JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7315–7320 Vol. 179, No. 23
0021-9193/97/$04.00+0

In Vitro Tn*7* Mutagenesis of *Haemophilus influenzae* Rd and Characterization of the Role of *atpA* in Transformation

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Received 1 May 1997/Accepted 19 September 1997

Haemophilus influenzae **Rd is a gram-negative bacterium capable of natural DNA transformation. The competent state occurs naturally in late exponential growth or can be induced by a nutritional downshift or by transient anaerobiosis. The genes *cya*, *crp*, *topA*, and *sxy* (*tfoX*) are known to function in the regulation of competence development. The phosphoenolpyruvate:carbohydrate phosphotransferase system functions to maintain levels of cyclic AMP necessary for competence development but is not directly involved in regulation. The exact signal(s) for competence and the genes that mediate the signal(s) are still unknown. In an effort to find additional regulatory genes, *H. influenzae* Rd was mutated by using an in vitro Tn*7* system and screened for mutants with a reduced ability to induce the competence-regulatory gene, *comA*. Insertions in *atpA*, a gene coding for the α subunit of the F_1 cytoplasmic domain of the ATP synthase, reduce transformation frequencies about 20-fold and cause a significant reduction in expression of competence-regulatory genes, while the expression of constitutive competence genes is only minimally affected. In addition, we found that an insertion in *atpB*, which encodes the *a* subunit of the F_0 membrane-spanning domain, has a similar effect on transformation frequencies.**

Haemophilus influenzae Rd is a gram-negative bacterium capable of natural DNA transformation. It achieves low levels of competence in late exponential growth phase or can be induced to high levels of competence by a shift to the starvation medium MIV (19) or by transient anaerobiosis (15).

The cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex plays a role in the regulation of competence development. Addition of 1 mM cAMP to early-exponential-growth-phase cells induces a low level of competence comparable to that seen spontaneously in late exponential growth phase (38). Mutations that inactivate the *H. influenzae* genes for CRP (*crp*) or adenylate cyclase (*cya*) prohibit the cells from becoming competent (8, 12). The *cya* mutant can be restored to wild-type levels of transformation by the addition of 1 mM cAMP to MIV (12). It is known that promoters which are regulated by the cAMP-CRP complex are also sensitive to DNA superhelicity (6) and that the *H. influenzae* homolog of topoisomerase I (*topA*) is required for full competence development (9).

Another competence-regulatory gene is *sxy* (also known as *tfoX*). Mutations which inactivate this gene abolish the ability of the cells to become competent (37, 39). However, certain point mutations of this gene (*sxy-1*) allow the cells to be competent all of the time, resulting in a transformation frequency 100-fold higher than that normally seen in exponential growth phase (28).

An operon of six genes, *comA* to *-F*, is induced during competence development (32, 34). A 26-bp palindromic competence-regulatory element in the promoter of *comA* is responsible for this induction (22, 34). This sequence has also been found upstream of several other genes (22, 35) (*rec2* [10], *comM* [17], *dprA* [22], and *pilA* to *pilD* [13, 35]) all of which are required for and induced during competence development (17, 22, 35). It is likely that this sequence is the binding site for a competence-specific, positive-acting transcription factor.

Previous studies have failed to identify the signal-receiving and -transducing genes of the competence system. Mutations in the *H. influenzae* two-component signal transduction systems had no significant effect on competence development. Mutations in *crr* or *ptsI* of the phosphoenolpyruvate:carbohydrate phosphotransferase system significantly reduce transformation frequencies, but *crr* and *ptsI* have not been shown to play a role in regulation of competence other than in maintaining adequate levels of cAMP (16, 24).

In an effort to find the genes responsible for mediating the competence-inducing signal, we mutated *H. influenzae* Rd DNA with the transposon Tn*7* (11), using an in vitro system with purified Tn*7* proteins (3). Use of Tn*7* as a mutagen overcame problems associated with other transposon systems. Since this is an in vitro system, the transposon does not need to be active in *H. influenzae*. Also, this system makes use of a gain-of-function TnsC mutant which removes the target specificity that Tn*7* usually has in in vitro reactions (31). After transforming mutant DNA into wild-type cells, we screened for mutants with a lowered ability to induce the competence gene *comA*. We found that a mutation in the ATP synthase of *H. influenzae* Rd has a moderate effect on transformation frequencies but significantly reduces the expression of induced competence genes while having little effect on expression of constitutive competence genes.

MATERIALS AND METHODS

Growth and transformation. *H. influenzae* (Table 1) was grown in brain heart infusion (BHI) supplemented with NAD (2 μg/ml) and hemin (10 μg/ml) at 37°C. Antibiotic concentrations were 10 μg/ml for kanamycin, 2.5 μg/ml for novobiocin, and 1 μg/ml for chloramphenicol (4). *Escherichia coli* (Table 1) was grown in Luria-Bertani broth at 37°C. Antibiotic concentrations were 100 μg/ml for carbenicillin, 25 μg/ml for kanamycin, and 25 μg/ml for chloramphenicol (29).

Two methods of inducing competence were used to assay the phenotypes of the mutants. The MIV procedure uses a nutritional downshift of a log-phase culture into an incomplete synthetic medium (19). Cells from an overnight plate were inoculated into 25 ml of supplemented BHI in a 250-ml flask (or 35 ml in

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotye and phenotype[a]	Origin or reference[b]
Strains		
E. coli DH5α	WT	NEB
H. influenzae		
KW20	WT	4
MAP7	KW20 Nv^r Sp^r	4
MGH100	KW20 *comA*::*lacZ kan* Kn^r	This study
MGH101	MGH100 *atpA*::*cat* Kn^r Cm^r	This study
IDA610	Insertion/duplication of *comA*, *comA*::*lacZ cat* Cm^r	This study
IDA620	IDA610 *crp*::*kan* Kn^r Cm^r	This study
IDA30	IDA610 *atpA*::*kan* Kn^r Cm^r	This study
IDR22	Insertion/duplication of *rec2*, *rec2*::*lacZ cat* Cm^r	This study
IDR30	IDR22 *atpA*::*kan* Kn^r Cm^r	This study
MGH11	IDA610 *atpA*::Tn7 Kn^r	This study
MGH30	KW20 *atpA*::*kan* Kn^r	This study
MGH31	KW20 *atpA*::*cat* Cm^r	This study
MGH40	KW20 *atpB*::*kan* Kn^r	This study
DYH15-1	KW20 *ptsI*::*lacZ kan* Kn^r	This study
JF590-15	KW20 *por*::*lacZ kan* Kn^r	33
MGH51	DYH15-1 *atpA*::*cat* Kn^r Cm^r	This study
MGH61	JF590-15 *atpA*::*cat* Kn^r Cm^r	This study
Plasmids		
pUC19, pUC18	Ap^r	NEB
pTZ18R	Ap^r	USB (Genscribe-Z kit)
pSU2718, pSU2719	Cm^r	25
pNK1210	Element 105 analog, WT transposase, Cm^r	23
pKK232-8	Promoterless *cat* Ap^r	Pharmacia
pLZK83-1	pTZ18R *lacZ kan* Kn^r Ap^r	4
pMGAZ1	pJF1040 *comA*::*lacZ cat* Cm^r	This study
pMGAZ2	pUC19 Δα*lacZ comA*::*lacZ cat* Ap^r Cm^r	This study
pMGRZ1	pJF1300 *rec2*::*lacZ cat* Cm^r	This study
pMGRZ2	pUC19 Δα*lacZ rec2*::*lacZ cat* Ap^r Cm^r	This study
pJF1040	pSU2718 *comA* Cm^r	35
pJF1300	pSU2719 *rec2* Cm^r	35
pGHICX59	pUC18 *atpA* Ap^r	TIGR
pGHIHM69	pUC18 *atpB* Ap^r	TIGR
pMG40	pGHICX59 *atpA*::*kan* Ap^r Kn^r	This study
pMG41	pGHICX59 *atpA*::*cat* Ap^r Cm^r	This study
pMG45	pGHIHM69 *atpB*::*kan* Ap^r Kn^r	This study
pMG11	pTZ18R *lacZ cat* Ap^r Cm^r	This study
pMG12	pTZ18R *cat* Ap^r Cm^r	This study

[a] Antibiotic abbreviations: Kn, kanamycin; Cm, chloramphenicol; Ap, ampicillin; Nv, novobiocin; Sp, streptomycin. WT, wild type.

[b] NEB, New England Biolabs; USB, United States Biochemical; TIGR, The Institute for Genomic Research.

a 500-ml flask) which was shaken at 180 rpm until cells reached an A_{600} of 0.2 to 0.25. Absorbance was measured in a 13- by 100-mm glass tube in a Spectronic 20 spectrophotometer. Samples of 15 ml (or 35 ml) were centrifuged, washed with 9 ml (or 20 ml) of MIV, centrifuged, and resuspended in 15 ml (or 35 ml) of MIV. Shaking was continued at 100 rpm for 100 min. Samples of 1 ml were incubated at 37°C for 30 min with excess (2.5 μg) *H. influenzae* Rd genomic DNA containing a marker for novobiocin resistance. Five milliliters of supplemented BHI was added, and cells were incubated with aeration for 1 h before plating on appropriate media.

In the transient anaerobiosis assay (15), cells were grown in 75 ml of supplemented BHI in a 500-ml flask until they reached an A_{600} of 0.5. Cultures were left standing at 37°C for 1 h. Shaking was then resumed for 20 min. Samples of 1 ml were incubated at 37°C for 30 min with 2.5 μg of *H. influenzae* genomic DNA as described above. Reaction mixtures were incubated for an additional 30 min at 37°C with aeration before plating on appropriate media.

Transformation frequency was measured as the number of novobiocin-resistant colonies divided by the total number of colonies.

DNA uptake was measured as described by Barcak et al. (4). In a 50-μl reaction, 36 μg of MAP7 genomic DNA was incubated with ~9 U of T4 polymerase for 20 min to allow retrograde digestion of the 3′ ends. ^{32}P-labeled dCTP and the other three nonradioactive deoxynucleoside triphosphates were then added, and the mixture was incubated for an additional 20 min to allow resynthesis. DNA was purified by using a Pharmacia Microspin G-25 column. Competent cells were incubated with approximately 4 μg of labeled DNA (~82,000 cpm) for 20 min at 37°C and pelleted. Pellets were Cerenkov counted. Cells were then washed with 0.5 M NaCl to remove DNA bound to the surface of the cells, repelleted, and recounted. Relative uptake efficiency of the mutant equals the mutant counts per minute divided by the wild-type counts per minute (4).

PCR and primers. PCRs were done either by using AmpliTaq enzyme and its accompanying buffer (containing $MgCl_2$) from Perkin-Elmer Cetus (Norwalk, Conn.) or by using the Expand High Fidelity PCR system (product no. 1732641; Boehringer Mannheim, Indianapolis, Ind.). Primers were synthesized at the Johns Hopkins University CORE DNA Analysis Facility.

DNA isolation and manipulation. Genomic DNA was prepared from 5-ml cultures grown to saturation as described by Barcak et al. (4). Small-scale plasmid preparations were done by the boiling method of Holmes and Quigley (20). Large-scale plasmid preparations, from 50 to 100 ml of cells, were performed with the Qiagen Midi-prep system.

DNA cloning and fragment manipulation were carried out according to standard protocols (2). Fragment purification was performed by using either a Qiaex kit (product no. 20021) or a PCR purification kit (product no. 28104), both from Qiagen.

Sequencing was performed at the Johns Hopkins University CORE DNA Analysis Facility.

Southern analysis. DNA was run and transferred to a nylon membrane according to standard procedures (2). Probes were labeled with the ECL direct nucleic acid labeling system (product no. RPN3005; Amersham). Hybridization, washing, and detection were performed as described in the protocol for the ECL labeling and detection kit (product no. RPN3004; Amersham).

Construction of the *lacZ cat* cassette. Plasmid pNK1210 was partially digested with *Bam*HI, and linear fragments were recovered. The staggered *Bam*HI ends were filled in, and then the linear fragments were digested to completion with *Bam*HI, resulting in excision of the *cat* gene with one blunt and one *Bam*HI end. Plasmid pLZK83-1 was cut at the unique *Xho*I site, made blunt, then cut with *Bam*HI. The fragment from pNK1210 containing *cat* and the fragment from pLZK83-1 containing *lacZ* were ligated to *Bam*HI-cut pTZ18R. The resulting plasmid is pMG11.

Construction of insertion/duplication strains. IDA610 and IDR22 were constructed as follows. The *lacZ cat* cassette was cut from pMG11 by digestion with *Bam*HI and inserted into the *Bgl*III site in the coding region of *comA* on pJF1040 (which carries *comA* in a 2.5-kb insert) and separately into the *Mlu*I site in the coding region of *rec2* on pJF1300 (which carries *rec2* on a 4.2-kb *Pst*I fragment insert), resulting in plasmids pMGAZ1 and pMGRZ1, respectively. The fragment of DNA containing *comA*::*lacZ cat* was excised from pMGAZ1 with a *Bam*HI/*Pst*I restriction digest. Similarly, the fragment of DNA containing *rec2*::*lacZ cat* was excised from pMGRZ1 by *Pst*I digestion. Each *H. influenzae* fragment was made blunt and independently ligated to blunted pUC19 from which α*lacZ* had been removed by digestion with *Nde*I and *Pvu*II. The resulting plasmids were pMGAZ2 and pMGRZ2 containing the *comA*::*lacZ cat* and *rec2*::*lacZ cat* insertions, respectively. Competent wild-type *H. influenzae* was transformed with either pMGAZ2, resulting in strain IDA610, or pMGRZ2, resulting in strain IDR22 (Fig. 1). Since pUC-derived plasmids cannot establish in *H. influenzae*, the only transformants obtained have inserted the entire plasmid into the chromosome at the location of either *comA* or *rec2*, which creates an insertion/duplication (Fig. 1). Southern analysis on genomic DNA from IDA610 and IDR22 confirmed that the correct construct existed.

In vitro mutagenesis. Tn*7* transposition reactions were performed in vitro, using a modification of the procedure of Bainton et al. (3). Transposition reactions contained purified TnsA, TnsB, and a gain-of-function TnsC mutant that allows Tn*7* transposition to occur with relatively little target site specificity (5, 30,

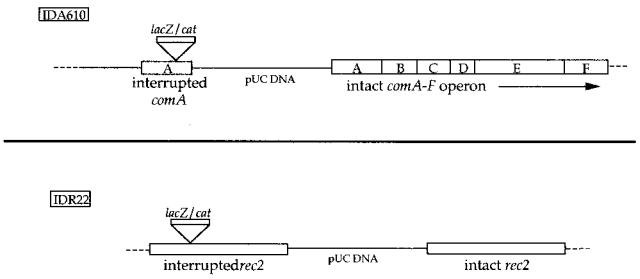

FIG. 1. Structures of insertion/duplication strains.

31). Transposition reactions also included a donor plasmid with a mini-Tn*7* element carrying a gene for kanamycin resistance (3) and, as a target, 0.9 or 0.3 μg of KW20 genomic DNA. After insertion, Tn*7* leaves 5-bp gaps at its ends which must be repaired prior to transformation (11). Mutated DNA was treated in a 30-μl reaction with Klenow enzyme and T4 ligase in the presence of 0.25 mM deoxynucleoside triphosphates and 1× ligase buffer to repair the gaps.

Screen. Mutagenized DNA was transformed into IDA610 by the natural DNA transformation pathway. Transformants were selected on kanamycin and plated to a density of about 100 colonies per plate. The plates were incubated at 37°C for 24 h and then replica plated to both a fresh kanamycin plate and one containing both MIV agar, to induce competence development in the transferred cells, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg/ml, to visualize the β-galactosidase activity in each colony. Although MIV does not support growth of colonies, the imprints of the transferred cells could be seen. Those imprints that had lighter or no blue color on the MIV–X-Gal plates were picked from the kanamycin replica.

Construction of specific insertions and double mutants. *E. coli* DH5α was used as the host for propagating all plasmids. We obtained clone pGHICX59, containing the wild-type *atpA* gene in pUC18, from The Institute for Genomic Research. We inserted a gene for kanamycin resistance (*kan*), and independently a gene for chloramphenicol resistance (*cat*), into the coding region of *atpA* at the *Nhe*I site, resulting in pMG40 and pMG41, respectively. The *H influenzae* DNA fragment containing *atpA*::*kan* was removed from pMG40 by restriction digestion with *Bsr*B1 and *Asp*718. This fragment was used to transform wild-type *H. influenzae* (KW20) and the *comA* insertion/duplication strain IDA610, resulting in MGH30 and IDA30, respectively. To construct IDR30, we transformed IDR22 with genomic DNA from MGH30 and selected for kanamycin resistance. To construct the *atpA ptsI* double mutant, the DNA fragment containing the *atpA*::*cat* construct was amplified from pMG41 by PCR, purified, and used to transform DYH15-1, which contains a *lacZ kan* cassette in *ptsI*. To construct the *por atpA* double mutant, we first transformed KW20 with genomic DNA from the *atpA*::*cat ptsI*::*lacZ kan* double mutant, selecting for chloramphenicol resistance. We checked that the resulting strain, MGH31, was not kanamycin resistant. We took genomic DNA from JF590-15, a mutant strain containing a *lacZ kan* gene in *por*, and transformed MGH31, selecting for kanamycin resistance. To construct a strain with the *atpA* mutation and a simple *lacZ kan* insertion in *comA*, with no duplication, we transformed MGH31 with genomic DNA from MGH100 and selected for kanamycin- and chloramphenicol-resistant colonies. Clone pGHIHM69, containing the *atpB* gene, was obtained from The Institute for Genomic Research. A *kan* cassette was inserted into the *Bsa*AI site in the coding region of *atpB*, resulting in plasmid pMG45. The *H. influenzae* fragment was removed from pMG45 by restriction digestion with *Xba*I and *Swa*I and used to transform competent KW20, selecting for kanamycin resistance, resulting in strain MGH40.

In all cases, correct strains were confirmed by PCR analysis in which primers flanking the insert sites were used to amplify the gene in question from both wild-type genomic DNA and insertion strain genomic DNA. If the construct is correct, the DNA from the mutant strain will result in a product larger than the wild-type DNA, which corresponds in size to the antibiotic resistance insert plus the wild-type fragment. If genomic DNA from a mutant strain that was already confirmed to be correct was used to transform another strain, that construct was not checked again in the new strain.

β-Galactosidase activity assay. β-Galactosidase activities were measured by using a permeabilized cell assay (26) with *o*-nitrophenyl-β-D-galactopyranoside as the substrate for the enzyme. Three-milliliter samples of cells were taken at time points throughout each competence-inducing procedure, pelleted, and either frozen at −20°C, until all samples could be tested together, or used immediately. To calculate the effect of the *atpA* mutation on induction of each gene, the fold increase in activity for each gene in both mutant and wild-type backgrounds was calculated, and then the mutant increase was divided by the wild-type increase to obtain the relative percent increase of the mutant.

RESULTS

Mutagenesis and screen. To find additional competence-regulatory genes, we looked for mutations which disrupt the induction of the competence-regulatory gene *comA*. Strain IDA610 (Fig. 1) contains both an intact *comA-F* operon and a copy of *comA* with a *lacZ cat* insertion. In this construct, the *lacZ* insertion allows β-galactosidase activity to be a measure of *comA* transcription, while the intact *comA-F* operon ensures that the cells will still be capable of natural transformation. MIV competence assays on the *comA* insertion duplication strain IDA610 confirmed that it has wild-type transformation levels. Also, β-galactosidase assays on samples of cells from the MIV competence-inducing procedure showed that the β-galactosidase activity rises 20- to 40-fold during competence induction, as expected based on Northern analysis of *comA* RNA levels during competence development (Fig. 2) (34). To ensure that a competence-regulatory mutation in IDA610 would reduce β-galactosidase activities, we inserted a *kan* cassette into *crp* in IDA610, resulting in strain IDA620. We found that the insertion in *crp* decreased β-galactosidase activities to background levels (Fig. 2).

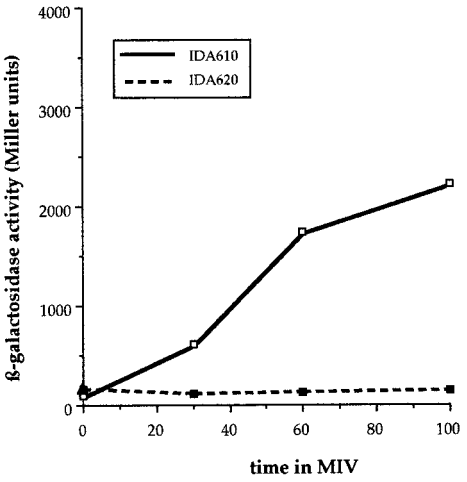

FIG. 2. Effect of a *crp* mutation on expression of β-galactosidase in IDA610.

H. influenzae genomic DNA mutated in vitro with Tn*7* was transformed into IDA610 by the natural transformation pathway which resulted in Tn*7* insertions in the IDA610 chromosome. As a pilot experiment to test this method, we screened approximately 2,000 potential mutant colonies for a decrease in *comA* induction by the MIV replica plating method. We picked 34 potential mutants, 20 of which remained after subsequent transformation screens. The mutant with the greatest transformation defect (~10-fold decrease from the wild-type level) was designated MGH11 and retained for further study.

In applying the screen, we found considerable variability in blue color development, using X-Gal assays with induced genes in *H. influenzae* which could result in a high frequency of false positives and negatives. In control platings of IDA620 (the *comA* insertion/duplication with an insertion in *crp*), while the majority of colonies were white, a few appeared blue. Despite this weakness of the screen, we were able to isolate the *atpA* mutant with its modest phenotype from a relatively small pool of potential mutants.

Identification of the mutant gene. We cloned a *Dra*I fragment containing the mutation. Sequence analysis of the ends of the insert fragment indicated that the Tn*7* insertion is within the *H. influenzae* homolog of *E. coli*'s *atpA* (HI0481; 94.7% similarity according to The Institute for Genomic Research *H. influenzae* database, accessible at http://www.tigr.org [14]). This gene is one of eight tandem genes in an operon that codes for the multisubunit enzyme ATP synthase. The operon has the same organization in *H. influenzae* as it has in *E. coli* except that *atpI* (a gene shown to be dispensable in *E. coli* [36]) is absent in *H. influenzae*. The *atpA* gene codes for the α subunit of the F_1 cytoplasmic domain (18).

Phenotype of an *atpA* mutation in different backgrounds. To remove variables that might be introduced by the presence of the transposon, we made new insertional mutants of *atpA* in wild-type cells (MGH30) and again into IDA610 (IDA30), using antibiotic resistance cassette insertions. The growth rate of MGH30 is about 75% of the wild-type level in early exponential growth phase. A similar effect on growth rate is ob-

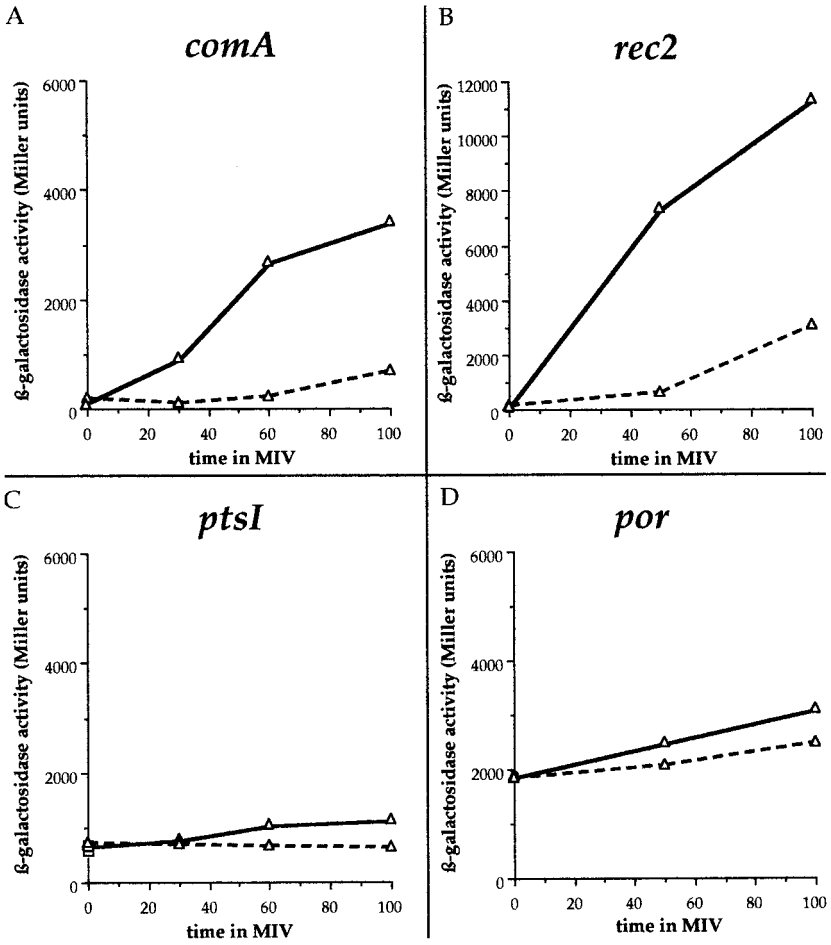

FIG. 3. Expression of four test genes in the MIV transformation assay. In all cases, curves shown are for a representative experiment, the solid lines represent strains that have wild-type *atpA*, and the dotted lines represent strains that have mutant *atpA*. Mutant and wild-type strains were tested in parallel on the same day. (A) *comA* expression in IDA610 (——) and IDA30 (– – –); (B) *rec2* expression in IDR22 (——) and IDR30 (– – –) (note that the scale on graph B is twice as large as on the other three graphs); (C) *ptsI* expression in DYH15-1 (——) and MGH51 (– – –); (D) *por* expression in JF590-15 (——) and MGH61 (– – –).

served in ATP synthase mutants of *E. coli* (21). MGH30 has a transformation frequency that is 10% of the wild-type level in the MIV assay (transformation frequencies, for the wild type 4×10^{-3} and 4×10^{-4} for the mutant) and 6.5% of the wild-type level in the transient anaerobiosis assay (transformation frequencies, 8.5×10^{-4} for the wild type and 5.5×10^{-5} for the mutant). Addition of 1 mM cAMP to the media improves transformation frequencies about twofold but does not restore them to wild-type levels. DNA uptake is also affected by a mutation in *atpA*. Uptake in MGH30 is approximately 1.7% of wild-type uptake in the MIV assay and approximately 2.3% of wild-type uptake in the transient anaerobiosis assay.

In addition, we compared β-galactosidase activities throughout the two competence-inducing procedures for IDA610 and IDA30 and found that the induction profile of *comA* was different in the *atpA* mutant than in the wild type. On average, the mutant experiences only 18% of the induction that the wild type does in MIV and 12.5% in transient anaerobiosis (Fig. 3A and 4A).

To determine if this is a specific effect on competence-induced genes or whether it is a general suppression of transcription, we checked the transcription of another gene induced during competence, *rec2*, and of two constitutively expressed genes required for competence, *ptsI* and *por*. To measure the activity of *rec2*, we used the insertion/duplication strain (IDR22) constructed similarly to IDA610 (Fig. 1). We found that an *atpA* mutant (IDR30) in the IDR22 background shows a similar decrease in induction (Fig. 3B and 4B).

We used MGH51 (*ptsI*::*lacZ kan atpA*::*cat*) and MGH61 (*por*::*lacZ kan atpA*::*cat*) to measure the activities of *ptsI* and *por*, respectively, in the *atpA* mutant background. We found that both of these constitutive genes were only minimally affected by the *atpA* mutation (Fig. 3C and D; Fig. 4C and D). These results indicate that the *atpA* mutation has a more marked effect on induced competence genes than on constitutive competence genes.

To ensure that the insertion/duplication construct does not affect the response of *comA* induction to the mutation in *atpA*, we placed a *cat* insertion into *atpA* in MGH100 (*comA*::*lacZ kan*), resulting in strain MGH101. We found that the mutation had a similar effect in the MGH100 background as it did in the IDA610 background (data not shown). Therefore, the presence of the insertion/duplication does not change the effect of an *atpA* mutation on *comA* expression.

Effect of an insertion into *atpB*. To test if the effect of *atpA* was due to that subunit alone or to a deficiency in the entire enzyme, we made a nonpolar (27) *kan* cassette insertion into the *H. influenzae* homolog of *atpB* (HI0485; 78.1% similarity),

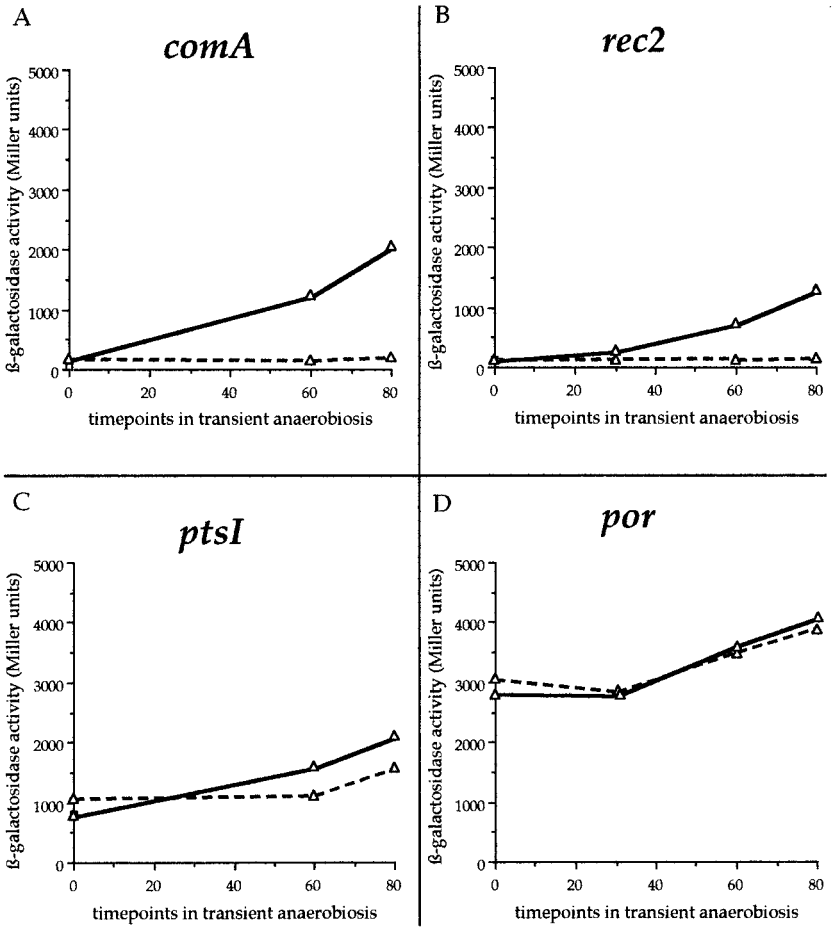

FIG. 4. Expression of four test genes in the transient anaerobiosis assay. In all cases, curves shown are for a representative experiment, the solid lines represent strains that have wild-type *atpA*, and the dotted lines represent strains that have mutant *atpA*. Mutant and wild-type strains were tested in parallel on the same day. (A) *comA* expression in IDA610 (——) and IDA30 (– – – –); (B) *rec2* expression in IDR22 (——) and IDR30 (– – – –); (C) *ptsI* expression in DYH15-1 (——) and MGH51 (– – – –); (D) *por* expression in JF590-15 (——) and MGH61 (– – – –).

which codes for the *a* subunit in the membrane spanning domain of the enzyme, resulting in strain MGH40. We found that this mutation has a phenotype very similar to that of the *atpA* mutation. The transformation frequency in an *atpB* mutant was, on average, 35% of the wild-type level in the MIV assay and 2.5% of the wild-type level in the transient anaerobiosis assay. Therefore, we conclude that the function of the whole enzyme, and not just a single subunit, affects the induction of the competence genes.

DISCUSSION

A mutation in the ATP synthase of *H. influenzae* Rd has a minor effect on transformation levels but a marked effect on the expression of induced competence genes. ATP synthase uses the energy from the proton motive force to generate ATP. The enzyme is composed of two large domains, a membrane-spanning proton channel (the F_0 domain) and a cytoplasmic ATPase (the F_1 domain). For every three protons that enter the cell through the channel, one molecule of ATP is generated from ADP and P_i. In addition, during anaerobic growth, under conditions of low membrane potential, or when there are high cellular ATP concentrations, the enzyme can reverse and use the energy from hydrolysis of ATP to pump protons out of the cell (1).

Mutations in ATP synthase are pleiotropic, and it is unlikely that ATP synthase is directly involved in competence development. Many metabolic changes occur in response to a mutation in the ATP synthase in *E. coli* (21), one of which is the depletion of ATP levels, which would lead to a lower cellular cAMP concentration which could affect competence development. Addition of cAMP to the transformation media only partially restored transformation levels, implying an additional effect due to loss of ATP synthase activity. It is possible that the signal for competence is the accumulation or depletion of a specific metabolite and that this mutation interferes with levels of that metabolite. Alternatively, the ATP synthase mutation may disrupt an aspect of metabolism that, though not the signal, is required for competence development. For example, it is known that near the end of MIV competence development, an increase of membrane potential is needed to achieve maximal competence (7). Mutants of *E. coli* in which the ATP operon has been deleted have increased membrane potential (21). Perhaps the absence of the ATP synthase causes an untimely or incorrect change in the membrane potential in *H. influenzae* which may interfere with either signal transduction or the DNA uptake machinery in the membrane. Further study to compare the physiology of wild-type and mutant ATP synthase in *H. influenzae* may indicate which, if any, other metabolic factors are involved in competence development.

ACKNOWLEDGMENTS

This work was funded by grant NP838C from the American Cancer Society and by grants GM48251 and GM53824 from the National Institutes of Health. H. O. Smith is an American Cancer Society Research Professor. N. L. Craig is an Investigator of the Howard Hughes Medical Institute.

We thank The Institute for Genomic Research for providing clones of the *H. influenzae* homologs of *atpA* and *atpB.*

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