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# Cyclic AMP and Cell Division in *Escherichia coli*

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**We examined several aspects of cell division regulation in *Escherichia coli* which have been thought to be controlled by cyclic AMP (cAMP) and its receptor protein (CAP). Mutants lacking adenyl cyclase (*cya*) or CAP (*crp*) were rod shaped, not spherical, during exponential growth in LB broth or glucose-Casamino Acids medium, and lateral wall elongation was normal; in broth, stationary-phase cells became ovoid. Cell mass was smaller for the mutants than for the wild type, but it remained appropriate for their slower growth rate and thus probably does not reflect early (uncontrolled) septation. The slow growth did not seem to reflect a gross metabolic disorder, since the mutants gave a normal yield on limiting glucose; surprisingly, however, the *cya* mutant (unlike *crp*) was unable to grow anaerobically on glucose, suggesting a role for cAMP (but not for CAP) in the expression of some fermentation enzyme. Both *cya* and *crp* mutants are known to be resistant to mecillinam, an antibiotic which inhibits penicillin-binding protein 2 (involved in lateral wall elongation) and also affects septation. This resistance does not reflect a lack of PBP2. Furthermore, it was not simply the result of slow growth and small cell mass, since small wild-type cells growing in acetate remained sensitive. The cAMP-CAP complex may regulate the synthesis of some link between PBP2 and the septation apparatus. The *ftsZ* gene, coding for a cell division protein, was expressed at a higher level in the absence of cAMP, as measured with an *ftsZ::lacZ* fusion, but the amount of protein per cell, shown by others to be invariable over a 10-fold range of cell mass, was independent of cAMP, suggesting that *ftsZ* expression is not regulated by the cAMP-CAP complex.**

Cell division in gram-negative bacilli such as *Escherichia coli* involves invagination of the three envelope layers—cytoplasmic membrane, peptidoglycan, and outer membrane—with concomitant formation at the cell center of a septum, which then provides the new hemispherical poles for the two daughter cells. This complex biochemical process is carried out by a number of enzymes. The rigid peptidoglycan layer responsible for maintaining the cylindrical shape of the cell is synthesized and cross-linked at least in part by penicillin-binding proteins (PBPs), some of which seem to be specifically involved in septation. Other septation functions have been identified genetically in *E. coli*, generally by *fts* (filamentation thermosensitive) mutations, although the molecular activities of these gene products remain to be elucidated.

During steady-state growth, *E. coli* cell division appears to be strictly regulated; septation takes place precisely at the center of the cell and at a precise cell mass whose value depends on the growth rate (9, 21). Virtually nothing is known about the molecular bases of this spatial and temporal regulation, and no specific regulatory functions have been clearly identified. Several observations, however, summarized below, have led to the tentative suggestion that cyclic AMP (cAMP) and its receptor protein (CAP) may regulate certain aspects of cell division in *E. coli*.

The cAMP-CAP complex is a positive transcriptional regulator of a number of catabolic operons in *E. coli* and as such plays a role in catabolite repression, whereby secondary carbon sources are not catabolized in the presence of glucose (4, 23, 32). The cAMP-CAP complex regulates other operons as well, both positively and negatively, and mutants lacking either adenylate cyclase (*cya*) or CAP (*crp*) have a highly pleiotropic phenotype in addition to their inability to utilize a broad range of normally well catabolized carbon sources. Several aspects of their phenotype seem to involve the regulation of cell division, suggesting that the cAMP-CAP complex regulates the transcription of one or more division genes. First, *cya* and *crp* strains have abnormal cell morphology; some authors have reported spherical cells rather than rods (15, 16), suggesting an excess of septation over lateral wall elongation, and cell mass is small. Second, *cya* and *crp* mutants are resistant to the β-lactam antibiotic mecillinam (1, 13). This antibiotic binds preferentially to PBP2 and prevents lateral wall elongation, leading to the formation of spherical cells (22, 30). Third, transcription of the cell division gene *ftsZ* has been reported to be higher in a *cya* mutant (9), possibly resulting in an excess of septation, since the FtsZ protein is thought to be limiting in cell division (37). Fourth, we have described a type of cell division that seems to require a functional cAMP-CAP complex: anucleate cells are formed whenever DNA synthesis is blocked in the absence of SOS-associated division inhibition, and the aberrant divisions giving rise to these cells are more frequent when the amount of cAMP-CAP complex is increased and abolished in *cya* and *crp* mutants (14). Finally, there is a mutant (*fic*) which stops dividing in the presence of exogenous cAMP at 43°C (35).

In the present study we reexamine these questions in an attempt to determine whether the cAMP-CAP complex is a direct regulator of some cell division function in *E. coli*.

## MATERIALS AND METHODS

**Bacterial strains.** For the present work, we chose the wild-type *E. coli* K-12 F⁻ λ⁻ strain GC2700, also known as FB8 and UTH1038, because it has not been subjected to the numerous cycles of mutagenesis typical of many laboratory strains. Isogenic *cya* and *crp* derivatives were constructed by P1 *vir*-mediated transduction. GC2700 was first transduced to *ilv*::Tn*5* or *cysG*::Tn*5* and selected on LB plates containing kanamycin (40 μg/ml). The former was then

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transduced to Ilv$^+$ *cya-854* (strain GC2793) and the latter to Cys$^+$ *crp-96* (strain GC2846); these transductants were Mal$^-$ Lac$^-$ Km$^s$. A. Ullmann kindly provided the donor strains as well as GC2700; the *cya-854* allele is a deletion (6), and the *crp-96* allele is nonreverting (26). Phage λ JFL100, which carries *lacZ* fused to the *ftsZ* promoters (9), was used to lysogenize strain GC2793 on a plate containing cAMP, to permit expression of the receptor. The lysogen (strain GC2961) was transduced to *cya*$^+$ (selection for Mal$^+$) to have an isogenic control (strain GC2962).

**Media and growth conditions.** M63 minimal salts medium (20) was used, containing 0.4% glucose or 0.2% acetate for aerobic growth and 1% glucose or 1% glycerol for anaerobic growth, with further supplements as indicated in the text; Casamino Acids, when used, was added at 0.05%. Complete medium was LB broth (20). Solid media contained 1.5% agar.

Cultures were grown aerobically in Erlenmeyer flasks with vigourous agitation. For anaerobic growth, plates were incubated in a Forma Scientific anaerobic glove box (model 1024) with a carbon dioxide-hydrogen-nitrogen (1:1:18) atmosphere.

All experiments were carried out at 37°C.

**Evaluation of cell number, volume, and morphology.** Bacteria were counted in a model ZI Coulter counter with a 30-μm orifice. Volume distributions were determined with a C1000 channelizer. Photomicrographs were taken through a Zeiss Jenamed phase contrast microscope; samples were fixed in 3.7% formaldehyde.

**Miscellaneous methods.** P1 transductions and β-galactosidase assays were performed as described by Miller (20).

## RESULTS

**Cell morphology.** Wild-type, *cya*, and *crp* bacteria were uniformly rod shaped during exponential growth in LB broth (Fig. 1) and in glucose-Casamino Acids medium (not shown). In stationary phase, when cell volume was smaller (Table 1), all cells remained rod shaped in glucose-Casamino Acids medium (not shown); in LB broth, the mutant cells were ovoid (Fig. 1). These were the only culture conditions in which we observed nearly spherical cells. As expected, addition of 1 mM cAMP to the medium partially corrected the phenotype of the *cya* mutant but had little effect on the *crp* and wild-type strains (Fig. 1, Table 1).

**Cell mass and growth.** During steady-state growth, cell division occurs at a precise cell mass, the absolute value of which increases with growth rate (9, 21). Mutants with smaller than normal cell mass could be affected in the temporal regulation of division, triggering septation too early in the cell cycle. To compare the cell mass of the wild-type, *cya*, and *crp* strains under steady-state conditions, we used very dilute exponential-phase cultures in which the cell number and the cell volume distribution could be measured with a Coulter counter. Overnight cultures were diluted $10^4$-fold in fresh medium. After 2.5 h, samples were withdrawn periodically for analysis. Cell number increased exponentially in all cultures for over 3 h, while the cell volume distribution remained essentially constant, indicating that the bacteria were in steady state. Under these conditions the *cya* and *crp* mutants had a smaller cell volume, both in glucose-Casamino Acids medium and in LB broth (Table 1). Addition of 1 mM cAMP partially corrected the volume of the *cya* mutant but did not affect that of the wild-type or *crp* strain (Table 1). During steady-state growth in glucose-Casamino Acids medium, the wild-type strain had a doubling time of 27 min, whereas the *cya* and *crp* mutants doubled in 45 and 44 min, respectively (Fig. 2).

TABLE 1. Modal volume of wild-type, *cya*, and *crp* strains[a]

| Phase and medium | 1 mM cAMP added | Modal cell volume (μm$^3$) | | |
|---|---|---|---|---|
| | | Wild type | *cya* | *crp* |
| Exponential phase | | | | |
| LB broth | − | 2.52 | 1.36 | 1.36 |
| | + | 2.48 | 2.09 | 1.32 |
| Glucose-Casamino Acids | − | 1.37 | 1.04 | 1.06 |
| Glucose | − | 1.14 | | |
| Stationary phase | | | | |
| LB broth | − | 0.78 | 0.50 | 0.43 |
| | + | 0.81 | 0.85 | 0.50 |
| Glucose-Casamino Acids | − | 0.85 | 0.70 | 0.66 |
| | + | 0.81 | 0.74 | 0.66 |

[a] The strains used were GC2700 (wild type), GC2793 (*cya*), and GC2846 (*crp*); data for LB broth are from the experiments described in the legend to Fig. 2.

The morphology of *cya* and *crp* mutant cells during steady-state growth was appropriate for their growth rate; wild-type cells growing in minimal glucose medium with a 51-min doubling time formed rods of similar volumes (Table 1). The smaller cell mass of the mutants is thus more likely to be a secondary result of their slower growth rate than a primary defect in the temporal regulation of division.

It is unclear why the *cya* and *crp* mutants grew more slowly than the wild-type strain. The *cya* mutant has been reported to synthesize low levels of 2-oxoglutarate dehydrogenase, a key enzyme of the tricarboxylic acid cycle (27). The enzyme level must be physiologically significant, however, since the mutants do not require succinate for growth on glucose, unlike *sucA* and *sucB* mutants which completely lack 2-oxoglutarate dehydrogenase. It has also been reported that *cya* mutants have defective electron transport (8). It is unlikely, however, that metabolism is grossly altered during aerobic growth, since the growth yield in limiting glucose was identical for wild-type, *cya*, and *crp* strains (not shown).

During anaerobic fermentation of glucose, the tricarboxylic acid cycle is effectively interrupted at the 2-oxoglutarate dehydrogenase step, and there is no electron transport, yet it has been reported that a *cya* mutant grows slowly under these conditions (24). Our *cya* mutant was totally unable to form colonies on minimal glucose plates incubated anaerobically, although the *crp* and wild-type strains grew well under these conditions. Normal growth was restored to the *cya* mutant by the addition of 1 mM cAMP to the medium. Growth was also observed in the presence of 0.04 M $KNO_3$, suggesting that nitrate respiration with concomitant electron transport could take place, with nitrate replacing oxygen as the terminal electron acceptor. Growth was similarly restored by the addition of 0.05% Casamino Acids; some amino acid presumably provides a terminal electron acceptor, since 0.05% Casamino Acids also permitted the wild-type strain to grow anaerobically on glycerol, which is not fermentable. On the other hand, the addition of 0.04 M fumarate to the medium did not permit the *cya* mutant to grow by fumarate respiration, although fumarate reductase synthesis does not require cAMP (33); this is consistent with the observation that *cya* mutants are unable to transport dicarboxylic acids (18). The wild-type strain grew well anaerobically on glycerol plates supplemented with nitrate or fumarate, but the *cya* and the *crp* mutants were unable to utilize glycerol, whether the terminal electron acceptor was nitrate, fumarate, or oxygen. This is consistent with the

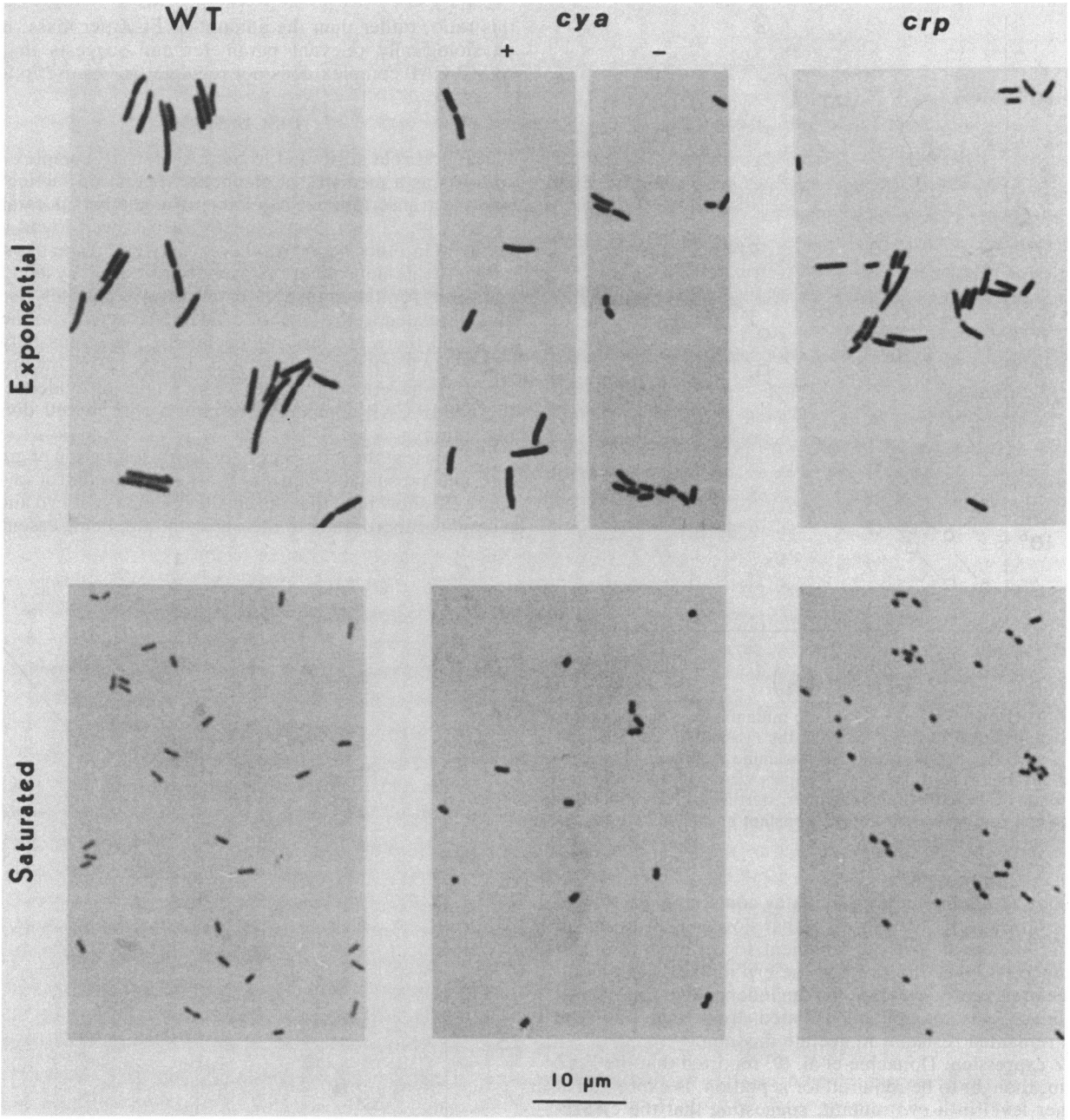


FIG. 1. Morphology of *cya* and *crp* mutants. Cultures of strains GC2700 (WT), GC2793 (*cya*), and GC2846 (*crp*) were grown to saturation in LB broth, and samples were fixed and photographed. These cultures were diluted $10^4$-fold in fresh LB broth with or without 5 mM cAMP and grown to exponential phase; samples were concentrated by filtration, fixed, and photographed. The only cAMP-containing culture shown is in the "+" panel of the *cya* mutant in exponential phase.

observation that glycerol kinase requires an active cAMP-CAP complex for expression, both aerobically and anaerobically (10).

**Mecillinam resistance.** Although *cya* and *crp* mutants are resistant to mecillinam (25 μg/ml), several observations indicate that PBP2, the principal target of this antibiotic, is still functional in the mutants. First, it is able to bind penicillin (13); second, the addition of mecillinam (1 μg/ml) to cultures of the *cya* and *crp* mutants induced spherical morphology, indicating that mecillinam is still able to inhibit PBP2 in these strains; third, exponentially growing mutant cells were rod shaped (Fig. 1), whereas *pbpA* mutants lacking PBP2 are spherical (28), and when cell division is blocked (for example, by thymine starvation) *cya* and *crp* mutants are able to elongate into filaments (not shown) (14).

As shown above, *cya* and *crp* mutants exhibit slow growth and small cell mass. Other mutations conferring mecillinam resistance are also associated with slow growth and small mass or spherical morphology (19, 38). It seemed possible that slowly growing cells, which are shorter than fast-growing cells, carry out less cell wall elongation and are therefore resistant to mecillinam (28a). To test this idea, we grew wild-type cells in acetate medium; the generation time was 100 min and the average cell volume was 0.7 $\mu m^3$. The

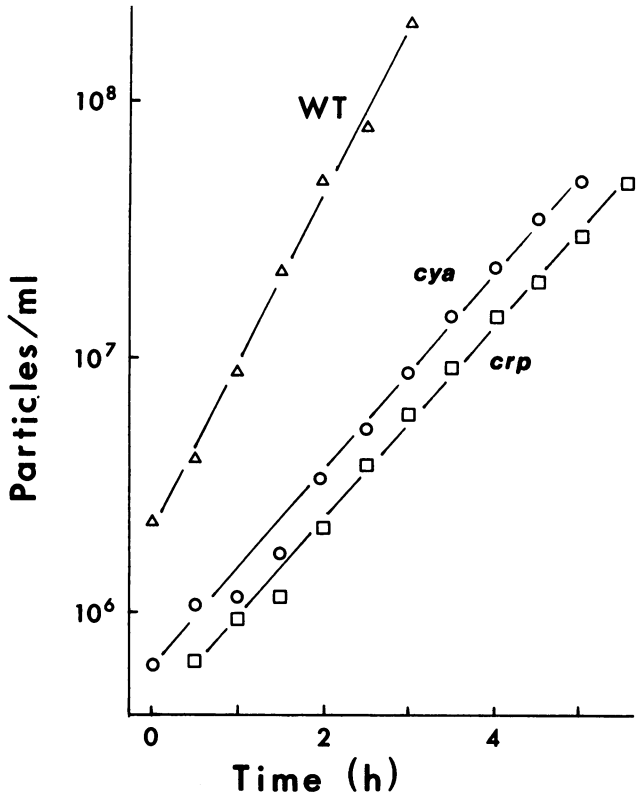


FIG. 2. Growth rate of *cya* and *crp* mutants. Overnight cultures of the wild-type (WT) strain GC2700, the *cya* mutant GC2793, and the *crp* mutant GC2846 in glucose-Casamino Acids medium were diluted $10^4$-fold in fresh medium. Samples were withdrawn periodically for analysis in the Coulter counter, starting 2.5 h after dilution; average cell volume was essentially constant in each culture for the duration of the experiment.

efficiency of plating on acetate plates containing mecillinam (10 μg/ml) was $3 \times 10^{-4}$, showing that slow growth and small size are not in themselves sufficient to confer mecillinam resistance. Neither the *cya* nor the *crp* mutant was able to use acetate as carbon source, but an independent rod-shaped mecillinam-resistant mutant isolated from the wild-type strain remained resistant in acetate medium.

**FtsZ expression.** Donachie et al. (9) reported that the FtsZ protein, thought to be required for septation, is expressed at a higher level in a *cya* mutant, suggesting that the cAMP-CAP complex may be a negative regulator of the *ftsZ* gene. Using an *ftsZ*::*lacZ* operon fusion placing β-galactosidase synthesis under control of the *ftsZ* promoters, the same authors studied the level of *ftsZ* transcription under different growth conditions; they reported (9) that over a 10-fold range of cell mass, a constant amount of FtsZ is synthesized per cell (or per septum). Since *cya* mutant cells are smaller than wild-type cells (Table 1), we measured the differential rate of synthesis of FtsZ per cell in *cya* and wild-type strains, using the same *ftsZ*::*lacZ* transcriptional fusion. The results (Fig. 3) showed that the two strains synthesized the same amount of β-galactosidase (and thus FtsZ) per cell, and the presence of exogenous cAMP in the *cya* culture did not affect this rate. The differential rate of FtsZ synthesis per mass was 1.7-fold higher in the *cya* mutant, whereas the average cell mass was 1.7-fold smaller.

The quantitative identity of the amount of FtsZ per cell in the presence or absence of cAMP reinforces the notion that this ratio, rather than the amount of FtsZ per mass, is the physiologically relevant parameter and suggests that the cAMP-CAP complex does not regulate *ftsZ* transcription.

## DISCUSSION

The first role attributed to the cAMP-CAP complex in *E. coli* was as a mediator of catabolite repression, acting as a positive transcriptional regulator of a number of catabolic operons (4, 23, 32). Subsequently, it has been found to be involved in other major regulatory networks. The phosphoenolpyruvate-dependent phosphotransferase system, responsible for the transport of numerous hexoses, is intimately related to the level of cAMP-CAP activity in the cell (25), and there are less well defined connections between cAMP-CAP and the stringent response (5, 11). In the present study we have examined the various lines of evidence that the cAMP-CAP complex may play a role in cell division regulation.

Cell mass at the moment of division is a function of growth rate and presumably reflects the temporal control of division. We show here that the smaller cell mass of *cya* and *crp* mutants is appropriate to their growth rate, being essentially

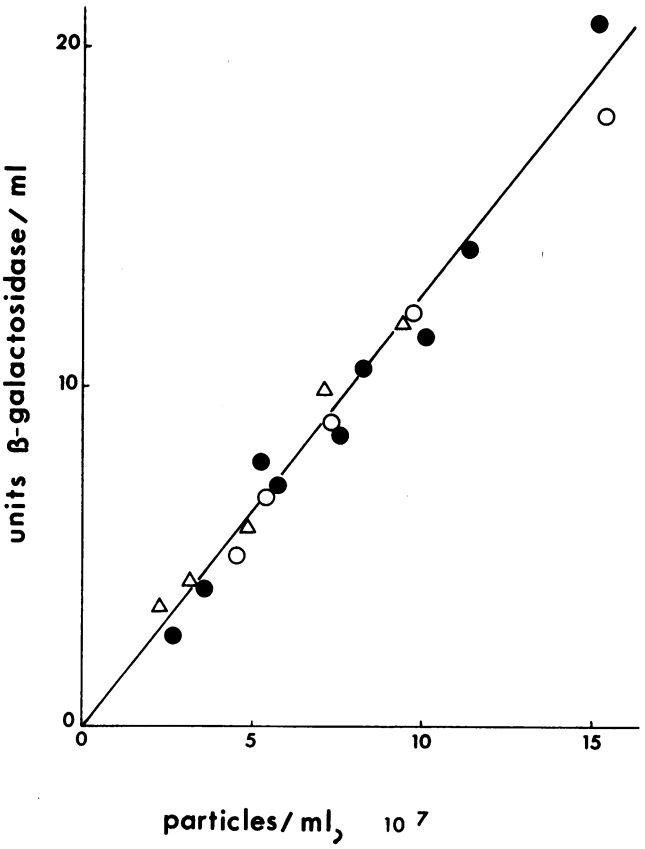


FIG. 3. Expression of the *ftsZ* gene in the presence or absence of cAMP. The *cya* mutant lysogenic for λ JFL100, carrying the *ftsZ*::*lacZ* fusion (strain GC2961), was diluted in glucose-Casamino Acids medium without cAMP (○) or with 1.0 mM cAMP (●). Samples were withdrawn periodically for counting and β-galactosidase assays. Doubling times were 48 and 31 min, respectively, and β-galactosidase specific activities per mass (20) were 85 and 55 units. The isogenic *cya* strain GC2962 (Δ) was analyzed similarly, with no exogenous cAMP; its doubling time was 27 min, and the β-galactosidase specific activity was 50 units.

the same as that of wild-type cells growing in a poorer medium at the same growth rate. This suggests that the mechanisms coupling cell division to growth rate function correctly during steady-state growth in the absence of a functional cAMP-CAP complex.

The slow growth of *cya* and *crp* mutants remains unexplained. Certain enzymes that are involved in the central metabolic pathways and known to have low levels of expression in the absence of cAMP (8, 27) may become growth rate limiting. In the course of our metabolic studies, we found that our *cya* mutant was unable to ferment glucose, although the *crp* mutant grew well anaerobically. This is presumably a direct result of the loss of adenyl cyclase, since the addition of exogenous cAMP restored growth. The *cya* defect seems to be specific to fermentation, without affecting anaerobic respiration. This suggests that cAMP is required for the expression of some fermentation enzyme but that its mediator is not CAP. It is interesting, however, that a *crp** allele, which permits CAP-promoted expression of certain catabolic operons in the absence of cAMP (26), restored fermentation ability to the *cya* mutant. It has been reported that cAMP, but not CAP, is required for the expression of fumarate reductase (34), but it was not clear that there was growth without cAMP or Casamino Acids (33). Fermentation ability is one of the few instances in which the *cya* and *crp* mutants exhibit different phenotypes. Other examples, observed in aerobic conditions, may be related to this. Whereas both mutants make small colonies on plates, as expected from their slower growth rate and lack of motility (39), those of the *cya* mutant are consistently smaller than those of the *crp* strain; this may reflect oxygen limitation at some stage of colony formation. Similarly, although both mutants have the same growth yield as the wild type in limiting glucose, the maximum yield in excess glucose is slightly higher for the *cya* mutant than for the *crp* and wild-type strains; oxygen limitation may result in carbon wasting in the latter strains via less efficient fermentative pathways.

The β-lactam mecillinam specifically inhibits PBP2 and induces spherical cell shape (22, 30). Certain resistant mutants have lost the ability to carry out cell wall elongation. These include mutations in *pbpA*, the PBP2 structural gene (28), and *rodA*, an adjacent gene of unknown function (29). Such mutants are spherical even in the absence of mecillinam and apparently grow by constant septation. The *cya* and *crp* mutants, on the other hand, have retained the ability to elongate: they are rod shaped during growth in the absence of mecillinam (Fig. 1) and form filaments when DNA synthesis is interrupted. Their mecillinam resistance is not solely due to their slow growth or small size, since the wild-type strain growing in poor medium remains sensitive.

It has been suggested that the lethality of mecillinam is due to an effect on septation (9). This could be brought about by protein-protein interactions. Evidence has recently been presented for direct interactions between the RodA protein and PBP3 (2) and between PBP3 and FtsA (31), both involved in septation, so the mecillinam-PBP2 interaction could conceivably affect the septation machinery. If so, this would imply that in the *cya* and *crp* mutants some component of the septation apparatus is qualitatively or quantitatively altered, at least in the presence of mecillinam. Further evidence for this comes from the observation that mecillinam stimulates septation at the nonpermissive temperature in the cell division mutant BUG-6 (7), which has been shown to carry a *crp*(Ts) mutation in addition to the *fts* allele (3).

The observation (9) that transcription of the *ftsZ* gene is increased in the absence of cAMP suggested a possible target for cAMP-CAP regulation of septation. The FtsZ protein, of unknown molecular activity, seems to be required early in the septation process (36). Its activity is limiting (37), and it has been suggested that FtsZ may be a septation regulator (12). The *ftsZ* gene itself is apparently subject to an unusual type of regulation; the amount of protein per cell (evaluated by means of an *ftsZ::lacZ* fusion) has been reported to be constant over a 10-fold range of cell mass (9). We show here that this regulation does not require cAMP; the higher specific activity in the *cya* mutant is exactly compensated for by its smaller cell size (Fig. 3). By this criterion, then, *ftsZ* expression is not regulated by the cAMP-CAP complex.

The cAMP-CAP complex has been shown to play a division-promoting role, probably involving the FtsZ protein, in the aberrant divisions that produce anucleate cells when DNA synthesis is arrested (14). The direct target of cAMP-CAP regulation has not yet been identified. We have recently isolated a mutant with a phenotype similar to that of the *cya* and *crp* strains (slow growth, small cell mass, and mecillinam resistance), although its cAMP-CAP complex remains functional (manuscript in preparation). We are currently studying the regulation of this gene and the relation of its product to PBP2 and the septation apparatus.

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