JOURNAL OF BACTERIOLOGY, Feb. 1989, p. 1199–1202
0021-9193/89/021199-04$02.00/0

Vol. 171, No. 2

Regulation of Luminescence by Cyclic AMP in *cya*-Like and *crp*-Like Mutants of *Vibrio fischeri*

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Mutants of *Vibrio fischeri* MJ-1 (wild type) apparently deficient in adenylate cyclase (*cya*-like) or cyclic AMP receptor protein (*crp*-like) were isolated and characterized. Compared with MJ-1, the mutants produced low levels of luminescence and luciferase. Addition of cyclic AMP restored wild-type levels of luminescence and luciferase in the *cya*-like mutant but not in the *crp*-like mutant. The results are consistent with the hypothesis that in *V. fischeri* cyclic AMP and cyclic AMP receptor protein are required for induction of the luminescence system.

Luminescence in *Vibrio fischeri*, a cardinal feature of its light organ symbiosis with monocentrid fish, requires autoinduction. During growth in culture, *V. fischeri* produces a specific, diffusible metabolite called autoinducer [*N*-(3-oxohexanoyl)homoserine lactone] which accumulates in the medium and induces synthesis of luciferase and other enzymes involved in luminescence when it reaches the critical concentration of a few molecules per cell (11, 20, 25, 30). In natural environments in which the *V. fischeri* population density can reach a high level, such as the fish light organ (10^9 to 10^{10} cells per ml of organ fluid), autoinducer presumably also accumulates and induces luminescence (29, 30). Recently, a 9-kilobase fragment of *V. fischeri* DNA containing genes specifying the luminescence enzymes and encoding regulatory functions necessary for their expression in *Escherichia coli* was isolated (12). The luminescence (*lux*) system contains seven genes in two divergently transcribed units (*luxR* and *luxICDABE*) (5, 12, 13). Expression of the *lux* genes in *E. coli* is autoinducible in a fashion analogous to that in *V. fischeri* (9, 12), with expression dependent on an apparently complex autoregulatory circuitry (9, 10, 12–14, 21).

Besides control by autoinduction, luminescence in *V. fischeri* is subject to an apparently atypical catabolite repression. Glucose represses luminescence transiently in batch culture; however, this repression is not reversed by cyclic AMP (cAMP), and prior growth of *V. fischeri* on glucose eliminates the repression (30). In contrast, in phosphate-limited chemostat culture, cAMP reversal of glucose repression has been demonstrated (16). Additionally, since the luminescence system is not involved in transport or catabolism of a carbon substrate, it is not obvious why cAMP should be involved in its control. Moreover, it has been postulated that glucose is the carbon source supplied to *V. fischeri* by its fish host (27).

Recently, studies with the cloned *V. fischeri lux* genes in *E. coli* adenylate cyclase (*cya*) and cyclic AMP receptor protein (CRP) (*crp*) mutants have addressed this controversy by demonstrating that autoinduction of luminescence requires cAMP and CRP, even though glucose repression of luminescence is not reversed by exogenous cAMP in the parental (cya^+ crp^+) strain (9, 10). The presence of a possible CRP-binding site within the *lux* regulatory region, identified by DNA sequence analysis (7, 15), supports these studies. However, evidence for cAMP-CRP control of luminescence obtained with *V. fischeri* regulatory mutants has not been reported.

Isolation and characterization of *V. fischeri cya*-like and *crp*-like mutants. To address questions of the role of cAMP-CRP in luminescence in *V. fischeri*, spontaneous mutants of a wild-type strain of *V. fischeri* (MJ-1) (30), apparently deficient in adenylate cyclase (*cya*-like) or CRP (*crp*-like), were isolated by a modification of the phosphomycin selection procedure (1, 23). *V. fischeri* MJ-1 was grown overnight with aeration at 22°C in the minimal medium of Nealson (26) supplemented with glucose (10 mM, final concentration), glucose 6-phosphate (20 mM), and DL-α-glycerophosphate (0.6 mM). Cells (0.1 ml; approximately 5×10^8 cells) were then plated on a selective medium, based on Luria-Bertani (LB) medium (31), which contained (per liter of tap water) 10 g of tryptone, 5 g of yeast extract, 0.34 M NaCl, 20 mM glycerol, 20 mM Tris hydrochloride (pH 7.5), and 15 g of agar (LB-salt medium [LBS]). The LBS was modified to include neutral red (30 $\mu g \cdot ml^{-1}$) (LBS-NR), galactose and maltose (25 mM each), and 1.5 mM phosphomycin. After 3 to 5 days of incubation, colonies that exhibited a phenotype consistent with a defect in adenylate cyclase or in CRP (i.e., slow growth, no fermentation of the sugars [color of colonies, gold to pale pink, compared with magenta for the wild-type strain], and little or no visible luminescence) were picked and purified on fresh selection plates. In a preliminary screening of 20 of these strains for their response to cAMP, 15 strains (*cya*-like) fermented galactose and maltose and produced high levels of luminescence on LBS-NR plates (without phosphomycin) when cAMP was added; 5 strains (*crp*-like) did not.

Representatives of each type of mutant (MJ-16 [*cya*-like] and MJ-46 [*crp*-like]) were examined in detail. Fermentation of carbon sources was tested on LBS-NR plates, with carbon sources filter sterilized and added to a final concentration of 25 mM to the autoclaved, cooled medium. Like MJ-1 (wild type), MJ-16 and MJ-46 fermented D-glucose, D-fructose, and *N*-acetylglucosamine in the absence of added cAMP; unlike the wild-type strain, however, the mutants did not ferment D-galactose, maltose, D-mannose, D-cellobiose, or D-glucose 6-phosphate. Addition of cAMP (10 μl, 0.5 M stock, spotted over cells grown on the LBS-NR plates for 12 h) restored the ability of MJ-16 to ferment this latter group of sugars but had no effect on their fermentation by MJ-46. Consistent with these results, MJ-16 and MJ-46

TABLE 1. Growth inhibition of *V. fischeri cya*-like and *crp*-like mutants by antibiotics

Antibiotic and concn	Inhibition zone (mm)[a]				
	MJ-1 (wild type)	MJ-16 (*cya*-like)		MJ-46 (*crp*-like)	
		−cAMP	+cAMP[b]	−cAMP	+cAMP[b]
Phosphomycin					
25 μg	2.0	0	1.0	0	0
250 μg	9.0	2.0	4.5	1.5	1.5
2.5 mg	13.0	5.0	10.0	5.0	5.0
Streptomycin					
25 μg	0.1	0	0.1	0	0
250 μg	4.0	2.0	3.0	3.2	3.2
2.5 mg	9.5	7.0	9.0	8.0	8.0

[a] Sterile filter paper disks were saturated with 25 μl of each antibiotic (at concentrations of 1, 10, and 100 mg/ml) and placed over lawns of cells spread on LBS plates. Inhibition zones, measured as the distance from the edge of the disk to the edge of visible growth, were determined after incubation of plates at 22°C for 72 h.

[b] Final concentration of 10 mM.

grew on minimal plates containing D-glucose, D-fructose, or *N*-acetylglucosamine, as did MJ-1, but unlike MJ-1, MJ-16 and MJ-46 did not grow on minimal plates containing D-galactose, maltose, D-mannose, D-cellobiose, D-glucose 6-phosphate, D-ribose, D-mannitol, glycerol, or DL-α-glycerophosphate. Thus, the *V. fischeri* mutants exhibited a pleiotropic carbohydrate-negative phenotype that in one strain (MJ-16) but not in the other (MJ-46) could be reversed by the addition of cAMP to the growth medium. The altered fermentation patterns of MJ-16 and MJ-46 make it unlikely that a mutation other than one in the putative *cya* or *crp* loci was affecting expression of the luminescence system (see below).

The antibiotics phosphomycin and streptomycin are thought to be transported by cAMP-CRP-controlled systems and have been used for the isolation and characterization of *cya* and *crp* mutants of *E. coli* and *Salmonella typhimurium* (1, 2, 23). In the absence of added cAMP, both antibiotics inhibited the growth of the mutants less than that of the wild-type strain (Table 1). The presence of cAMP, however, substantially increased the inhibition of MJ-16 but did not increase the inhibition of MJ-46 (Table 1).

Estimates of cAMP levels in the mutants and in the wild-type strain were determined by the method of Gilman (17), as modified by Botsford (3), with a binding-protein preparation from beef kidney (6). To extract cAMP, *V. fischeri* cells were grown in LBS-glucose broth to an optical density at 660 nm of 0.5 (approximately 3×10^8 cells · ml^{-1}, 100 μg of protein · ml^{-1}); pelleted by microcentrifugation (room temperature; 11,000 × *g* 1 min), rapidly suspended without washing in 0.4 ml of hot (95°C) sodium acetate buffer (50 mM, pH 4.0), and heated at 95°C for 10 min. The cell debris was then pelleted, and the extract was transferred to a clean tube and frozen at −20°C until it was assayed. cAMP was detected in MJ-1 (wild type) and MJ-46 (*crp*-like) at levels of 2.7 and 0.5 pmol per 10^7 cells, respectively, but was not detected in MJ-16 (*cya*-like). For comparison, *E. coli* PD100 (*cya*$^+$ *crp*$^+$), PD200 (Δ*cyaA*), and PD300 (Δ*crp*) (9) prepared similarly were found to contain 1.7, 0, and 9.3 pmol of cAMP per 10^7 cells, respectively. Thus, *V. fischeri* appears to contain cAMP, and at least for the wild-type strain and the *cya*-like mutant, the pattern of the presence and absence of cAMP is consistent with that found in *E. coli*. The detected levels of cAMP are higher than those typically reported for *E. coli* (e.g., reference 3), probably because substantial amounts of extracellular cAMP were associated with the unwashed cell pellets.

The mutants reverted spontaneously to the wild-type phenotype (i.e., utilization of sugars and production of luminescence) at frequencies of approximately 10^{-7} (MJ-16) and 10^{-8} (MJ-46), which are consistent with the defects being single point mutations.

Control of cellular luminescence and luciferase synthesis by cAMP. Compared with MJ-1 (wild type), the mutants produced a very low level of luminescence. To examine the luminescence response of the mutants to added nucleotides, cultures were grown for 12 to 18 h on LBS plates, and 10 μl of the appropriate nucleotide solution (0.5 M stock) was then spotted over the cells. Responses were noted periodically from 2 to 6 h and again 12 h after addition of the nucleotide by visual observation of the plates in a well-darkened room (10-min dark adaptation). Addition of cAMP restored a high level of luminescence in the *cya*-like mutant but had no effect in the *crp*-like mutant. The response to cAMP was specific; addition of adenine, adenosine, AMP, ADP, ATP, cGMP, dibutyryl cGMP, or dibutyryl cAMP did not stimulate luminescence in either MJ-16 or MJ-46.

To quantify the cAMP response and to examine the luminescence behavior of the mutants, cultures were grown in LBS broth containing D-glucose (10 mM) and supplemented with cAMP (10 mM) and autoinducer [*N*-(3-oxohexanoyl)homoserine lactone, 0.2 μM], in 3-ml volumes, as described previously (9). The light-measuring equipment and standard to calibrate the equipment have been described previously (9, 18), as have the procedures for measuring luminescence of broth cultures (9).

In the absence of added cAMP, the *cya*-like mutant (MJ-16) produced a low level of luminescence that increased with cell density during growth (Fig. 1). With added cAMP, however, with or without added autoinducer, luminescence reached levels similar to induced levels produced by the wild-type strain (Fig. 1). When autoinducer was added with cAMP, this fully induced level of luminescence was reached sooner (Fig. 1), presumably because the added autoinducer eliminated the delay that occurs while autoinducer produced by the cells accumulates to the critical concentration necessary for induction (5 to 10 nM) (20). Addition of autoinducer alone provided some stimulation of luminescence, but the maximum level of luminescence was about 100-fold less than the fully induced level reached in the presence of cAMP (Fig. 1).

For the *crp*-like mutant (MJ-46), addition of cAMP did not stimulate luminescence. Regardless of the presence of added cAMP, luminescence in this strain was low and was similar to that of the *cya*-like mutant grown in the absence of added cAMP, both with and without added autoinducer (Fig. 2).

Extracts of cells of MJ-16 and MJ-46 taken from the growth experiments described above were used to determine luciferase activity, as described previously (9, 10), with cells harvested from the cultures at an optical density at 660 nm of 1.4. Cell extracts were prepared as described previously (9), except that complete lysis of cells was obtained with one freeze-thaw cycle. The results were consistent with those for luminescence. Autoinducer alone effected a small increase in luciferase activity in both mutants, but luciferase levels approaching those of the wild-type strain were produced only by the *cya*-like mutant when grown in the presence of added cAMP (Table 2). Thus, cAMP and CRP appear to be

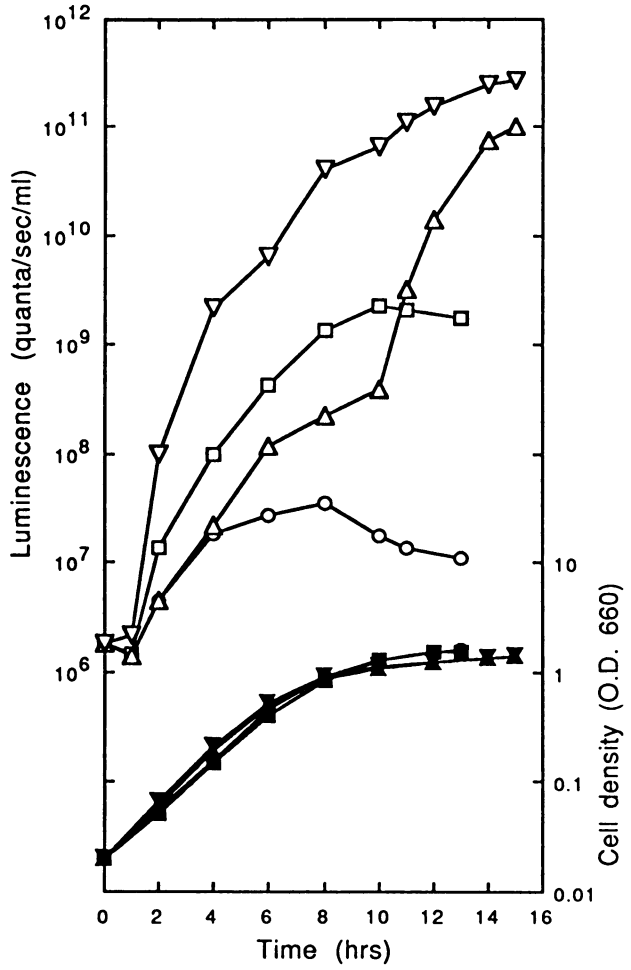

FIG. 1. Effects of cAMP and autoinducer on in vivo luminescence during growth of *V. fischeri* MJ-16 (*cya*-like). Open symbols, luminescence; solid symbols, cell density. Symbols: △ and ▲, cAMP added to the medium; □ and ■, autoinducer added; ▽ and ▼, autoinducer and cAMP added; ○ and ●, no addition. O.D. 660, Optical density at 660 nm.

required for induction of luminescence and luciferase synthesis in *V. fischeri*.

These results confirm studies with *E. coli cya* and *crp* mutants containing recombinant *lux* plasmids in which cAMP and CRP were shown to be required for induction of the *lux* system (9, 10) and studies with the wild-type *V. fischeri* in which cAMP control of luminescence was implicated (16). With regard to the altered patterns of carbohydrate fermentation and antibiotic sensitivity, the *V. fischeri* mutants are similar to *E. coli* and *S. typhimurium cya* and *crp* mutants. Phenotypically, MJ-16 appears to be deficient in adenylate cyclase, and MJ-46 appears to be deficient in CRP. Furthermore, the luminescence and luciferase responses of the mutants to cAMP and autoinducer (Fig. 1 and 2 and Table 2) are remarkably similar to the responses of *E. coli cya* and *crp* mutants containing the cloned *lux* genes on recombinant plasmids (9, 10). However, despite these similarities, in the absence of information on adenylate cyclase and CRP, the identification of these mutants as *cya*-like and *crp*-like should be considered tentative. This is particularly the case for MJ-46, since its *crp*-like identification is based on negative results and since, unlike *E. coli crp* mutants (4),

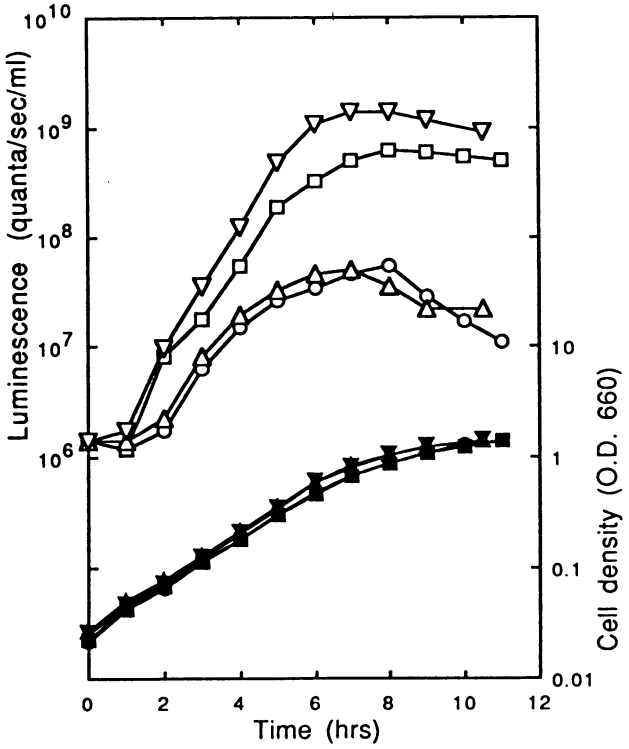

FIG. 2. Effects of cAMP and autoinducer on in vivo luminescence during growth of *V. fischeri* MJ-46 (*crp*-like). Symbols are as in the legend to Figure 1. O.D. 660, Optical density at 660 nm.

it does not appear to overproduce cAMP. A pattern of responses like that of MJ-46 could derive from a mutation producing a very active 3′,5′-cAMP phosphodiesterase. At present, methods for genetic analysis of *V. fischeri* are not sufficiently developed to permit a genotypic characterization of these mutants.

Catabolite repression of luminescence in a closely related species, *Vibrio harveyi*, has been described elsewhere (28), and a mutant of this species that requires cAMP for induction of luminescence and for utilization of a variety of carbohydrates has been isolated (32). DNA sequence analysis of the cloned *V. harveyi lux* genes has revealed a consensus CRP-binding site in the putative *lux* regulatory region (24). Thus, similarities may exist in cAMP-CRP control of the *lux* systems of *V. fischeri* and *V. harveyi*. For other species of luminous bacteria, less information is available, but control of *lux* gene expression may involve cAMP and CRP (8, 19, 22).

Previous studies on the control of luminescence in *V. fischeri* have been hampered by the lack of regulatory

TABLE 2. Luciferase activity in *V. fischeri cya*-like and *crp*-like mutants

Addition(s) (concn)	Luciferase activity (10^5 U)[a]		
	MJ-1 (wild type)	MJ-16 (*cya*-like)	MJ-46 (*crp*-like)
None	2,300	45	40
Autoinducer (0.2 μM)	ND[b]	135	65
cAMP (10 mM)	ND	1,000	20
Autoinducer and cAMP	ND	1,800	60

[a] Normalized to an optical density at 660 nm of 1.0.
[b] ND, Not determined.

mutants. Except for natural isolates of *V. fischeri* deficient in autoinducer synthesis (25), the *cya*-like and *crp*-like mutants characterized here are the first regulatory mutants of this species to be described. Further studies of these mutants may lead to an understanding of the apparently atypical catabolite repression of luminescence in this species and should facilitate studies of *lux* gene regulation and other cAMP-CRP-controlled functions in *V. fischeri*.

I thank T. Melton for suggesting the phosphomycin selection technique, J. Ray and U. Mueller for technical assistance, C. Herman and P. Newcomer for assistance with cAMP assays, and A. Eberhard for pure autoinducer.

This work was supported by contract N00014-87-K-0727 from the Office of Naval Research.

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