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A 3′,5′ Cyclic AMP (cAMP) Phosphodiesterase Modulates cAMP Levels and Optimizes Competence in *Haemophilus influenzae* Rd

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Changes in intracellular 3′,5′ cyclic AMP (cAMP) concentration regulate the development of natural competence in *Haemophilus influenzae*. In *Escherichia coli*, cAMP levels are modulated by a cAMP phosphodiesterase encoded by the *cpdA* gene. We have used several approaches to demonstrate that the homologous *icc* gene of *H. influenzae* encodes a functional cAMP phosphodiesterase and that this gene limits intracellular cAMP and thereby influences competence and other cAMP-dependent processes. In *E. coli*, expression of cloned *icc* reduced both cAMP-dependent sugar fermentation and β-galactosidase expression, as has been shown for *cpdA*. In *H. influenzae*, an *icc* null mutation increased cAMP-dependent sugar fermentation and competence development in strains where these processes are limited by mutations reducing cAMP synthesis. When endogenous production of cAMP was eliminated by a *cya* mutation, an *icc* strain was 10,000-fold more sensitive to exogenous cAMP than an *icc*$^+$ strain. The *icc* strain showed moderately elevated competence under noninducing conditions, as expected, but had subnormal competence increases at onset of stationary phase in rich medium, and on transfer to a nutrient-limited medium, suggesting that excessive cAMP may interfere with induction. Consistent with this finding, a *cya* strain cultured in 1 mM cAMP failed to develop maximal competence on transfer to inducing conditions. Thus, by limiting cAMP levels, the *H. influenzae* cAMP phosphodiesterase may coordinate its responses to nutritional stress, ensuring optimal competence development.

The cyclic nucleotide 3′,5′ cyclic AMP (cAMP) is a central mediator of the responses of enteric bacteria to changes in the nutritional environment. Expression of a large number of cAMP-controlled genes is elevated when intracellular cAMP levels peak at the onset of starvation (24) or stationary phase (38).

In enteric bacteria, intracellular levels of cAMP are determined by the relative rates of its synthesis, excretion, and degradation (4). Adenylate cyclase, which synthesizes cAMP, has only basal activity during exponential growth on preferred sugars and is stimulated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) when these sugars are depleted from the medium. cAMP is excreted when preferred sugars are restored (7), but the regulatory significance of excretion is unclear and awaits isolation of excretion-defective mutants. Finally, cAMP levels can be reduced by cAMP-specific phosphodiesterases, which catalyze cleavage of 3′,5′-cAMP to 5′-cAMP. Although the regulatory role of cAMP phosphodiesterases has been unclear (9), Imamura et al. recently demonstrated that the cAMP phosphodiesterase produced by the *Escherichia coli cpdA* gene modulates intracellular cAMP levels and proposed that such phosphodiesterases may regulate expression of cAMP-dependent genes (20).

In *Haemophilus influenzae*, cAMP regulates development of natural competence, and cells mutant in the adenylate cyclase gene (*cya*) or the cAMP receptor protein (CRP) gene (*crp*) cannot become competent (11, 14). Although direct measurement of cAMP concentrations in *H. influenzae* has not been achieved (14), competence is undetectable during exponential growth, when cAMP levels are expected to be low. It develops spontaneously when exogenous cAMP is added or when endogenous cAMP levels are expected to be high, for example, at the onset of stationary phase in rich medium. The highest levels of competence are seen when exponential-phase cells are transferred to the nutrient-limited medium MIV (18), when intracellular cAMP levels are expected to peak.

Competent cells can take up several hundred kilobases of DNA from their environment. Some of this DNA may be homologously recombined into the chromosome; nonrecombined DNA is degraded, and the nucleotides are reused (35). The requirement of cAMP for competence induction suggests that *H. influenzae*'s ability to take up DNA from the environment may be a response to nutritional stress, with the degraded DNA serving as a source of nucleotides. To explore this possibility, we are investigating the regulation of intracellular cAMP levels in *H. influenzae* and their relationship to competence.

We have previously shown that the synthesis of cAMP by adenylate cyclase is regulated by a simple fructose-specific PTS and that this in turn regulates competence development (22). Efflux of cAMP has not been studied. *H. influenzae* does, however, possess a *cpdA* homolog, *icc* (15, 20). Below, we show that *icc* encodes a cAMP phosphodiesterase which functions together with adenylate cyclase to fine-tune intracellular cAMP levels and to modulate competence development (and other cAMP-dependent processes) according to growth rate and/or nutrient availability.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Tables 1 and 2. Strain RR801 was derived from a *ptsI*::*kan crr*::*cat* strain obtained from M. Gwinn (17). Double-mutant strains RR819, RR820, and RR821 were constructed by transforming strains RR668, RR745, and RR801 to spectinomycin resistance with limiting RR812 chromosomal DNA.

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source and/or reference
H. influenzae Rd		
KW20	Wild type	1
RR812	*icc*::*spc*	This study
RR668	*cya*::mini-Tn*10kan*	14
RR745	*ptsI*::mini-Tn*10kan*	22
RR801	*crr*::*cat*	This study
RR819	*ptsI*::mini-Tn*10kan icc*::*spc*	This study
RR820	*crr*::*cat icc*::*spc*	This study
RR821	*cya*::mini-Tn*10kan icc*::*spc*	This study
MAP7	*str kan nov nal spc vio stv*	J. Setlow (6)
E. coli		
W3110	Wild type	R. Redfield
SH8150	*cpdA*::*kan*	H. Niki (20)
GM2163	*dam*	New England BioLabs

Culture conditions. *H. influenzae* cells were grown aerobically at 37°C in brain heart infusion broth (BHI; Difco) supplemented with hemin and NAD (sBHI), with the recommended concentrations of antibiotics (6). Additional hemin was applied to sBHI plates greater than 24 h old. *E. coli* strains were routinely grown aerobically in Luria-Bertani broth at 37°C, with the recommended concentrations of antibiotics (5).

Competence and transformation. Spontaneous competence development by *H. influenzae* was followed during growth in sBHI, while maximal competence was induced by transfer of exponential-phase cells to MIV starvation medium (18). Competence was assessed as transformation frequency. *E. coli* cells were made competent by transfer to cold $CaCl_2$ and transformed by standard procedures (5).

Sugar fermentation assays. Sugar fermentation by *H. influenzae* was assayed in Difco phenol red broth (PRB) supplemented with NAD, hemin, 10% BHI, and 1% sugar to be tested. After overnight growth in loosely capped tubes, results were scored by measurement of culture pH. Sugar fermentation by *E. coli* was scored on Difco MacConkey agar plates containing 1% lactose or maltose.

Cloning and mutagenesis of *icc*. The availability of the full *H. influenzae* genome sequence (36) has simplified the construction of directed mutations in genes of interest. A 3-kb region of KW20 chromosomal DNA containing the *icc* operon (genes HI 0398 and HI 0399) (15) was amplified by PCR using the following forward and reverse primers (*H. influenzae* genome positions are given in parentheses): 5′-GCGTAAATCGCCAAGTGACGG-3′ (bp 419090 to 419111) and 5′-GCATTCCGTTCAACTTGGGC-3′ (bp 422142 to 422122). The resulting fragment was cloned into the vector pGEM-T (Promega) to generate pCMP. A gel-purified spectinomycin resistance gene (*spc*) cassette from *Bam*HI-cut plasmid pKRP13 was ligated into *Bcl*I-cut pCMP (prepared from the *dam E. coli* strain GM2123) (Fig. 1), giving pCMP::spc. MIV-competent KW20 cells were transformed to spectinomycin resistance with *Apa*I-linearized pCMP::spc. Southern blotting of genomic DNA from the new *icc*::*spc* strain RR812 with *spc* and *icc* PCR product probes confirmed that it contained a single cassette insertion in the *icc* gene.

β-Galactosidase assay. Cells were grown in M9 minimal glucose medium (5) supplemented with 0.5% Casamino Acids and 1 mM isopropyl-β-D-thiogalactopyranoside. β-Galactosidase activity was assayed by using a modification (33) of the method described by Miller (25).

RESULTS

Analysis of the *icc* gene. The gene now known to encode the *E. coli* cAMP-specific phosphodiesterase was originally characterized as affecting only the expression of the *lacZ* gene (19), and the gene sequence was submitted to GenBank under the name *icc*. Imamura et al. subsequently demonstrated that this gene encodes a 3′,5′-cAMP-specific phosphodiesterase (20) and renamed it *cpdA*. The existence of an *H. influenzae icc* homolog was noted (15, 20).

The predicted 274-amino-acid Icc protein shows 53.3% identity and 71.3% similarity to its *E. coli* homolog, CpdA (36). Gapped TBLASTN (3) searching of the GenBank databases and available bacterial genome sequences detected putative Icc homologs with extensive amino acid sequence identities (E [expect value] < 1e-30) in only three other bacterial species, all members of the gamma subdivision of the proteobacteria (31) (Table 3). In addition, cAMP-specific phosphodiesterases have been purified from the proteobacteria *Serratia marcescens* (30), *Salmonella typhimurium* (2), and *Klebsiella aerogenes* (10). As expected, the *icc*-containing organisms with complete genome sequences available also contain adenylate cyclase (*cya*) homologs: *H. influenzae*, *E. coli*, *Pseudomonas aeruginosa*, and *S. typhimurium*. These findings also suggest that 3′,5′-cAMP phosphodiesterases may be limited to the proteobacteria.

The *icc* gene may belong to a conserved nucleotide-processing operon. The upstream gene HI 0398 is separated from *icc* (HI 0399) by only 13 bp (Fig. 1). Similarly, an HI 0398 homolog is found immediately upstream of the *icc* homologs of *P. aeruginosa* and *Actinobacillus actinomycetemcomitans* and is separated by one gene from the *E. coli icc* homolog *cpdA* (36). HI 0398 and its homologs all contain a signature sequence common to members of the MutT family of proteins, whose members hydrolyze nucleoside diphosphate linkages in various substrates (29). Although we found no putative *icc*-specific promoters (by sequence comparison with the *E. coli* promoter consensus), we identified two potential promoters upstream of the HI 0398 initiation codon: P1 at bp 419321 to 419351 and P2 at bp 419417 to 419445.

We identified a putative binding site for CRP immediately 5′ to P2 (5′-TTTTGTGACTCACTTCAAACTC-3′ at bp 419394 to 419416) by comparison with the *E. coli* CRP-binding consensus (7) (Fig. 1). This finding suggests that expression of *icc* is not constitutive but may be induced as cAMP levels rise.

Demonstration of a functional *icc*-encoded phosphodiesterase. To determine whether the *E. coli* and *H. influenzae* cAMP phosphodiesterase homologs have similar activities, we compared the effects of overexpression of each on cAMP-dependent processes in *E. coli*. Expression of the *E. coli* lactose and maltose utilization operons is cAMP dependent, and Imamura et al. have shown that overexpression of *cpdA* reduces transcription of *lacZ* and catabolism of lactose analogs (20). As judged by colony color on MacConkey agar plates supplemented with 1% lactose or maltose, plasmids carrying the *E. coli* and *H. influenzae* homologs of *cpdA* reduced fermentation of both sugars by *E. coli* W3110 to similar degrees. Disruption of the *icc* gene by gene cassette insertion in pCMP::spc abolished this effect, while vector controls pACYC184 and pGEM*sxy* had no effect on sugar fermentation (23).

β-Galactosidase activity is a more sensitive indicator of intracellular cAMP concentration in cells expressing different phosphodiesterase homologs. We found that pCMP, like pAX923 (20), reduced β-galactosidase activity (presented as mean ± standard error of the mean [SEM]) by at least 75% (Fig. 2). These data confirm that the *H. influenzae* gene *icc* encodes a cAMP phosphodiesterase.

Regulation of *H. influenzae* cAMP levels by *icc*. To determine whether the *icc* gene limits intracellular cAMP levels in *H. influenzae*, we constructed an *icc* mutant strain and assessed the

TABLE 2. Plasmids used in this study

Plasmid	Insert/vector	Source (reference)
pKRP13	*spc* cassette	G. J. Phillips
pCAT19	*cat* cassette	C. Fuqua
pGEM*sxy*	*sxy*/pGEM-T	L. Bannister
pGEM+	Control insert/pGEM-T	This study
pAX923	*cpdA*/pACYC184	H. Niki (20)
pCMP	*icc*/pGEM-T	This study
pCMP::spc	*icc*::*spc*/pGEM-T	This study

TABLE 3. Eubacterial species whose genomes contain Icc homologs

Organism	Icc homolog[a]		No. of aa	E value[b]	Source/accession no. (reference)
	% Identical	% Similar			
Actinobacillus actinomycetemcomitans[c]	64.2	78.2	271	1e-106	QUAGCT (37)
Escherichia coli	53.3	71.3	231	4e-77	GenBank/D16557 (16)
Pseudomonas aeruginosa[c]	27.7	46	260	1e-34	PAGP (32)

[a] Gapped TBLASTN (3) searches of the completely sequenced genomes of the following bacterial species found no sequences with significant identity to Icc: *Borrelia burgdorferi* (36), *Chlamydia trachomatis* (12), *Helicobacter pylori* (36), *Neisseria meningitidis* (34, 36), *Synechocystis* strain PCC 6803 (21), *Treponema pallidum* (36), *Bacillus subtilis* (8), *Mycoplasma genitalium* (36), and *Streptococcus pyogenes* (37).
[b] Likelihood that an identified sequence match could have occurred by change.
[c] Sequencing and/or analysis of the genome is incomplete.

effects on known cAMP-dependent processes: ribose fermentation and competence development.

We have previously shown that utilization of ribose by *H. influenzae* is cAMP dependent. PRB assays demonstrated that *icc* disruption has little effect on ribose fermentation in a wild-type background (Fig. 3). We predicted, however, that if the *icc* mutation prevents degradation of cellular cAMP, it should restore ribose fermentation to strains with low cAMP (*ptsI* or *crr*) but not to a strain totally lacking cAMP (*cya*). As expected, an *icc cya* strain remained unable to ferment ribose, but *icc ptsI* and *icc crr* strains fermented ribose at almost wild-type levels, presumably because they cannot degrade the small amounts of cAMP that they synthesize (Fig. 3).

Development of competence for natural transformation is also cAMP dependent and is our most sensitive indicator of changes in intracellular cAMP levels in *H. influenzae* (e.g., Fig. 4). Wild-type *H. influenzae* strain KW20 reaches transformation frequencies (TF) as high as 10^{-2}. The *cya* mutation completely abolishes competence development (TF $< 10^{-7}$) (14), while *ptsI* strains show a ~200-fold reduction in competence (TF $= 7 \times 10^{-5}$) (22). As expected, competence remained undetectable (TF $< 10^{-7}$) in an *icc cya* strain, but disruption of the phosphodiesterase gene in a *ptsI* background boosted competence 10-fold (TF $= 7.6 \times 10^{-4}$) (23). These data confirm that *H. influenzae* cells lacking the *icc*-encoded phosphodiesterase have increased intracellular cAMP levels.

Sensitivity of *H. influenzae icc* strains to exogenous cAMP. Alper and Ames (2) found that the presence of a *cpd* mutation in *S. typhimurium* strains lacking endogenous cAMP (*cya* mutants) reduced 10-fold the exogenous cAMP required for expression of catabolic operons. We examined the cAMP sensitivity of *H. influenzae cya* strains carrying *icc*$^+$ and *icc* alleles by assaying the sensitivity of competence development to exogenous cAMP. As expected, the *icc* strain was much more sensitive to exogenous cAMP: the lowest concentration tested (10 nM) increased competence 10,000-fold in this strain but only 3-fold in the *icc*$^+$ strain (Fig. 4). These data imply that the *H. influenzae icc* strain is unable to degrade exogenously supplied cAMP.

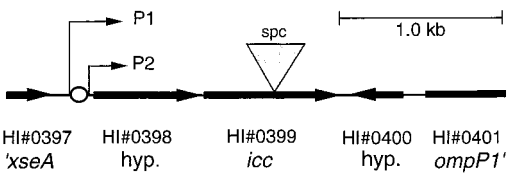

FIG. 1. The 3-kb *H. influenzae icc* operon fragment cloned in pCMP. *xseA*, exonuclease VII, large subunit; *ompP1*, outer membrane protein; hyp., hypothetical protein; P1 and P2, putative promoter sites; open circle, putative CRP-binding site; inverted triangle, location of *spc* cassette insertion at bp 1573 (a *Bcl*I site) of pCMP::spc.

Regulation of competence development by a cAMP phosphodiesterase. We found that RR812 developed spontaneous competence unusually early during exponential growth in rich medium (Fig. 5). This observation is consistent with a model in which Icc regulates intracellular cAMP concentrations throughout growth, not merely when cAMP levels are high.

Our simplest model predicted that cAMP phosphodiesterase inactivation should increase intracellular cAMP concentrations and that an *icc* strain should achieve at least wild-type levels of competence. It was therefore surprising to discover that the *icc* strain failed to reach wild-type levels of competence in both rich and nutrient-limited media (Fig. 5 and 6). We subsequently determined that this was not due to a simple toxic effect of high intracellular cAMP, since up to 10 mM exogenous cAMP did not significantly affect *H. influenzae* viability. Moreover, *icc* disruption itself does not reduce viability of this strain—it grows with a normal doubling time in batch culture. Instead, we reasoned that the timing of an increase in intracellular cAMP relative to growth rate or nutrient availability might be significant in the induction of maximal competence. To investigate this, we assessed competence of a *cya* strain provided with 1 mM exogenous cAMP either continuously or only after transfer of cells to starvation medium. We found that *cya* cells that encounter a dramatic increase in cAMP coincident with nutrient limitation develop wild-type competence levels. However, cells cultured in 1 mM cAMP prior to transfer to MIV show delayed and reduced compe-

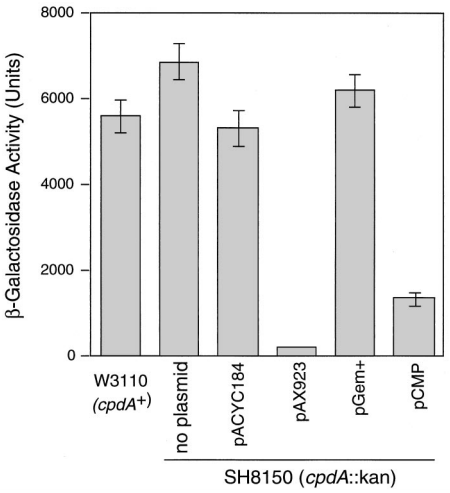

FIG. 2. β-Galactosidase activity in *E. coli* cells carrying *cpdA*/*icc* plasmids. Activity is in Miller units (25). Mean values from three replicates are shown; error bars represent SEM. (SEM for pAX923 is too small to be visible on this scale.)

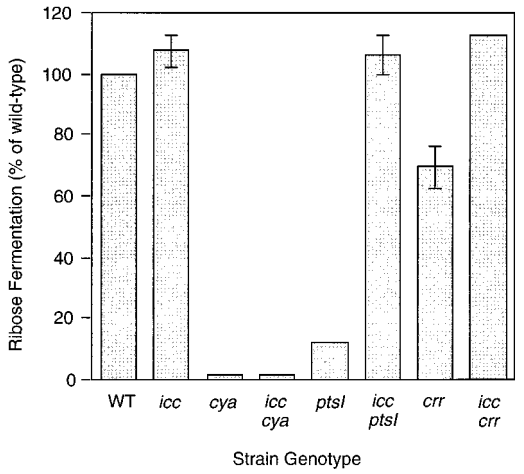

FIG. 3. Effect of *icc* disruption on ribose fermentation in wild-type (WT), *cya*, and PTS-disrupted backgrounds. A 100-μl aliquot of exponentially growing wild-type or mutant strains was inoculated into 5 ml of supplemented PRB plus 1% ribose. After rotation of the cultures overnight, we measured the pH of each culture and calculated the pH change (ΔpH). The degree of ribose fermentation of each mutants strain was expressed as a percentage of wild-type ΔpH (wild-type *H. influenzae* cultures in 1% ribose showed an average pH drop from 7.47 to 5.43). Mean values from three replicates are shown. Error bars represent SEM. (SEM values for *cya*, *icc cya*, *ptsI*, and *icc crr* strains are too small to be visible on this scale.)

tence, similarly to the *icc* strain (Fig. 6). These data suggest that the timing of a cAMP increase is critical in competence induction and that Icc regulates this timing in *H. influenzae*.

DISCUSSION

Why bacteria limit increases in cellular cAMP. To optimize transcription of cAMP-dependent genes involved in nutrient transport and catabolism, bacterial cells must closely regulate cAMP levels throughout growth. Because cAMP is a very stable molecule, active mechanisms for cAMP elimination are needed to quickly reduce cAMP concentrations whenever conditions improve. It has also been suggested that cAMP

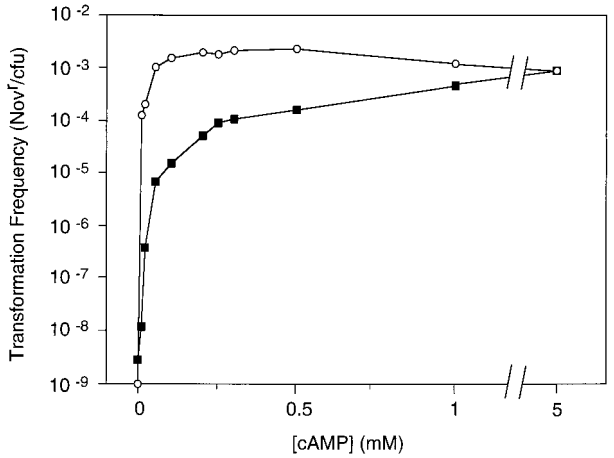

FIG. 4. cAMP sensitivity of *icc cya* (○) and *icc*$^+$ *cya* (■) *H. influenzae* strains. Strains were grown to mid-exponential phase in sBHI, and competence was induced by transfer of cells to MIV medium containing 0 to 5 mM cAMP, as described. Cells were incubated with MAP7 chromosomal DNA (1 μg ml^{-1}) for 30 min; transformation frequency was calculated as the number of novobiocin-resistant (Nov^r) transformants divided by the total number of cells. Representative results are shown.

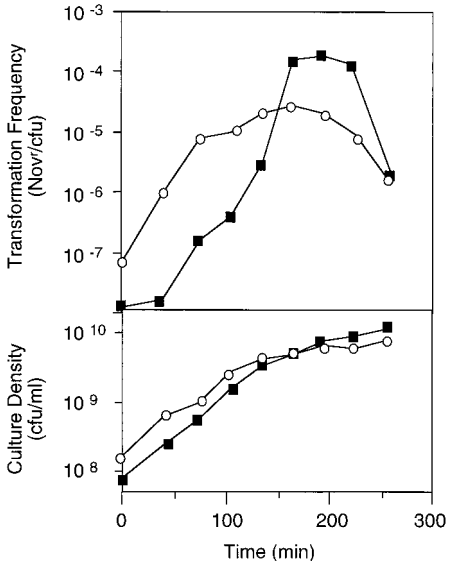

FIG. 5. Spontaneous competence of RR812 (*icc*) (○) and KW20 (■) in sBHI. Transformation frequencies were assayed as described in the legend to Fig. 4. Representative results are shown. Nov^r, novobiocin resistant.

phosphodiesterases may protect cells from the transcriptional repression that can result from excessive cAMP (2). Such repression may be caused by the cAMP-CRP complex directly: although this complex is better known as a transcriptional activator, it can also function as a transcriptional repressor (7). In addition, excessive cAMP may prevent efficient transcription of cAMP-dependent genes by overloading the CRP complex: the CRP protein has two identical subunits, each of which binds one molecule of cAMP, and the CRP-cAMP$_2$ complex has much lower affinity for CRP-binding sites than the CRP-cAMP$_1$ complex (7).

Uncontrolled cAMP increases may repress transcription of competence genes. It was puzzling that disruption of the *H. influenzae* phosphodiesterase gene reduced maximal competence levels in both stationary phase and MIV (Fig. 5 and 6), even though ribose fermentation data (Fig. 3) implied that intracellular cAMP concentrations were raised. However, competence

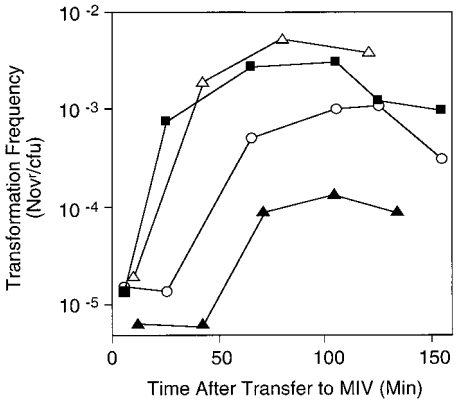

FIG. 6. MIV-induced competence of RR812 (*icc*) and KW20 compared with RR668 (*cya*) precultured with or without 1 mM cAMP. Transformation frequencies were assayed as described in the legend to Fig. 4. Filled squares, KW20; open circles, RR812 (*icc*); open triangles, RR668 transferred to MIV plus 1 mM cAMP; filled triangles, RR668 (*cya*) cultured in sBHI plus 1 mM cAMP before transfer to MIV plus 1 mM cAMP. Representative results are shown. Nov^r, novobiocin resistant.

is also thought to require at least one cAMP-independent regulatory event (14), and the uncontrolled increase in intracellular cAMP in the *icc* strain may interfere with necessary coordination between the cAMP-dependent and cAMP-independent signals. Thus, one role of the cAMP phosphodiesterase may be to regulate the timing of increases in intracellular cAMP.

The biological function of competence. In *H. influenzae*, competence is induced by conditions of nutrient limitation and absolutely requires an increase in cAMP, an established indicator of nutritional stress. Ferenci and colleagues have demonstrated that *E. coli* cAMP levels peak during the transition to starvation, allowing transient hunger-response expression of cAMP-dependent genes (13, 26–28). The pattern of competence development by *H. influenzae* suggests that it may also be a hunger response. We have previously shown that adenylate cyclase activity is influenced by the PTS, which responds to changes in carbon source availability. We have now shown that an *H. influenzae* cAMP phosphodiesterase participates in regulation of cAMP levels and maximal competence development.

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