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Competence development by *Haemophilus influenzae* is regulated by the availability of nucleic acid precursors

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Summary

DNA uptake by naturally competent bacteria provides cells with both genetic information and nucleotides. In *Haemophilus influenzae*, competence development requires both cAMP and an unidentified signal arising under starvation conditions. To investigate this signal, competence induction was examined in media supplemented with nucleic acid precursors. The addition of physiological levels of AMP and GMP reduced competence 200-fold and prevented the normal competence-induced transcription of the essential competence genes *comA* and *rec-2*. The rich medium normally used for growth allows only limited competence. Capillary electrophoresis revealed only a subinhibitory amount of AMP and no detectable GMP, and the addition of AMP or GMP to this medium also reduced competence 20- to 100-fold. Neither a functional stringent response system nor a functional phosphoenolpyruvate:glycose phosphotransferase system (PTS) was found to be required for purine-mediated repression. Added cAMP partially restored both transcription of competence genes and competence development, suggesting that purines may reduce the response to cAMP. Potential binding sites for the PurR repressor were identified in several competence genes, suggesting that competence is part of the PUR regulon. These observations are consistent with models of competence regulation, in which depleted purine pools signal the need for nucleotides, and support the hypothesis that competence evolved primarily for nucleotide acquisition.

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Introduction

The evolutionary function of natural competence is controversial. Although DNA uptake can lead to genetic exchange, the benefits are rare, unpredictable and likely to be small at best (for example, see Redfield *et al*., 1997). Because *de novo* synthesis of nucleotides is expensive (Stouthamer, 1979) and mucosal surfaces contain significant DNA (300 μg ml^{-1} in the respiratory mucus of healthy humans; Matthews *et al*., 1963), DNA uptake may be an efficient supplement to nucleotide salvage for mucosal commensals such as *Haemophilus*, *Neisseria* and *Streptococcus* (Redfield, 1993). Most of the DNA taken up by *Haemophilus influenzae* is degraded to nucleotides and rapidly reused for DNA synthesis; $<$15% is incorporated into the chromosome by homologous recombination (Pifer and Smith, 1985). Any DNA that does undergo recombination also contributes to the nucleotide pool, because the strand it displaces is degraded.

In most naturally competent bacteria, regulation of competence is not inconsistent with a nutritional function, as competence is induced by nutrient depletion, by increasing culture density and/or by the onset of stationary phase (Solomon and Grossman, 1996). We are examining the role of nutritional signals in *H. influenzae*. In *H. influenzae* cultures, about 1% of cells become competent as growth in rich medium slows at the approach of stationary phase, and all cells become competent when exponentially growing cells are abruptly shifted from rich medium to a starvation medium called MIV (Herriott *et al*., 1970). Induction of competence absolutely requires the catabolite regulator protein (CRP) and its cofactor cyclic AMP (cAMP), which combine to provide a general signal of energy insufficiency (Chandler, 1992; Dorocicz *et al*., 1993; Macfadyen *et al*., 1996). Adding cAMP to exponentially growing cultures induces only the partial competence normally seen when growth slows; full competence requires transfer to MIV medium even in the presence of cAMP. This suggests that a regulatory signal distinct from cAMP is produced in MIV medium and needed for full competence induction (Dorocicz *et al*., 1993). If nucleotides are the primary benefit of DNA uptake, this signal should arise from the depletion of intracellular nucleotide pools. The experiments described below were designed to determine whether supplying *H. influenzae* cells with nucleic acid

precursors affects competence development and to investigate how the observed effects are regulated.

Results

Purine ribonucleosides inhibit competence development

Transformation was measured after wild-type cells were transferred to the competence-inducing starvation medium MIV or to MIV supplemented with one of the following: hypoxanthine, uracil, ribonucleoside monophosphates (NMPs) or triphosphates (NTPs) or deoxyribonucleoside monophosphates or triphosphates (dNMPs or dNTPs). The test concentration of 5 mM of each precursor or nucleotide was chosen to approximate physiologically relevant concentrations (1–5 mM; Peterkofsky and Gollop, 1993). Figure 1 shows that the presence of 5 mM NMPs reduced competence several hundredfold, whereas dNMPs, dNTPs, NTPs and free bases had little effect on competence.

The effect of individual ribonucleoside monophosphates on competence development was then tested, revealing that the NMP effect seen in Fig. 1 was caused by AMP and GMP (Fig. 2). The addition of adenosine and guanosine also reduced competence by at least 100-fold, although adenosine had a less severe effect than AMP. However, the free purine bases adenine and guanine (Fig. 2) or free ribose (not shown) reduced competence less than threefold. The inhibition of competence by both AMP and GMP was concentration dependent and clearly evident at concentrations as low as 0.1 mM of either nucleotide (Fig. 3).

The MIV starvation medium used in the above experiments normally contains no nucleotides or precursors (Herriott *et al*., 1970). In the brain–heart infusion (BHI)-based medium used for growth of *H. influenzae* (supplemented BHI, sBHI), only a small fraction of cells become competent, suggesting that sBHI might contain purine nucleotides that inhibit competence. As no data were available, we used capillary electrophoresis to examine nucleotide concentrations in BHI (Britz-McKibbin *et al*., 2000). The only nucleotide detectable was adenosine-5′-monophosphate. Multiple samples ($n = 6$) of BHI extracts were analysed, and the peak areas of AMP were measured, normalized with an internal standard and extrapolated with an AMP standard plot. The calculated

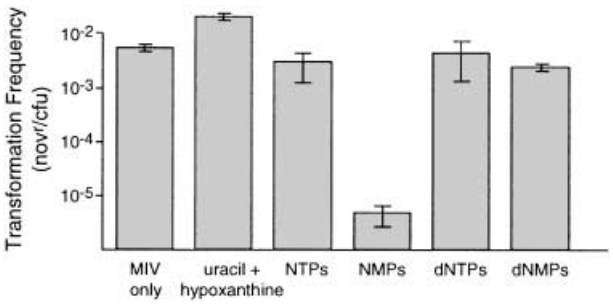

Fig. 1. Competence development by *H. influenzae* in MIV medium supplemented with nucleotides or nucleotide precursors. Wild-type cells in the early exponential phase of growth in rich medium were transferred to MIV medium supplemented with either uracil and hypoxanthine (5 mM each, 10 mM total) or nucleotides (5 mM each A, G, C and U nucleotides or A, G, C and T deoxyribonucleotides; 20 mM total). NTP, ribonucleoside triphosphates; NMP, ribonucleoside monophosphates; dNTP, deoxyribonucleoside triphosphates; dNMP, deoxyribonucleoside monophosphates. Mean data from three replicates are shown. Error bars represent the standard error of the mean (SEM).

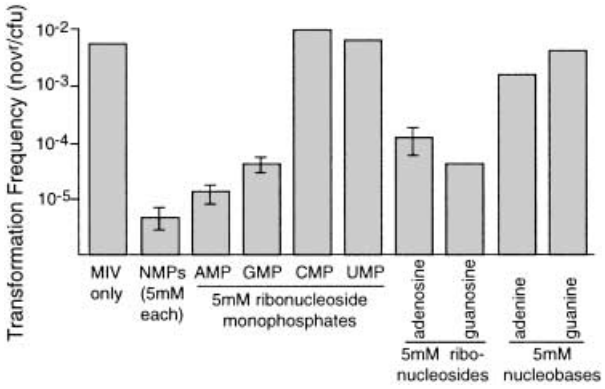

Fig. 2. Competence development by *H. influenzae* in MIV medium supplemented with ribonucleoside monophosphates, ribonucleosides or purine bases. Wild-type *H. influenzae* cells in the early exponential phase of growth in rich medium were transferred to MIV medium supplemented with ribonucleoside monophosphates (NMP), purine ribonucleosides or their respective bases. Mean data from three replicates are shown. Error bars represent SEM.

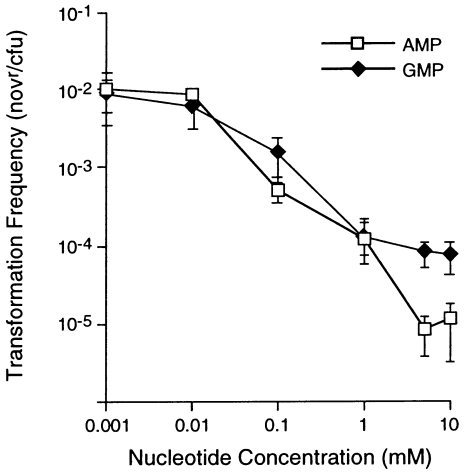

Fig. 3. Concentration dependence of competence on purine ribonucleoside monophosphates. Wild-type *H. influenzae* cells in the early exponential phase of growth in rich medium were transferred to MIV medium supplemented with different concentrations of AMP or GMP. Mean data from three replicates are shown. Error bars represent SEM.

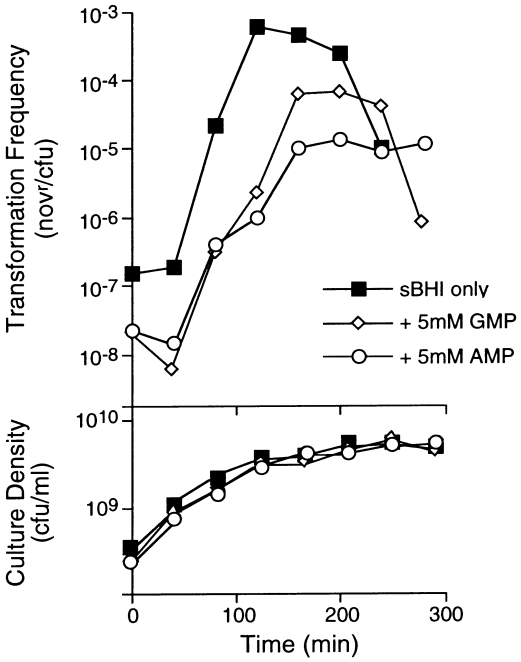

Fig. 4. Competence development by *H. influenzae* in rich medium supplemented with AMP or GMP. Cultures were sampled at intervals during growth in sBHI supplemented with 5 mM AMP or GMP. Competence assays were repeated three times with independent cultures, and each experiment gave essentially similar results. Representative data are shown.

average concentration of AMP in BHI was 40 μM, which would be too low to inhibit competence development detectably in MIV. Levels of GMP, CMP and UMP were below the detection threshold of ≈1 μM, as were nucleoside di- and triphosphates. These results suggest that the low competence developed in sBHI is not the result of inhibitory concentrations of nucleotides. However, this analysis could not rule out the presence of inhibitory concentrations of purine nucleosides, which capillary electrophoresis could not discriminate from other constituents of the medium.

Independent evidence that the low competence normally developed in rich medium is not the result of inhibition by endogenous purine nucleotides or nucleosides was obtained by nucleotide supplementation. Figure 4 shows that purine nucleotides inhibited competence development in sBHI almost as strongly as they did in MIV.

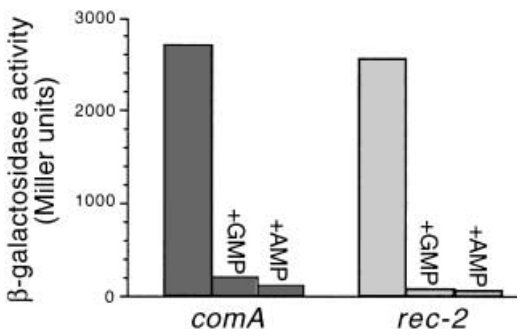

Fig. 5. Expression of *comA::lacZ* and *rec-2::lacZ* fusions in nucleotide-supplemented MIV. Exponentially growing cultures of strains IDA610 (*comA::lacZ*) and IDR22 (*rec-2::lacZ*) were transferred to MIV with and without 5 mM AMP or GMP, and β-galactosidase production was measured after 100 min.

AMP and GMP reduce competence by reducing transcription of competence genes

To determine whether added AMP and GMP acted directly on the machinery of DNA uptake or altered the transcription of competence genes, we tested their effect on the expression *lacZ* operon fusions to the essential competence genes *rec-2* and *sxy and* to the *comA* gene of the *comABCDEF* operon (Gwinn *et al*., 1997; Bannister, 1999). Genes of the *com* operon are essential for DNA binding (Tomb *et al*., 1991), and *rec-2* is required for translocation of bound DNA into the cytoplasm (McCarthy, 1989). Expression of the *comA* and *rec-2* fusions is known to be induced 30- to 60-fold by transfer to MIV medium (Gwinn *et al*., 1997), and Fig. 5 shows that the addition of AMP or GMP decreased this induction by 15- and 38-fold for *comA and* 42- and 63-fold for *rec-2*. The *sxy* gene product is required for the transcription of *comA* and *rec-2*, but it is not itself induced by competence (Zulty and Barcak, 1995; Ma and Redfield, 2000); purine nucleotides did not significantly affect its expression (data not shown).

Nucleotide effects are not mediated by the phosphoenolpyruvate:glycose phosphotransferase system (PTS) or the stringent response

In *H. influenzae*, as in *Escherichia coli*, the sugar transporters of the PTS respond to shortage of preferred sugars by stimulating adenylate cyclase to produce cAMP, which activates transcription of cAMP/CRP-regulated genes. Induction of competence and transcription of both *comA* and *rec-2* requires CRP and cAMP (Gwinn *et al*., 1997), and mutations knocking out components of the PTS reduce competence by reducing the production of cAMP (Macfadyen *et al*., 1996; Macfadyen, 1999). If AMP and GMP affect competence by inhibiting PTS-dependent stimulation of cAMP production, we might expect cells carrying PTS knock-out mutations to be immune to the nucleotide effects. However, AMP and GMP caused the same proportional reduction in competence in all our PTS mutants as that in wild-type cells (Fig. 6). This suggests that AMP and GMP do not affect PTS-dependent control of cAMP levels, but act by

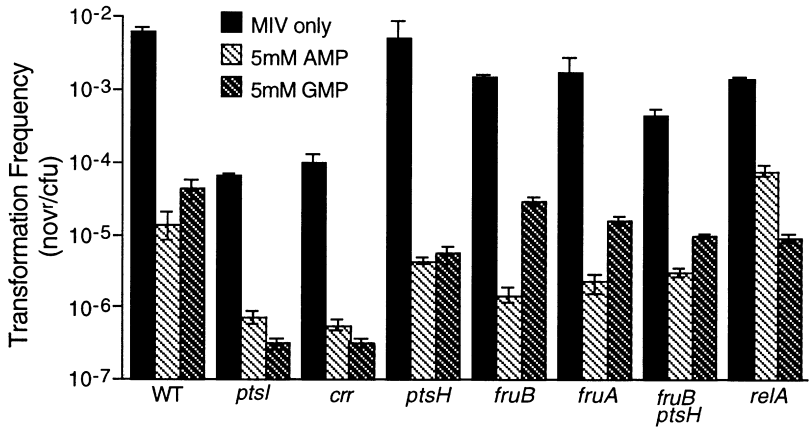

Fig. 6. Competence of various mutant strains in MIV with and without AMP or GMP. Early exponential phase cells were transferred to MIV nutrient-limited medium plus 5 mM AMP or GMP, and transformation frequency was measured at 100 min after transfer. Mean values from three replicates are shown. Error bars represent SEM. The enzymes missing from each PTS mutant are: *ptsI*, EI; *crr*, $EIIIA^{Glc}$; *ptsH*, HPr; *fruB*, DTP; *fruA*, $EIIB'BC^{Fru}$; *fruB ptsH*, DTP HPR.

interfering with another process. In *E. coli*, the *relA* gene product mediates the stringent response to amino acid starvation by synthesizing the signal molecule (p)ppGpp from GTP and ATP. This depletes ATP and GTP pools by 30–40% (Peterkofsky and Gollop, 1993) and might link changes in pools to induction of competence. We have constructed an *H. influenzae relA* knock-out mutant, and Fig. 6 shows that its response to added GMP is the same as that of wild-type cells, although it is not as strongly inhibited by AMP.

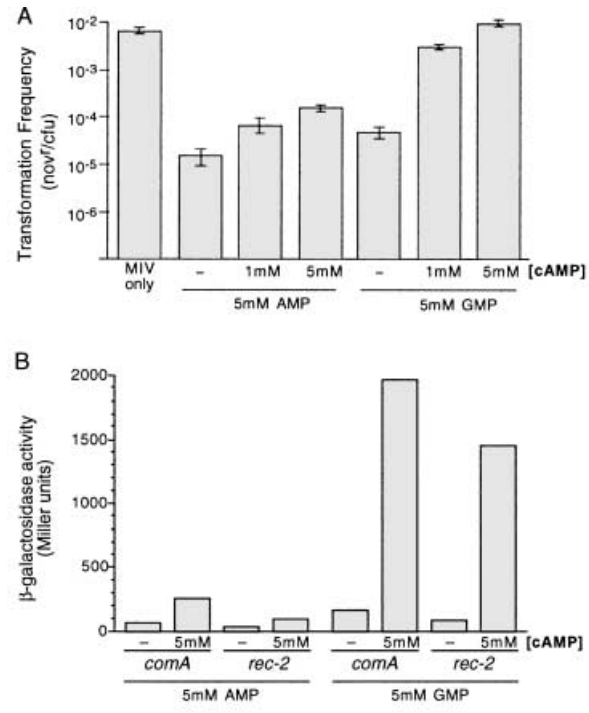

Fig. 7. Exogenous cAMP partially restores competence and transcription of *comA* and *rec-2*.
A. Wild-type *H. influenzae* cells in the early exponential phase of growth in rich medium were transferred to MIV medium supplemented with 5 mM AMP or GMP plus 0, 1 or 5 mM cAMP, as indicated. Mean data from three replicates are shown. Error bars represent SEM.
B. Exponentially growing cultures of strains IDA610 (*comA::lacZ*) and IDR22 (*rec-2::lacZ*) were transferred to MIV with and without 5 mM AMP or GMP and with and without 5 mM cAMP, and β-galactosidase production was measured after 100 min.

cAMP stimulates competence in the presence of inhibiting nucleotides

Purine nucleotides could act by reducing the intracellular cAMP concentration, so we tested whether added cAMP could restore competence and competence-specific transcription. Figure 7A shows that 5 mM cAMP increased the competence of AMP-inhibited cells by about 10-fold and completely restored competence to GMP-inhibited cells. This suggests that the availability of purine nucleosides influences either intracellular cAMP levels or the cell's ability to respond to increasing cAMP. However, because 1 mM cAMP is sufficient to restore full competence to *cya* cells in MIV (Dorocicz *et al*., 1993), but insufficient to overcome inhibition by AMP and GMP, these nucleotides are unlikely to act simply by inhibiting cAMP production. This is confirmed by the effect of cAMP on the transcription of competence genes, shown in Fig. 7B. Cyclic AMP at 5 mM restored GMP-inhibited transcription to 60–70% of its uninhibited level, but raised AMP-inhibited transcription only slightly to 4–10% of its uninhibited level.

Discussion

The above results show that purine nucleotides repress the transcription of competence genes, suggesting that competence may develop only when purine pools are depleted. This finding raises both regulatory and evolutionary questions.

First, how is this regulation brought about? The simplest hypothesis is that some competence genes are regulated by the PurR repressor, which permits transcription

only when purine pools are depleted (Zalkin and Nygaard, 1996). In *E. coli*, the PUR regulon controls the 10 operons required for synthesis of AMP and GMP and also contributes to the regulation of *pyrC* and *pyrD* for pyrimidine nucleotide synthesis, *codBA* for cytosine transport and salvage, *prs* for PRPP synthetase and *glyA* and the *gcv* operon genes for one-carbon metabolism (Zalkin and Nygaard, 1996). The *H. influenzae* PurR protein (HI#1635) has 73% similarity and 56% identity to its *E. coli* counterpart, and *H. influenzae* homologues of *E. coli* PUR regulon genes have candidate PurR operators that match the *E. coli* consensus (Mironov *et al*., 1999).

In principle, PurR could either repress *comA* and *rec-2* transcription directly or prevent synthesis of a master regulatory gene. The latter is unlikely because expression of Sxy, the only known competence-specific regulator, was not affected by AMP and GMP. Examination of the promoter sequences identified an excellent match to the PurR consensus in the *rec-2* promoter, 54 nucleotides upstream of the start codon, and a weaker match in the *comABCDEF* promoter, 47 nucleotides upstream of its start codon. A potential PurR site was also found in the promoter of the *dprA* gene, whose regulation and function resemble that of *rec-2* (Karudapuram *et al*., 1995). These genes are all essential for competence, and repression by PurR would fully account for the inhibition of competence by purine nucleotides. However, the possibility remains that non-transcriptional effects also contribute to the inhibition of transformation, either in the cytoplasm or by interfering directly with DNA uptake at the cell surface. We are now directly testing the role of PurR by knocking out *purR* and by site-directed mutagenesis of candidate operator sites.

Unlike purine biosynthetic genes, transcription of *comABCDEF* and *rec-2* is strongly dependent on cAMP and CRP (Zulty and Barcak, 1995; Gwinn *et al*., 1997), probably mediated by binding of CRP to a conserved promoter sequence called the competence regulatory element (CRE) (Karudapuram and Barcak, 1997; Gwinn *et al*., 1998; Macfadyen, 2000). This joint regulation by cAMP and a gene-specific signal is typical of members of the global cAMP/CRP regulon. An increase in cAMP indicates that energy resources must be conserved or supplemented, and gene-specific signals indicate the utility of each transcription unit (Collado-Vides *et al*., 1991). For competence genes, this appears to be the need to supplement purine pools. The *H. influenzae* genome encodes all the enzymes and transporters needed for the uptake of free nucleosides and processing of these into nucleotides and deoxynucleotides (Fleischmann *et al*., 1995). However, none of these genes appears to be regulated by PurR (Mironov *et al*., 1999), suggesting that nucleotide salvage in *H. influenzae* is the default strategy, supplemented by *de novo* purine synthesis when the energy supply is adequate and by DNA uptake when both energy supply and salvage are inadequate. This may reflect the availability of nucleotides and precursors in its mucosal environment. The ability of excess cAMP partially to overcome inhibition by AMP and GMP suggests that the two DNA-binding proteins (PurR and CRP) may interact, and that binding of one can weaken binding of the other.

The only known co-repressors of the *E. coli* PurR orthologue are guanine and hypoxanthine (Zalkin and Nygaard, 1996), so it was somewhat surprising that, in *H. influenzae*, purine bases were much less effective inhibitors of competence than purine nucleotides and nucleosides. This is probably caused by differences in transport processes. Although *H. influenzae* has a homologue of the *E. coli* nucleoside transporter *nupC*, it lacks genes encoding transport proteins for purine bases. In *E. coli* and presumably in *H. influenzae*, nucleotides are converted to nucleosides in the periplasm, transported by NupC and rapidly metabolized in the cytoplasm to hypoxanthine and guanine respectively (Zalkin and Nygaard, 1996). Thus, the *H. influenzae* salvage pathway may have evolved to use exogenous nucleotides and nucleosides more efficiently than purine bases, perhaps because bases are not abundant in host mucus.

Placement of competence in the PurR regulon would strengthen the hypothesis that its primary function is the acquisition of nucleotides rather than genetic information. However, several perplexing questions remain. First, why is competence not regulated by the depletion of deoxyribonucleotide pools? Several reasons can be suggested. Both bacterial and eukaryotic cells (such as polymorphonuclear leucocytes and epithelial cells in the mucosal environment) contain substantially more RNA than DNA, and RNA released by cell lysis is likely to be degraded by mucosal nucleases. Thus, DNA-rich mucosal environments may be even richer in ribonucleotides. Furthermore, *H. influenzae* lacks any sensor of intracellular deoxyribonucleotide or deoxyribose concentration. Finally, regulation by deoxyribonucleotides may be redundant because the different pools are easily equilibrated – excess ribonucleotides are not a problem, because the demand is high, and deoxyribonucleotides obtained from DNA will relieve the pressure on the ribonucleotide pool to provide them for DNA synthesis.

Secondly, why is DNA uptake not regulated by pyrimidine pools as well as purine pools? *H. influenzae* lacks a homologue of *E. coli*'s CytR repressor or other genes that could sense the depletion of pyrimidine pools. In enteric bacteria, the regulation of both purine and pyrimidine nucleotide synthesis and catabolism is largely controlled by intracellular levels of purines and purine nucleosides (Neuhard and Kelln, 1996), suggesting that regulation by pyrimidines is redundant.

Table 1. Bacterial strains used in this study.

Strain	Relevant genotype	Source or reference
KW20 (Rd)	Wild type	Alexander and Leidy (1951)
RR745	ptsI::miniTn*10kan*	Macfadyen *et al.* (1996)
RR817	*ptsH*::miniTn*10kan*	Macfadyen (1999)
RR801	*crr*::cat	Macfadyen *et al.* (1998)
RR798	*fruB*::kan	Macfadyen (1999)
RR813	Δ*fruA*::spc	Macfadyen (1999)
RR823	*ptsH*::miniTn*10kan fruB*::kan	Macfadyen (1999)
RR822	*relA*::spc	Macfadyen (1999)
RR839	*lacZ* fusion to *sxy*	Bannister (1999)
IDA610	*lacZ* fusion to *comA*	Gwinn *et al.* (1997)
IDR22	*lacZ* fusion to *rec-2*	Gwinn *et al.* (1997)

Thirdly, *H. influenzae* cells preferentially take up DNA fragments that carry a specific short sequence, the uptake signal sequence or USS (Danner *et al.*, 1980). This sequence is very abundant in the *H. influenzae* genome and has been assumed to exist to prevent the uptake of foreign DNAs. However, preferential binding of the DNA uptake machinery to a common sequence could result from selection for high-affinity binding, and this binding creates a form of molecular drive that can cause further increases in the abundance of the sequence (Redfield, 1991).

The discovery that competence development is regulated by the availability of nucleic acid precursors strengthens the proposition that *H. influenzae* takes up DNA primarily to obtain nucleotides. This is also seen in *E. coli* strains carrying null mutations in homologues of the *H. influenzae* competence genes *comJ* and *comE* (Tomb *et al.*, 1991; Dougherty and Smith, 1999), which are absolutely required for DNA binding and uptake in *H. influenzae*. These *E. coli* mutants are unable to catabolize exogenous DNA, although normal growth and nucleotide uptake and salvage are unimpaired. The mutant phenotype also confirms the importance of DNA catabolism for survival under more natural conditions, as the mutants are outcompeted by the wild type after 2 days co-incubation at stationary phase (S. Finkel and R. Kolter, submitted).

Experimental procedures

Bacterial strains, plasmids and culture conditions

All *H. influenzae* strains used in this study are descendants of Alexander and Leidy's (1951) original Rd strain and are listed in Table 1. Unless otherwise indicated, gene number assignments (HI#) and identifications are as assigned for this strain by Fleischmann *et al.* (1995) and The Institute for Genome Research (Rockville, MD, USA). Fusions of *lacZ* to the *comA* and *rec-2* genes were obtained from Michelle Gwinn and Mark Chandler; the fusion of *lacZ* to *sxy* was obtained from Laura Bannister (Bannister, 1999). *H. influenzae* cells were grown aerobically at 37°C in BHI broth (Difco) supplemented with haemin and NAD (sBHI), with the recommended concentrations of antibiotics where needed (Barcak *et al.*, 1991). Additional haemin was applied to sBHI plates >24 h old.

Chemicals

Borax, hydroxy-β-cyclodextrin, nucleosides, nucleotides and nucleobases were obtained from Sigma. The internal standard phenyltrimethylammonium chloride (PTAC) was from Sigma-Aldrich Canada. Membrane filters (0.22 μm) were from Millipore.

Competence and transformation

Spontaneous competence development by *H. influenzae* was monitored during growth in sBHI with and without test nucleosides. A single colony was inoculated into 50 ml of sBHI and incubated with shaking at 37°C. Once the OD_{600} reached ≈ 0.1, aliquots were removed at intervals and incubated, rolling, with a saturating concentration of DNA from the standard antibiotic-resistant strain MAP7 (1 μg ml^{-1}) at 37°C for 20 min. DNase I (Boehringer Mannheim) was added to a concentration of 1 μg ml^{-1}, and the mixture was rolled at 37°C for a further 10 min (Barcak *et al.*, 1991). To induce maximal competence, exponential phase cells were transferred to MIV starvation medium (Herriott *et al.*, 1970) supplemented where appropriate with test nucleosides or nucleotides. Cells at an OD_{600} of 0.2–0.3 were collected by filtration in 100 ml analytical test filter funnels (0.2 μm pore size; Nalgene) (Williams *et al.*, 1996), resuspended in MIV and incubated with gentle shaking (100 r.p.m.) at 37°C. To assay competence, 200 μl aliquots were incubated, rolling, with 1 μg ml^{-1} MAP7 DNA at 37°C for 30 min. sBHI (800 μl) was then added, and the cultures were incubated with rolling at 37°C for a further 30 min. Transformation frequency was assessed by scoring the frequency of Nov^r transformants (Barcak *et al.*, 1991).

Non-growth medium for competence induction

MIV medium for maximal competence induction was prepared as described by Herriott *et al.* (1970); it contained 80 mM NaCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KH_2PO_4, pH 7.4, and per litre: 1.0 g of fumarate, 0.006 g of citrulline, 0.02% of Tween-80, 4.0 g of aspartic acid, 0.2 g of glutamic acid, 0.004 g of cysteine, 0.01 g of tyrosine, 0.02 g of phenylalanine, 0.03 g of serine, 0.02 g of alanine and 0.5 g of casamino acids.

Analysis of nucleotides in BHI

The separation buffer (BHE) consisted of 160 mM borate, pH 9.6, containing 100 mM hydroxy-β-cyclodextrin and 2 mM EDTA. All solutions were filtered through 0.22 μm membrane filters before analysis. The stock adenosine-5′-monophosphate was made up in 0.9% NaCl solution at 100 μg ml^{-1}; serial dilutions were prepared in 0.9% NaCl solution for the construction of the standard plot of AMP.

An aliquot (100 μl) of the BHI medium containing the

internal standard PTAC (1 mg ml^{-1}) was extracted with perchloric acid (15 μl of a 60% solution) at room temperature. After centrifugation at 13 K for 5 min, the solution was neutralized with 6 N KOH to pH 7 (about 30 μl). The precipitate was collected by centrifugation at 13 K for 10 min at room temperature. The supernatant was diluted with an equal volume of deionized water and filtered through a 0.22 μm membrane before CE analysis.

The analysis was carried out using a Beckman P/ACE 5500 automated system equipped with a UV absorbance detector and a photodiode-array detector (PDA). Uncoated capillaries (Polymicro Technologies) were fused silica, effective length 50 cm, internal and external diameters of 75 μm and 360 μm respectively. Before each analysis, the capillary was rinsed with methanol for 2 min, deionized water for 1 min, sodium hydroxide (1 M) for 2 min followed by the separation buffer for 3 min. The sample was injected using 0.5 p.s.i. of pressure for 30 s. Application of 30 kV normal polarity at 18°C enabled the separation to be achieved in less than 30 min. UV detection was monitored at 254 nm, and PDA detection was from 200 nm to 300 nm.

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