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# The *Haemophilus influenzae* Adenylate Cyclase Gene: Cloning, Sequence, and Essential Role in Competence

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**Competence for transformation in *Haemophilus influenzae* is stimulated by cyclic AMP (cAMP) and requires the cAMP-dependent catabolite regulatory protein CRP. Thus, understanding the control of competence will require understanding how cAMP levels are regulated. As a first step, we have cloned the *H. influenzae* adenylate cyclase gene (*cya*) by complementing the Lac$^-$ phenotype of Δ*cya Escherichia coli*. Its sequence specifies an 843-amino-acid protein which has significant identity to other known bacterial adenylate cyclases (41 to 43% and 61% identical to the *cya* genes of enteric bacteria and of *Pasteurella multocida*, respectively). As seen in other bacterial *cya* genes, there is evidence for regulation similar to that demonstrated for *E. coli*: the presence of a strong consensus CRP binding site within the promoter of the gene may provide feedback control of cAMP levels by repressing *cya* transcription, and translation may be limited by the weak ribosome binding site and by initiation of protein synthesis with GUG rather than AUG or the UUG used in other bacterial *cya* genes. We confirmed the essential role of cAMP in competence by constructing and characterizing *H. influenzae cya* mutants. This strain failed to develop competence either spontaneously or after transfer to a competence-inducing medium. However, it became as competent as its wild-type parent in the presence of exogenous cAMP. This result suggests that the failure of exogenously added cAMP to induce optimum competence in wild-type cells is not due to a limitation to the entry of cAMP into the cells. Rather, it strongly favors models in which competence induction requires both an increase in intracellular cAMP and a second as yet unidentified regulatory event. *H. influenzae* strains mutant in *cya* or *crp* were unable to ferment xylose or ribose. This confirms that *H. influenzae*, like *E. coli*, uses cAMP and CRP to regulate nutrient uptake and utilization and lends increasing support to the hypothesis that DNA uptake is a mechanism of nutrient acquisition.**

Although it is almost 50 years since bacterial transformation revealed that DNA carries genetic information (3), we still know very little about how or why some bacteria are able to take up exogenous DNA. Specifically, we do not know how transformable bacteria decide to become competent, we do not know how DNA is translocated across the cell envelope, and we do not know why these transformation systems ever evolved in the first place. In order to answer the last of these questions, we are investigating the first, guided by the principle that the regulatory mechanisms controlling competence development evolved to maximize its benefits and minimize its costs.

DNA uptake could have evolved to provide cells with nutrients, with templates for DNA repair, or with novel genetic information (24, 31, 35). In *Haemophilus influenzae*, the strongest evidence in favor of a primarily nutritional function is the involvement of cyclic AMP (cAMP) and the cAMP-binding regulatory protein CRP, which mediate catabolite repression in *Escherichia coli*. *H. influenzae* cultures cannot normally be transformed during exponential growth in rich media, but addition of 1 mM cAMP causes transformation frequencies to rise dramatically, from $\leq 10^{-8}$ to $10^{-4}$ per cell (39). However, because this competence is 100-fold lower than that induced by transfer of cells to a starvation medium, the possibility remained that cAMP had only a minor role in competence.

The importance of cAMP in competence was established when a transposon insertion that entirely prevents competence was found to disrupt a gene homologous to the *E. coli crp* gene (9). This gene encodes the cAMP-dependent DNA-binding protein CRP, which regulates expression of operons controlling many diverse pathways, especially those involved in nutrient acquisition and utilization. Because CRP acts as a transcriptional regulator only when it has bound cAMP and because in *E. coli* CRP levels are not normally limiting, CRP-regulated promoters are thought to be primarily controlled by the intracellular concentration of cAMP. cAMP levels in turn are controlled at several steps: transcriptional and translational regulation of *cya*, activation of adenylate cyclase by the phosphotransferase system (PTS), and possibly active excretion and/or enzymatic breakdown of cAMP (7).

In *H. influenzae*, the CRP-dependent induction of competence by exogenous cAMP implies that intracellular cAMP levels may regulate competence. If this is correct, then in order to understand how cells decide to become competent we need to find out how cAMP concentrations are controlled. As a first step, we have cloned and sequenced the *H. influenzae cya* gene and have constructed and characterized *cya* mutants.

## MATERIALS AND METHODS

**Sources.** Bacterial strains are listed in Table 1. All *H. influenzae* strains are descendants of Alexander and Leidy's original Rd strain (2). The *H. influenzae-E. coli* shuttle vector plasmid pSU2718 was obtained from M. Chandler (8), and λ-1316 (carrying miniTn*10kan*) was obtained from N. Kleckner (38). The λGEM-12 library of *H. influenzae* chromosomal DNA used in this study has been described elsewhere (4).

**Culture conditions.** *H. influenzae* strains were routinely

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TABLE 1. Bacterial strains used for this study

| Bacterium and strain | Genotype | Source or reference |
|---|---|---|
| *E. coli* | | |
| CA8306-nal | *thi Δcya nal* | F. O'Gara, 19 |
| DH5α | *supE44 ΔlacU169*(f80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1* | D. Hanahan |
| NM554 | *araD139 Δ(ara-leu)7696 Δ(lac)174 galU galK hsdR strA recA13* | N. Murray |
| *H. influenzae* Rd | | |
| KW20 | Wild type | 2 |
| KW26 | *str nov vio thy* | J. Setlow, 5 |
| MAP7 | *str nal kan nov stv spc vio* | J. Setlow, 4 |
| RR668 | *cya*::miniTn*10kan* | This study |
| JG98 | *tfo98*::miniTn*10kan* | M. Chandler, 36 |
| RR648 | *sxy-1*::miniTn*10kan* | 38a |

grown at 37°C in Difco brain heart infusion broth (BHI) supplemented with NAD (2 μg/ml) and equine hemin (10 μg/ml) (sBHI). Sugar fermentation was assayed with Difco phenol red assay broth supplemented with NAD, hemin, 10% BHI, and 1.0% of the sugar to be tested; the results were scored after overnight growth in loosely capped tubes. Antibiotics for the *H. influenzae* experiments were used in broth and in 1.2% agar plates at the following concentrations: novobiocin, 2.5 μg/ml; kanamycin, 7.0 μg/ml; and chloramphenicol, 2.0 μg/ml. Additional hemin (250 μl at 1 mg/ml) was applied to sBHI plates more than 24 h old. Unless indicated, media for strains with miniTn*10kan* insertions contained kanamycin.

*E. coli* strains were routinely grown in Luria-Bertani broth and plates (34). Complementation of the Lac$^-$ phenotype of Δ*cya E. coli* was scored on Difco MacConkey lactose plates. Antibiotics were used in *E. coli* plates or broth cultures at the following final concentrations: ampicillin, 50 μg/ml; kanamycin, 10 μg/ml; and chloramphenicol, 25 μg/ml.

**Competence and transformation.** In *H. influenzae*, maximal competence was induced by transfer to MIV starvation medium (18). Cells were collected from 15 ml of exponentially growing culture at an optical density at 600 nm ($OD_{600}$) of 0.25 and rinsed with and resuspended in 15 ml of MIV. The culture was then shaken at 37°C. When the time course of spontaneous competence in sBHI was to be monitored, cultures were maintained in exponential growth ($OD_{600} \leq 0.05$) for at least 2 h to allow competence to stabilize before the first sample was taken. *H. influenzae* competence was assayed by incubating 1 ml of culture (in sBHI or MIV) with 1 μg of MAP7 chromosomal DNA for 15 min at 37°C. Excess DNA was then degraded with DNase I for 5 min, and the culture was diluted and plated on sBHI to score total CFU and on sBHI plus novobiocin to select for transformants. *E. coli* cells were made competent by transfer to cold $CaCl_2$ and transformed by standard procedures (34). Strain CA8306-nal transformed poorly, perhaps because it is restriction positive.

**DNA uptake and binding assays.** MIV-treated *H. influenzae* cultures were incubated with 1 μg of $^3$H-labelled KW26 DNA (prelabelled in vivo with [$^3$H]thymidine to 7,000 cpm/μg) and with DNase I as described above. For measurements of DNA binding, the DNase I was omitted. Cells were then chilled and collected by centrifugation at 4°C, resuspended in 1 ml of cold 1 M NaCl, and centrifuged again. The cells were resuspended in 100 μl of $H_2O$, lysed by addition of 10 μl of 10% sodium dodecyl sulfate, and counted in 1 ml of Aquasol (Dupont).

**Transposon mutagenesis.** The *recA sup*$^0$ strain NM554 was first transformed with the *H. influenzae cya* plasmid pID1, and a transformant carrying a monomeric plasmid was infected with λ1316, which carries miniTn*10kan*, as described by Way et al. (38). A pool of plasmids with transposon insertions was purified from the resultant Cm$^r$ Kan$^r$ colonies. CA8306-nal was transformed with this pool and plated on MacConkey kanamycin-chloramphenicol plates. Several Kan$^r$ Cm$^r$ Lac$^-$ colonies were chosen, and their plasmids were characterized by restriction mapping. The *cya* mutant strain RR668 was then constructed by transforming KW20 to kanamycin resistance with the insert of one mutant plasmid, with a limiting DNA concentration to prevent formation and integration of multimeric fragments. Similar methods were used to generate additional *cya*::miniTn*10kan* mutations in pRJR124 and transform them into the *H. influenzae* chromosome.

**DNA sequencing.** Double-stranded plasmid DNA was isolated by alkaline lysis (34), prepared for dideoxy chain-termination sequencing with Magic Miniprep kits from Promega, and sequenced with a T7 sequencing kit, deaza G/A mixes and M13 reverse and universal primers from Pharmacia, and other specifically designed oligonucleotide primers.

**Nucleotide sequence accession number.** The *H. influenzae cya* nucleotide sequence reported here has been submitted to GenBank under the accession number L23824.

## RESULTS

**Cloning and mapping *cya*.** We initially cloned the *H. influenzae cya* gene by screening an *H. influenzae* plasmid library for complementation of the Lac$^-$ phenotype of *E. coli* CA8306-nal. Because this strain cannot produce cAMP, it is unable to ferment catabolite-repressed sugars and gives white colonies on MacConkey lactose indicator plates. Accordingly, we constructed an *H. influenzae* KW20 genomic library of *Sau*3A partial-digestion fragments in the shuttle vector pSU2718, transformed the library into CA8306-nal, and isolated our initial *cya* plasmid, pID1, from a red colony on a MacConkey lactose-chloramphenicol plate. Subcloning and preliminary sequencing identified an open reading frame in pID1 with an amino acid sequence homologous to the catalytic domains of other bacterial *cya* genes.

Unfortunately, when we probed a Southern blot of KW20 chromosomal DNA with pID1, we learned that its insert was a chimera; the segment downstream of *cya* was derived from another part of the chromosome and had probably been ligated to the *cya* fragment during construction of the library. Consequently, we reisolated the *cya* gene from an *H. influenzae* λGEM-12 library (4) by plaque hybridization, using as a probe the 1.0-kb *Xba*I-*Kpn*I subfragment containing the central segment of the *cya* gene (Fig. 1C). The presence of an intact chromosomal segment in this phage (λ15-8 in Fig. 1B) was confirmed by restriction mapping and Southern blot hybridization. A 7.7-kb *Eco*RI-*Sac*I fragment was subcloned from λ15-8 into pGEM-7, forming the *cya*$^+$ plasmid pRJR124 (Fig. 1C). This plasmid strongly complements the *cya* mutation in CA8306-nal and was used to obtain the complete sequence of *cya*.

**Sequence analysis.** Figure 2 shows the sequence of the *H. influenzae cya* gene and its deduced amino acid sequence. Alignment of the amino acid sequence to those of other bacterial adenylate cyclases is shown in Fig. 3. As expected, the *H. influenzae* protein is more closely related (61% identity) to that of *Pasteurella multocida* (25) (both are in the family *Pasteurellaceae*) than to those of enteric bacteria (41 to 43% identity). The alignment has no major nonhomologous seg-

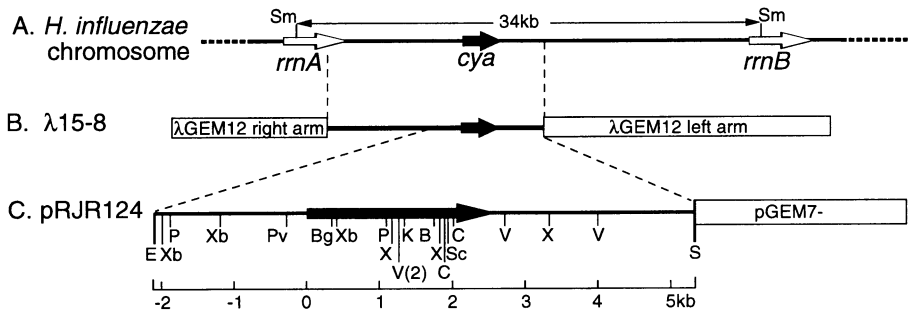


FIG. 1. Chromosomal and cloned fragments of the *H. influenzae cya* gene. (A) The chromosomal segment containing *cya*. Sm, *Sma*I sites. (B) Phage λ15-8, containing a 15-kb *cya* fragment. (C) pRJR124, the *cya* fragment cloned from λ15-8 into pGEM7−. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I; Sc, *Sca*I; V, *Eco*RV; X, *Xho*I; Xb, *Xba*I. The arrows indicate the direction of transcription.

ments; mismatches and short gaps occur mainly in regions previously seen to align poorly (25). However, the *P. multocida* and *H. influenzae* proteins lack an 18- to 22-amino-acid tail which is present but poorly conserved in *E. coli* and *Erwinia chrysanthemi*.

The TTG initiation codons of the other bacterial *cya* genes shown in Fig. 3 align with a GTG codon in the *H. influenzae cya* sequence. This is likely to be the true initiation codon, as the nearest 3′ alternatives are 42 bp (TTG) and 300 bp (ATG) downstream and there is an in-frame stop codon 12 bp 5′ of the TTG, which would terminate translation initiating upstream. Analysis of the region upstream of the initiation codon identified a poor potential ribosome binding site (TAGGGA) at bp −12 to −7. The two strongest matches to the *E. coli* promoter consensus sequence (enclosed in dashed boxes in Fig. 2) both agree with the most important (underlined) bases of the consensus TTGACAN$_{17}$TATAAT (17).

Like its *E. coli* homolog, the *H. influenzae cya* promoter contains a sequence very similar to the *E. coli* CRP binding site consensus, which could mediate cAMP-dependent repression of *cya* transcription. The *H. influenzae* CRP site (bp −89 to −68 [solid box in Fig. 2]) is potentially very strong, with 18 of 22 identities to the *E. coli* CRP consensus AAATGTGATCTA GATCACATTT (7). There is a second possible CRP binding site between bases −27 and −48. Because it has only 11 of 22 matches to the consensus, CRP binding is likely to be weaker but may still be significant.

We were unable to identify any potential stem-loop structures that could serve as transcription terminators in the 120 bp we have sequenced 3′ of the *cya* stop codon. Nor did we find any perfect copies of the *H. influenzae* 11-bp DNA uptake signal sequence AAGTGCGGTCA (15), which may participate in termination (21). However, there is a 10-of-11 match to the uptake sequence at bp 967, and two matches to the 9-bp version of the sequence (AAGTGCGG) occur at bp 370 and 2140.

**Chromosomal location of *cya*.** The chromosomal location of the *H. influenzae cya* gene was initially estimated by probing pulsed-field gel filters (22) with a 1.0-kb *Xba*I-*Kpn*I subfragment containing the central segment of the *cya* gene (Fig. 1C). The probe hybridized to bands corresponding to the 34-kb *Sma*I K fragment and the comigrating 32-kb *Apa*I O and P fragments (results not shown), placing *cya* in the 30-kb region between *rrnA* and *rrnB*, at 340 to 370 kb on the revised chromosomal map (32). We were able to use the *Sma*I site in the miniTn*10kan* transposon insertion present in the *cya* mutant strain RR668 (described below) to precisely locate *cya* within this segment. *Sma*I digests of RR668 chromosomal DNA showed the expected loss of the 34-kb *Sma*I K fragment and the appearance of new fragments consistent with a transposon insertion 10 kb from either the *Sma*I site in *rrnA* or that in *rrnB*. Probing pulsed-field gel filters with the upstream part of λ15-8 showed hybridization to those restriction fragments containing 23S rRNA genes (22), indicating that *cya* is at 350 kb on the *H. influenzae* map and is transcribed in the same direction as the *rrnA* and *rrnB* operons (Fig. 1A).

**Competence studies.** To investigate the role of adenylate cyclase in competence development, we used transposon mutagenesis to construct *H. influenzae cya* mutant strains. We first mutagenized pID1 with miniTn*10kan* (38) in the *E. coli recA sup*$^0$ host NM554 and identified an insertion-bearing plasmid that failed to complement CA8306-nal. Restriction mapping and sequencing located the insertion at bp 917 in the putative catalytic domain of *cya*. This mutated *cya* gene was then transformed into the *H. influenzae* KW20 chromosome, and the *cya* mutant strain RR668 was selected by screening for kanamycin-resistant transformants. Southern blot analysis confirmed that RR668 carries a simple gene disruption of *cya*.

As shown in Fig. 4, wild-type cultures are not competent during exponential growth but develop moderate competence at the onset of stationary phase (open squares in Fig. 4A) and become fully competent after transfer to the starvation medium MIV (open squares in Fig. 4C). In contrast, the *cya* mutant failed to develop competence at all. The transformation frequency in MIV was about $10^{-7}$; this is $10^5$-fold lower than that of the wild type.

The presence of exogenous cAMP causes wild-type cultures to become moderately competent during exponential growth but does not change the levels of competence seen in stationary phase or in MIV. The response of the *cya* mutant to exogenous cAMP confirmed that its competence defect is caused solely by its inability to produce cAMP. In the presence of cAMP, wild-type cultures and mutant cultures developed competence at exactly the same rate, and showed identical transformation frequencies (Fig. 4). In the absence of added cAMP, strain RR668 showed no detectable DNA binding or DNA uptake after MIV treatment, confirming that the defect is in the development of competence rather that in a later recombination step. Addition of cAMP restored both DNA binding and DNA uptake (data not shown).

The *cya* mutation had little effect on cell growth rate in the rich medium sBHI. The slower growth exhibited by strain RR668 (Fig. 4B) is at least partly because of the presence of kanamycin in its culture medium, as a parallel culture without

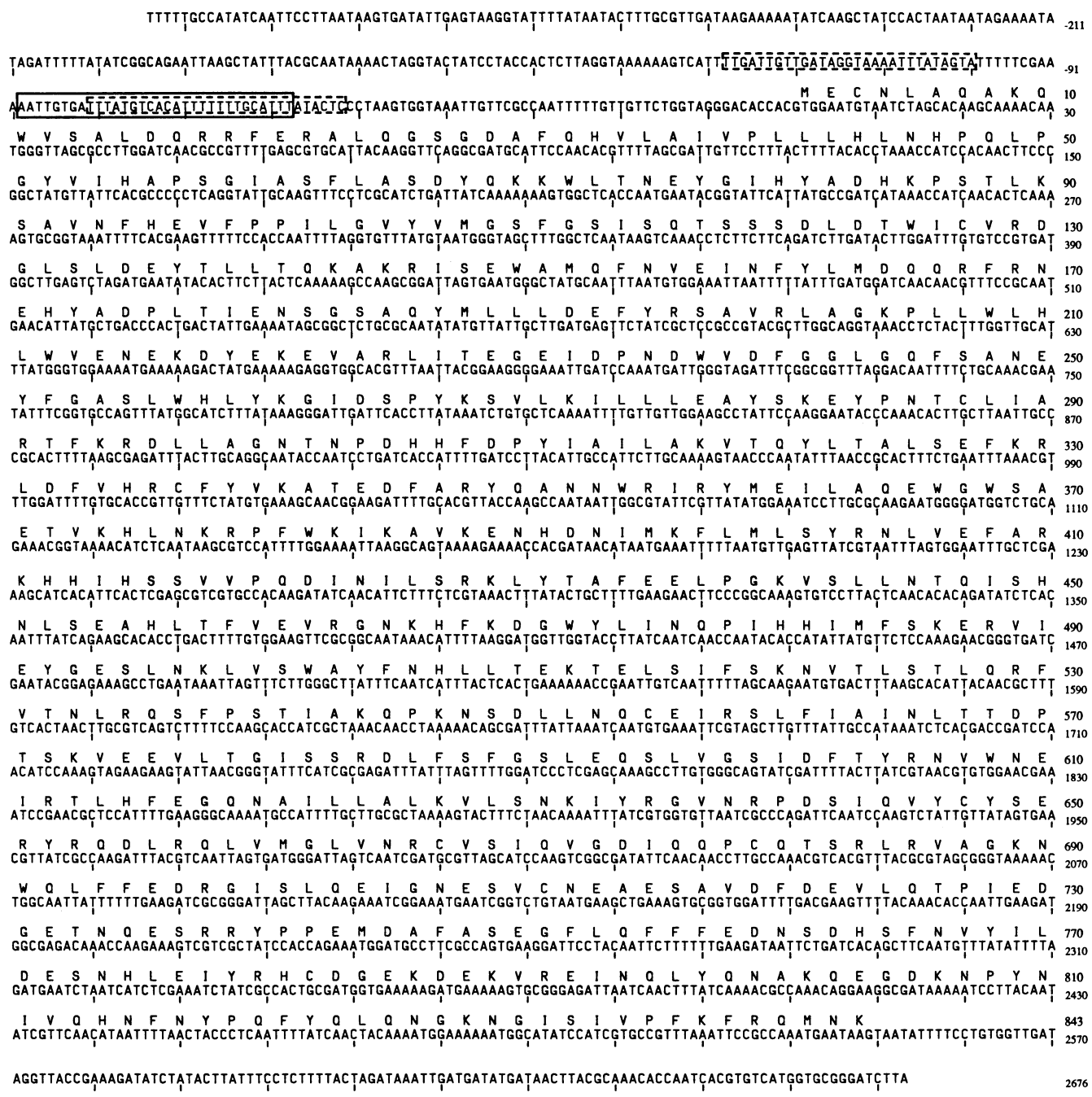

FIG. 2. Nucleotide and deduced amino acid sequence of the *cya* gene. Nucleotide 1 is the first G of the GTG initiation codon. Two possible promoter sequences are enclosed in dashed boxes, and the strong CRP consensus site is enclosed in a solid box.

kanamycin grew almost as fast as the parent (data not shown), and all the cultures in sBHI reached the same final density. After transfer to MIV, wild-type cells usually complete one cell doubling before ceasing division (Fig. 4D), but the *cya* mutant abruptly ceased cell division unless cAMP was present.

We have also examined several *H. influenzae* strains carrying miniTn*10kan* insertions in the putative regulatory domain of *cya* (at approximately bp 1100, 1300, 1600, and 2100). Although these insertions did not completely eliminate adenylate cyclase activity in *E. coli* (CA8306-nal with these plasmids gave white colonies with red centers), in *H. influenzae* they all caused competence defects as severe as that of the catalytic-domain insertion described above (data not shown).

**Role of cAMP and CRP in catabolite regulation.** To determine whether cAMP and CRP play a role in sugar uptake and metabolism in *H. influenzae*, we grew cultures of wild-type and mutant strains in phenol red broth supplemented with various sugars. The phenol red serves as an indicator of the drop in pH caused by sugar fermentation. Although the wild-type strain KW20 grew well in phenol red broth supplemented only with

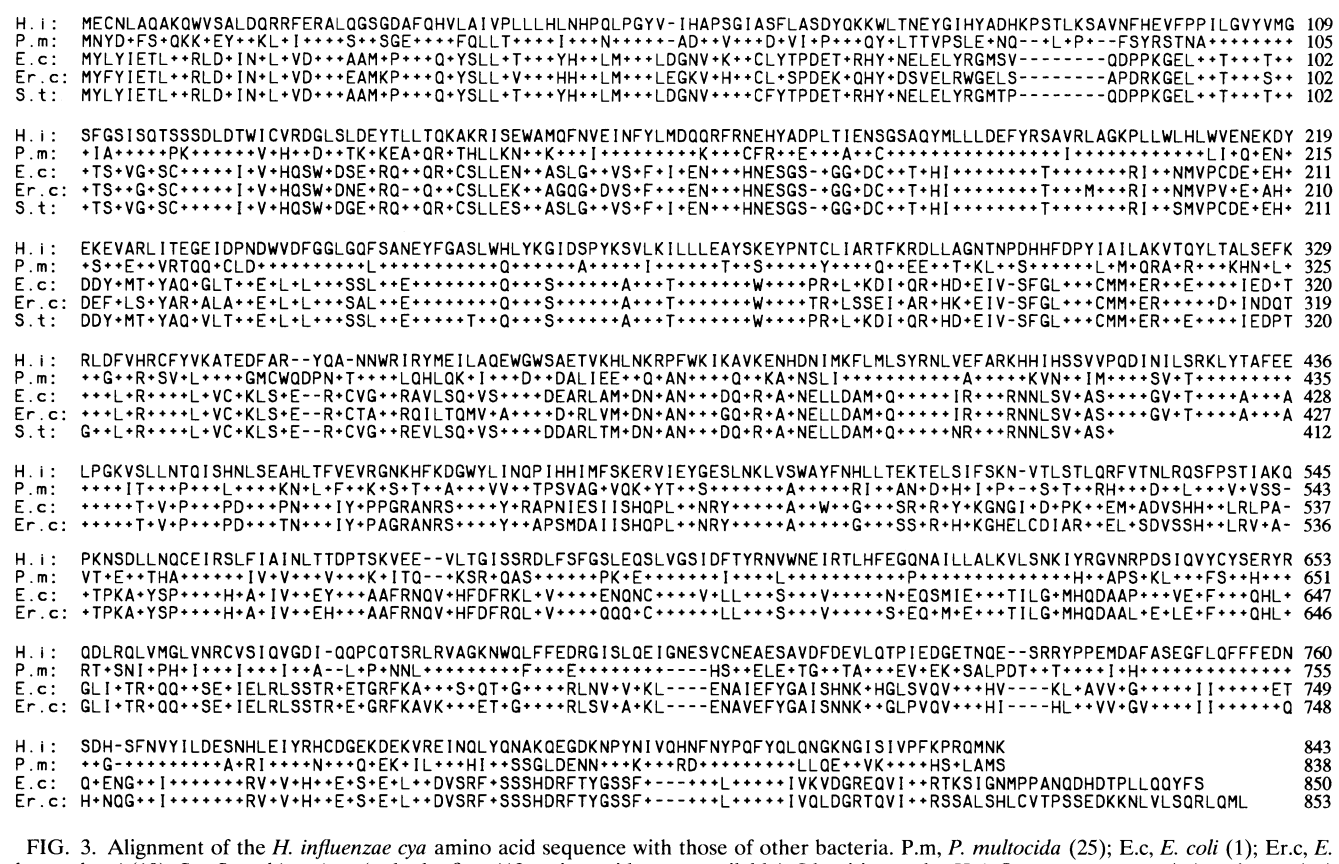


FIG. 3. Alignment of the *H. influenzae cya* amino acid sequence with those of other bacteria. P.m, *P. multocida* (25); E.c, *E. coli* (1); Er.c, *E. chrysanthemi* (10); S.t, *S. typhimurium* (only the first 412 amino acids were available). Identities to the *H. influenzae* sequence (+) and gaps (−) are indicated.

hemin and NAD, *cya* and *crp* mutants grew poorly unless the broth was also enriched with 10% BHI. Consequently, the enriched medium was used for all strains.

Apart from glucose, the only sugars *H. influenzae* is reported to use are galactose, ribose, maltose, and xylose (20). A previous report of deoxyribose fermentation was in error (19a). Table 2 shows that, as expected, wild-type *H. influenzae* fermented glucose, ribose, xylose, and galactose but not lactose (we have not been able to detect maltose fermentation by any of our strains). The *crp* and *cya* mutants were unable to ferment ribose or xylose and gave an intermediate color change with galactose. Addition of cAMP restored sugar fermentation to the *cya* strain but had no effect in other strains. To demonstrate that the failure to ferment sugars is not a general property of nontransformable cells and to control for the presence of kanamycin in the mutant cultures, we tested strains JG98 and RR648. These strains contain miniTn*10kan* insertions that prevent competence but have no known effects on metabolism; both strains fermented the sugars as well as did KW20.

## DISCUSSION

The results here show that production of cAMP by adenylate cyclase is essential for competence development. This result is not unexpected, because Chandler has shown that competence requires CRP and we know that in other bacteria CRP is active only when complexed with cAMP. The present confirmation allows us to begin looking at how intracellular cAMP levels are themselves controlled and how changes in these levels interact with other cellular processes to bring about competence.

**Control of cAMP concentration.** In *E. coli* and *Salmonella typhimurium*, the major *cya* promoter is repressed by CRP binding to a site centered on the −10 promoter element (12, 26), thus providing feedback from intracellular cAMP levels. Expression is also limited by inefficient translation: the gene has a poor ribosome binding site, uses UUG as a start codon, and contains a high proportion of rare codons in its first 20 amino acids (1). Presumably this poor translation helps cells maintain a low but stable level of adenylate cyclase, which is necessary if basal cAMP concentrations are to be maintained at only a few hundred molecules per cell (6). In *E. coli*, modulation of cAMP levels in response to nutrient availability is thought to depend on activation of adenylate cyclase by $EnzymeIII^{Glc}$ of the PTS (23) and possibly on degradation and excretion of cAMP. Because adenylate cyclase retains some catalytic activity even after C-terminal deletion of up to 70% of the *cya* gene, the protein is thought to consist of an N-terminal catalytic domain and a larger C-terminal regulatory domain which mediates interactions with $EnzymeIII^{Glc}$ (33).

Our sequence analysis suggests that some of these controls also act in *H. influenzae*. The *H. influenzae* CRP site is in a position to interfere with transcription from either of the possible promoters, especially because its near-perfect match to the *E. coli* CRP-binding consensus predicts very tight binding (11). Feedback repression of *cya* transcription is likely to be essential for maintaining low basal adenylate cyclase levels; we do not yet know whether it also has a role in

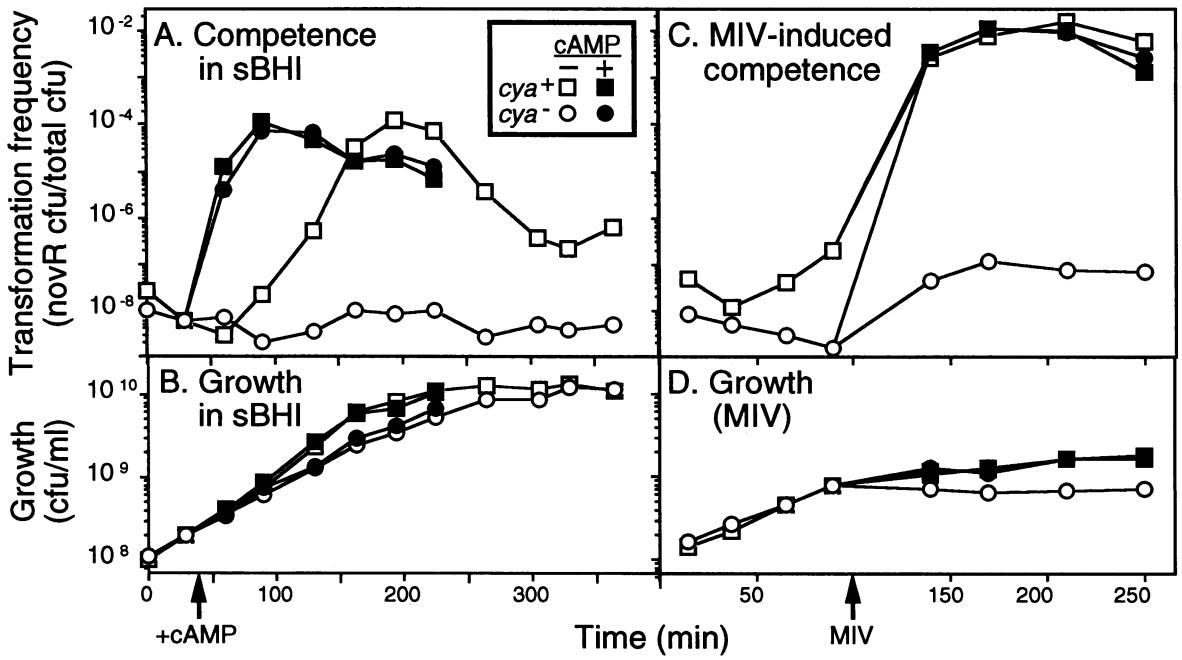


FIG. 4. Time courses of competence development in sBHI and MIV with or without cAMP. Total and novobiocin-resistant (novR) CFU counts were determined after transformation with MAP7 DNA. (A and B) Exponentially growing cultures of KW20 in sBHI (squares) and RR668 in sBHI plus kanamycin (circles) were split at an optical density at 600 nm of 0.05, and one portion of each was supplemented with 1 mM cAMP (solid symbols). (C and D) Similar exponentially growing cultures at an optical density at 600 nm of 0.2 were transferred to MIV with (solid symbols) and without (open symbols) 1 mM cAMP. The times of addition are indicated (arrows).

competence development. GTG appears to serve as the *cya* initiation codon. In *E. coli*, TTG is the least efficient of the three known initiation codons; replacing it with GTG or ATG increases *cya* translation twofold or sixfold, respectively (27). We do not know how efficiently *H. influenzae* can use each of these codons. It may be that, because the *H. influenzae* genome is very AT rich, GTG is its rarest initiation codon and thus helps limit translation of *cya*. Codon usage in the first 20 amino acids does not appear to differ significantly from that of other *H. influenzae* genes (35a, 37). In *H. influenzae*, the presence of a PTS has not been demonstrated. However, both the putative catalytic and regulatory domains of the *H. influenzae* adenylate cyclase are homologous to those of the *P. multocida* enzyme, which has been shown to be subject to PTS regulation in *E. coli* (25). Because control of adenylate cyclase is probably a key regulator of competence, we are at present investigating whether *H. influenzae* has phosphoenol pyruvate-dependent protein phosphorylation and whether the presence of glucose or other sugars affects the development of competence.

TABLE 2. Sugar fermentation by wild-type and mutant *H. influenzae*

| Strain (genotype) | Sugar provided[a] | | | | | |
|---|---|---|---|---|---|---|
| | None | Glucose | Ribose | Xylose | Galactose | Lactose |
| KW20 (wild type) | − | + | + | + | + | − |
| RR668 (*cya*) | − | + | − | − | + (weak) | − |
| RR668 with cAMP | − | + | + | + | + | − |
| RR540 (*crp*) | − | + | − | − | + (weak) | − |
| RR540 with cAMP | − | + | − | − | + (weak) | − |
| JG98 (*tfo98*) | − | + | + | + | + | − |
| RR648 (*sxy-1*) | − | + | + | + | + | − |

[a] +, fermentation; −, no fermentation.

We have tried repeatedly to measure intracellular cAMP in *H. influenzae* by radioimmunoassay, but the values we have obtained were too low to be considered reliable, probably because removing extracellular cAMP from the cells washes out some intracellular cAMP. The cell volume is only 1/10 that of *E. coli*, so that under nonactivating conditions each *H. influenzae* cell may contain only 20 to 30 molecules of cAMP. The high surface-to-volume ratio of these tiny cells and the possible presence of an active cAMP efflux system (13) may permit rapid loss of cAMP during washing.

**How cAMP might control competence.** As is true of catabolite-regulated operons in *E. coli*, there is good reason to believe that induction of competence in *H. influenzae* requires more than just an increase in intracellular cAMP concentration. Wise and coworkers (39) first observed that addition of cAMP only raised transformation frequencies to the level that develops spontaneously at the onset of the stationary phase, which is about 100-fold lower than that seen after transfer of exponentially growing cultures to MIV. One possible explanation is that extracellular cAMP cannot raise intracellular cAMP to fully inducing levels. Zoon et al. (40) investigated this by examining the increase in lactate dehydrogenase activity caused by cAMP addition and by transfer to MIV. They found that both treatments increased lactate dehydrogenase about threefold (presumably by relief of catabolite repression) and concluded that relief of catabolite repression was not itself a sufficient condition for the development of competence.

We were able to test this more rigorously by examining the effect of exogenous cAMP on competence development by our *cya* mutants. The *cya* mutations reduced MIV-induced compe-

tence 100,000-fold. Addition of cAMP to the MIV brought the mutants' transformation frequencies up not just to the level characteristic of cAMP-induced wild-type cells in rich medium but to the maximal competence observed with wild-type cells in MIV. This confirms that exogenous cAMP can yield intracellular cAMP concentrations adequate for maximal development of competence and suggests that the failure of cAMP to induce similar competence in rich medium reflects the need for an additional regulatory event.

This cAMP-independent event must be induced in all cells when cultures are transferred to MIV, but it may also occur spontaneously in the small fraction of cells that develop competence under other conditions (e.g., at the onset of stationary phase in rich medium and after addition of cAMP). It is well established that most of the cells in MIV-induced cultures are competent (shown by analysis of the frequency of cotransformation with genetically unlinked markers [14]). We have applied a similar analysis to the competence induced by cAMP and at onset of stationary phase. Our results indicated that only about 1 cell in 100 is fully competent (transforms as efficiently as an MIV-treated cell) under these conditions. These competent cells account for all of the transformants; the remaining 99% of the cells in the culture do not transform at all (10a). This implies that competence is an all-or-nothing state, and might be explained by a model in which the cells that become competent in late log phase or after cAMP addition are those in which the cAMP-independent regulatory event has occurred spontaneously.

Halling has pointed out that because small cells may contain very few molecules of key metabolites and regulators, stochastic fluctuations in the concentrations (or states) of these molecules may be very important (16). *H. influenzae* cells are very small, and random fluctuations in basal cAMP concentrations could explain not only the competence of some cells in wild-type cultures at stationary phase or in the presence of cAMP (by spontaneous activation of the cAMP-independent regulator in about 1% of cells) but also the hypercompetent phenotype of cells carrying a mutation in the *sxy-1* gene (29) (by activation of CRP in cells whose other regulator is already activated by the *sxy-1* mutation). Analysis of the frequency of cotransformation by this hypercompetent mutant under less than fully inducing conditions revealed that its transformants also arose from a small fraction of fully competent cells in a background of noncompetent cells (unpublished results). It may be that the effect of the *sxy-1* mutation is to permanently activate this second regulator, so that activation of CRP (by an increase in cAMP) leads to competence.

**The biological function of competence.** Our goal is to understand how naturally transformable bacteria benefit from their ability to take up DNA. The potential benefits of producing recombinant progeny are unpredictable and infrequent and likely to be overshadowed by the more frequent costs associated with DNA uptake and the risk of acquiring deleterious alleles, especially if, as is likely, the available homologous DNA is derived from cells that have died (28). We feel that the best indicator of transformation's function is its mode of regulation, because the genes that regulate competence development themselves will have evolved to optimize the cell's use of transformation. By this analysis, the use of transforming DNA as templates for DNA repair appears unlikely to be the primary function of transformation because we have found that competence is not induced by DNA damage (30).

The experiments described above show that cAMP and CRP regulate competence. In enteric bacteria, cAMP and CRP do not control competence (in fact, these bacteria are not known to have natural transformation systems at all) but do regulate the uptake and catabolism of many sugars, amino acids, and nucleosides, as well as ferric iron uptake, bioluminescence, pilus adhesion, and chemotaxis (7). The fermentation defects of our *cya* and *crp* mutants show that in *H. influenzae* cAMP and CRP do indeed control sugar utilization.

The essential role of cAMP and CRP in competence suggests that DNA uptake may contribute to the cell's carbon-energy budget. By far the simplest contribution would be the nutrients contained in the DNA itself. While the energy contained in the amounts of DNA taken up under laboratory conditions may not be significant, the nucleotides might be. *H. influenzae* is unable to synthesize its own purines and lives on the mucosal surfaces of the human respiratory tract, an environment that contains extremely high concentrations of host DNA. We are currently investigating whether these cells can obtain their nucleotides by taking up intact extracellular DNA.

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