effect on growth similar to that of 5 μM EDDA (Fig. 3), yet had no significant effect on the cell density at which luciferase was induced (Fig. 4). In addition, growth in low-salt medium slows growth, but does not alter the OD at which induction occurs (data not shown). Finally, it appears that, in fact, there is not a single extracellular critical concentration of autoinducer for induction, since when autoinducer production was compared between SWC cultures with 0 and 5 μM EDDA with the HB101(pJE411) bioassay of conditioned medium, the control culture induced at about 50 ng of autoinducer per ml, yet the EDDA culture induced at about 25 ng of autoinducer per ml (Fig. 5). Therefore, the iron does not act solely by affecting growth, although reduced growth may have some influence on the iron effect.

Autoinducer-iron interaction. In a second model, autoinducer molecules might bind iron, thus inactivating themselves. Autoinducer might act as a siderophore when iron is present or as a sensing mechanism to turn on luminescence when iron is absent, a possible selective advantage under low iron conditions (Haygood Ph.D. thesis). If this were the case, the addition of iron to autoinducer should reduce production of β-galactosidase in response to autoinducer in the HB101(pJE411) bioassay. The addition of 0 to 200 μg of FAC per ml to tubes containing 100 ng of autoinducer per ml had no significant effect on induction (Table 1). In a similar experiment under anaerobic conditions, the addition of 0 to 200 μM $FeSO_4$ gave the same result (Table 1). Thus neither Fe(III) nor Fe(II) appears to directly affect autoinducer activity. This hypothesis would also predict that EDDA should increase the apparent autoinducer concentration measured by the bioassay by binding iron in the medium; it does not (Table 1, Fig. 6). Finally, autoinducer does not appear to act as a siderophore, since in a siderophore bioassay (12) concentration disks containing autoinducer did not stimulate growth on SWC plates containing 10 to 40 μM EDDA in the overlay, but disks with FAC did (data not shown).

Autoinducer production. Models can be postulated in which iron represses autoinducer production, including the following: (i) iron stabilizes a polymeric siderophore, which breaks down to form autoinducer; (ii) iron represses the enzymatic conversion of an autoinducer precursor to active autoinducer; or (iii) iron accelerates breakdown of autoinducer. The first alternative seems unlikely, since *E. coli* strains containing plasmid pJE202 produce autoinducer and show autoinduction (5). Autoinducer synthesis in these

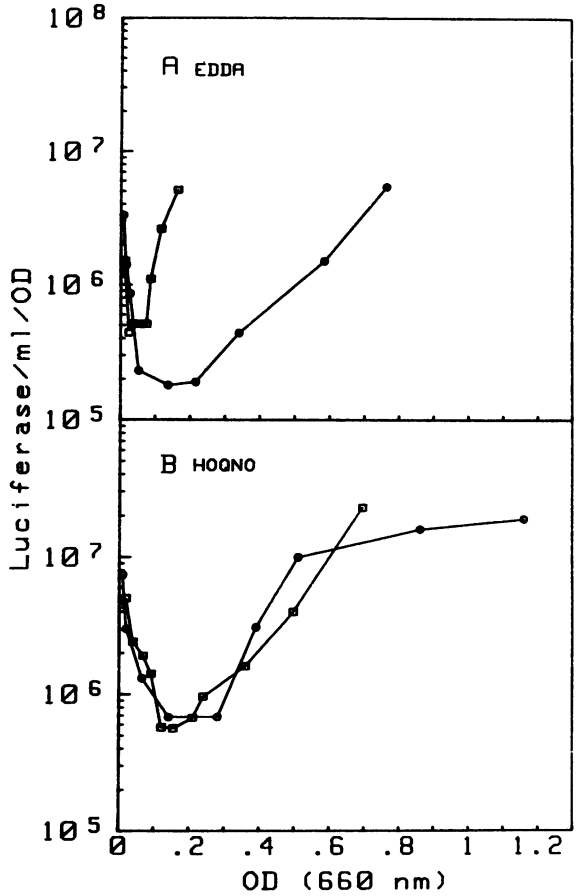

FIG. 4. Effect of EDDA and HOQNO on luciferase induction in MJ1 in SWC. (A) SWC contained (○) 0 and (□) 5 μM EDDA. (B) SWC contained (○) 0 and (□) 10 μM HOQNO.

clones is governed by a single gene (*luxI*) (6), presumably using precursors provided by *E. coli*. If, as in the second alternative, iron acts directly on the *luxI* gene product, iron stress (EDDA) should cause premature induction of luciferase in *E. coli* strains containing pJE202 as in MJ1. Experiments with ED8654(pJE202), with wild-type iron uptake, and RW193(pJE202), with impaired uptake, showed that EDDA had no effect on induction of luciferase, although EDDA slightly increased luciferase expression in RW193(pJE202) (Fig. 7 and 8). This implies that iron does not act on the *luxI* gene product directly or on the product of that protein. It is possible that in MJ1 iron might repress the production of autoinducer precursors or accelerate the breakdown of autoinducer, thus indirectly affecting autoinducer production. However, the autoinducer production experiment described above (Fig. 5) does not show a significantly faster rate of autoinducer production before induction in the EDDA culture than in the control. Thus it does not appear that iron represses autoinducer production.

Autoinducer transport. The means by which autoinducer exits and enters the cell in *V. fischeri* is unknown. No genes dedicated to transport exist on pJE202, yet *E. coli* strains containing the plasmid show apparently normal induction. This result could be accounted for if LuxI resides in the cytoplasmic membrane, exporting autoinducer as it is synthesized or LuxR resides in the membrane, responding to extracellular autoinducer (or both). If this is the case, neither of these components can be responsible for iron regulation, since autoinducer production (governed by LuxI) before induction is unaffected by iron (Fig. 5), and induction in *E. coli* containing pJE202, which bears both *luxI* and *luxR*, is also unaffected by iron (Fig. 7 and 8). If, on the other hand, transport functions are substituted in *E. coli*, the possibility exists that these functions are iron regulated in *V. fischeri* like phenylalanine, tyrosine, and tryptophan transport are iron regulated in *E. coli* (2). (i)If autoinducer exits and enters the cell by passive diffusion through a single component, iron could keep the pore open and lack of iron could close it. Low iron conditions would result in intracellular accumulation of autoinducer, with induction at a lower cell density and lower extracellular autoinducer concentration. However, if this were the case, accumulation of autoinducer in the culture medium should be shut down under low iron conditions; this is clearly not the case (Fig. 5). (ii) A model

TABLE 1. Effect of iron on β-galactosidase production by *E. coli* HB101(pJE411)

	Experimental conditions	Autoinducer (100 ng/ml)	β-Galactosidase (U)
Aerobic	FAC, 0 μg/ml	+	192
	FAC, 20 μg/ml	+	207
	FAC, 50 μg/ml	+	165
	FAC, 100 μg/ml	+	184
	FAC, 200 μg/ml	+	154
Anaerobic	$FeSO_4$, 0 μM	+	151
	$FeSO_4$, 50 μM	+	141
	$FeSO_4$, 100 μM	+	131
	$FeSO_4$, 200 μM	+	141
Aerobic	EDDA, 0 μM	−	29
	EDDA, 2 μM	−	32
	EDDA, 5 μM	−	25
	EDDA, 10 μM	−	30

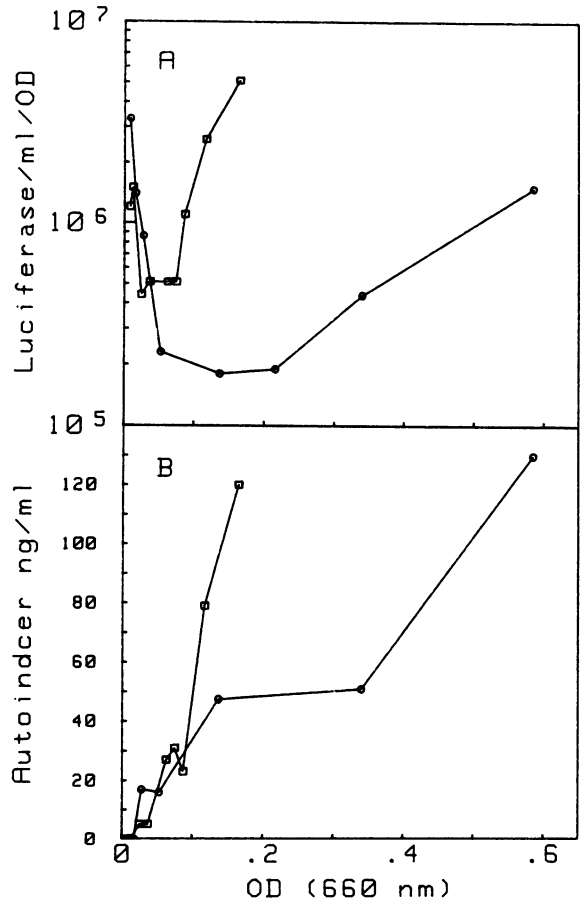

FIG. 5. Effect of EDDA on autoinducer production by MJ1 in SWC containing (○) 0 and (□) 5 μM EDDA. (A) In vitro luciferase activity as a function of growth. (B) Autoinducer concentration (ng/ml) in conditioned media from the cultures in panel A, measured as β-galactosidase production by HB101(pJE411) (see Fig. 6).

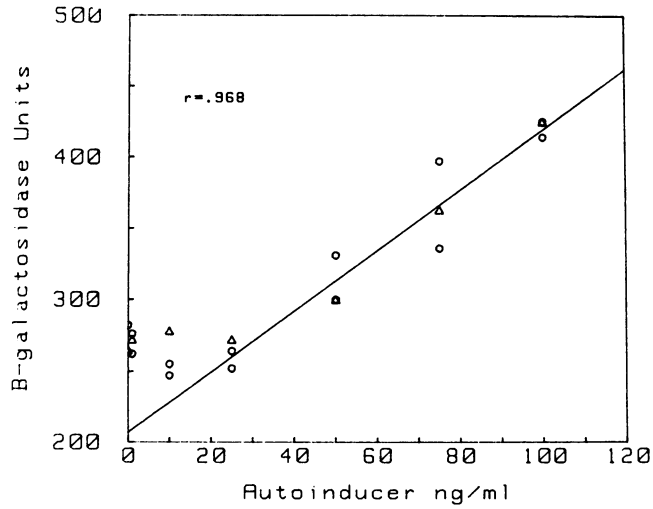

FIG. 6. Effect of EDDA on β-galactosidase production by HB101(pJE411) in response to synthetic autoinducer in SWC containing (○) 0 and (△) 5 μM EDDA. Regression was performed on the linear portion of the curve, from 25 to 100 ng of autoinducer per ml.

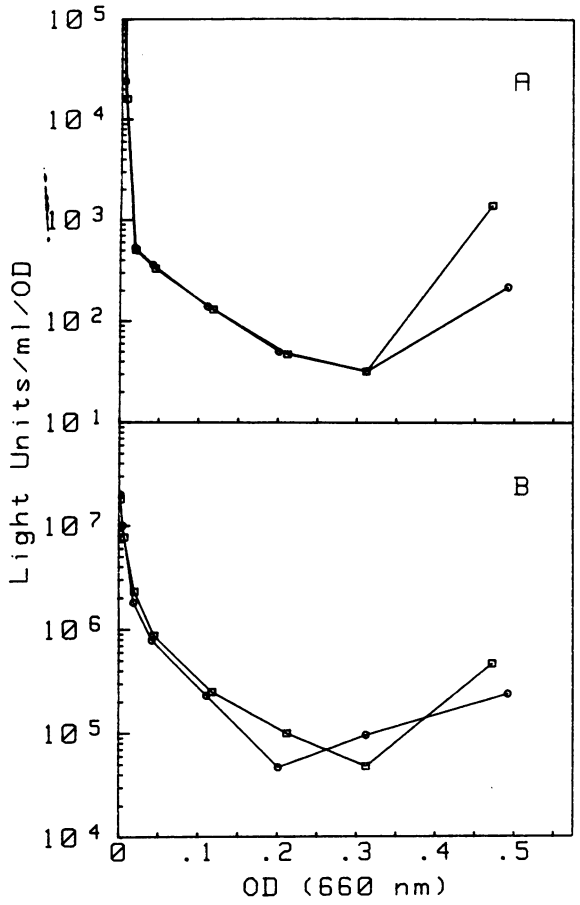

FIG. 7. Effect of EDDA on induction of luminescence and luciferase in *E. coli* ED8654(pJE202) (wild type for iron assimilation) in nutrient broth with (○) 0 and (□) 10 μM EDDA. (A) In vivo luminescence as a function of growth. (B) In vitro luciferase activity as a function of growth. Growth is not significantly impaired by 10 μM EDDA in this strain.

in which autoinducer export is iron regulated, but response to autoinducer occurs at the cytoplasmic membrane or via unregulated import, would likewise affect the appearance of autoinducer in the medium before induction. (iii) In the final model (Fig. 9), autoinducer export, perhaps via LuxI, is not iron regulated, but import (which could occur by passive diffusion through a regulated component) is iron repressed. This hypothesis was not tested directly, but several results are consistent with it, and none is contradictory. First, in the autoinducer production experiment, the induction by EDDA culture at a lower extracellular autoinducer concentration than in the control (Fig. 5) is consistent with this hypothesis, because, if autoinducer transport is iron repressed in the control, the EDDA culture could have an intracellular autoinducer concentration at induction equal to the control, even though the extracellular autoinducer concentration was lower than in the control culture. Some evidence exists indicating that some transport functions are substituted in *E. coli*. The *E. coli tonB* mutant RWB7 is dim when transformed with pJE202, and RWB7(pJE202) acts as a donor, but not a recipient, of autoinducer (J. Engebrecht, personal communication). Given that *tonB* is a specific transport component (15) the effect of the absence of *tonB* on luminescence and the absence of transport functions coded by pJE202 strongly suggest that autoinducer transport functions are provided by *E. coli*. TonB is located in the cytoplasmic membrane; the means by which autoinducer passes through the outer membrane is unknown, since *tonA*, *fep*, and *ompR* mutants containing plasmid pJE202 are unaffected in luminescence (J. Engebrecht, personal communication). The final test of this hypothesis must be to measure uptake of radioactively labeled autoinducer into cells grown with and without EDDA.

Direct iron effects on transcription of the *lux* operon. Iron could act entirely independently of autoinducer by affecting transcription directly or via an independent effect on the regulatory protein LuxR. If iron acts without the involvement of autoinducer and if it acts directly or through LuxR, EDDA, in the absence of autoinducer, would be expected to increase β-galactosidase production in HB101(pJE411). EDDA does not have a significant effect in the absence or presence of autoinducer (Table 1, Fig. 6). Thus it seems unlikely that iron affects transcription directly. This conclusion is supported by the lack of an EDDA effect on luciferase induction in ED8654(pJE202) or RW193(pJE202) (Fig. 7 and 8).

Indirect mechanisms. The above experiments do not, however, rule out the possibility that iron acts via other mechanisms not present in *E. coli*. Such mechanisms include

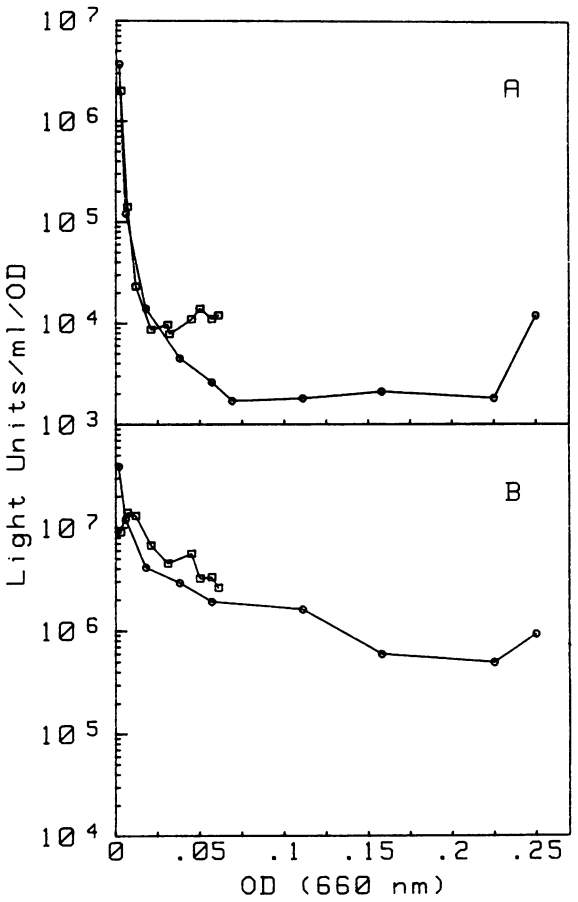

FIG. 8. Effect of EDDA on induction of luminescence and luciferase in *E. coli* RW193(pJE202) (defective in iron assimiliation) in nutrient broth with (○) 0 and (□) 10 μM EDDA. (A) In vivo luminescence as a function of growth. (B) In vitro luciferase as a function of growth. In this strain, 10 μM EDDA causes cessation of growth at OD 0.06.

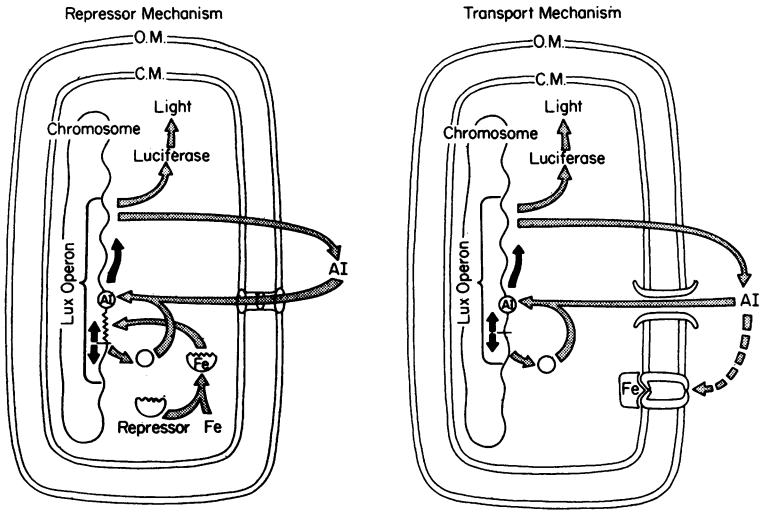

FIG. 9. Schematic representation of models of iron repression of luciferase synthesis. Abbreviations: OM, outer membrane; CM, cytoplasmic membrane. The repressor mechanism model shows one example of a mechanism acting independently of autoinduction. A repressor, coded by a gene outside the *lux* operon, binds iron and then binds to the DNA, preventing luciferase production even though sufficient autoinducer (AI) and LuxR (O) are present. In the autoinducer transport mechanism, when intracellular iron is high, it prevents entry of autoinducer into the cell by allosteric modification of a transport protein. When iron is low, autoinducer is low, autoinducer enters the cell, and induction proceeds.

(i) a mechanism mediated by a change in redox balance due to backup of the electron transport chain under iron stress; (ii) an iron-regulatory protein (Fig. 9) such as that postulated to account for the *fur* mutation in *E. coli*; and (iii) an iron-regulated tRNA modification mechanism like that regulating amino acid uptake and biosynthesis in *E. coli*. The first mechanism is unlikely since HOQNO, which might be expected to have an effect on electron transport similar to EDDA, does not affect induction of luciferase (Fig. 4). Any mechanism truly independent of autoinduction should cause a genuine autoinducer (*luxI*) mutant in *V. fischeri* to increase transcription of the lux operon under iron stress even in the absence of autoinducer. Well-characterized, stable *V. fischeri luxI* mutants do not yet exist. Another approach to this problem would be to make β-galactosidase fusion transposon mutants in luminescence in MJ1, as has recently been done in other marine vibrios (1). Such mutants can be obtained and maintained by selection for antibiotic resistance coded for by the transposon. Choosing a mutant with the correct phenotype (autoinducer donor negative, recipient positive) and using the available information concerning the restriction sites in the *lux* operon and a *lux* probe such as pJE202 (5), it would be possible to identify a transposon insertion in the *luxI* gene. Such a transposon mutant could be used as in the HB101(pJE411) bioassay. If β-galactosidase production in such a mutant were increased in the presence of EDDA, it would conclusively demonstrate that iron acts independently of autoinduction; it also would suggest that regulation of autoinducer transport is not the sole basis of the iron effect.

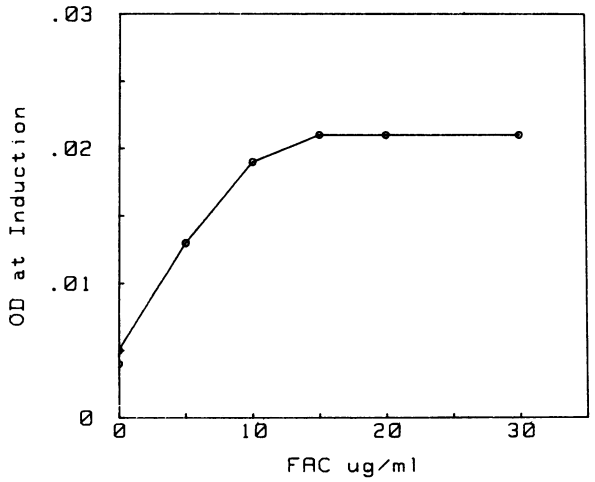

FIG. 10. Effect of FAC on the OD at induction of MJ1 in ASG.

Iron is not the only nutritional factor affecting autoinduction. In minimal medium, increasing FAC increases the cell density of induction up to an OD of 0.02, but beyond about 15 μg of FAC per ml it has no further effect (Fig. 10). In complex media, the typical cell density of induction is much higher (OD 0.2 to 0.3 in SWC), consistent with the higher iron content of such media, but the addition of more iron (up to 100 μg/ml in SWC) does not further delay induction (data not shown). These data suggest that another factor in complex medium cooperates with iron in delaying induction. These results could be explained if iron exerted its effects after transport into the cell and transport, perhaps by means of a siderophore, were limited by availability of certain amino acids. Thus, in minimal media, above 15 μg of FAC per ml the ability of the cells to transport iron is saturated.

In summary, six models of action of iron have been considered. Two possibilities (which are not mutually exclusive) are most consistent with the data: (i) iron may regulate autoinducer transport (Fig. 9), and (ii) iron may act indirectly, and independently of autoinduction, to regulate transcription of the *lux* operon, perhaps via a repressor (Fig. 9).

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