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The *Haemophilus influenzae sxy-1* Mutation Is in a Newly Identified Gene Essential for Competence

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The *sxy-1* mutation of *Haemophilus influenzae* causes a 100- to 1,000-fold increase in spontaneous natural competence. We have used mapping and sequencing to identify this mutation as a G-to-A transition in an open reading frame adjacent to the *rec-1* locus. This mutation substitutes valine for isoleucine at amino acid 19 of the protein specified by this gene (now named *sxy*). A multicopy plasmid containing the wild-type *sxy* gene confers constitutive competence on wild-type cells. Cells carrying this plasmid exhibit, in all stages of growth, DNA uptake levels and transformation frequencies as high those normally seen only after full induction of competence by starvation; deletion of part of the *sxy* gene from the plasmid abolishes this effect. In contrast, a transposon insertion in *sxy* entirely prevents both DNA uptake and transformation, indicating that *sxy* encodes a function essential for competence. These findings suggest that *sxy* may act as a positive regulator of competence. However, because cells carrying the transposon-inactivated *sxy*::Tn allele grow slowly under conditions that do not induce competence, *sxy* may also have a role in noncompetent cells.

Under growth-limiting conditions, *Haemophilus influenzae* cells become competent to bind and take up double-stranded DNA fragments and will recombine these fragments into any homologous sites in their chromosomes. This competence for transformation is tightly regulated—fully inducing conditions can increase baseline transformation frequencies by at least 6 orders of magnitude. However, we know very little about how this regulation works. Although development of maximum competence is triggered by transfer to a starvation medium, attempts to define the inducing signals by manipulating culture conditions have not been very informative, and most of our understanding comes from genetic analysis. A number of different mutations affecting competence and transformation have been identified and characterized, but the only ones known to be in regulatory genes are in homologs of *Escherichia coli's cya* and *crp* genes. These genes encode the enzyme adenylate cyclase and the cyclic AMP (cAMP)-binding regulatory protein CRP (cAMP receptor protein), which together regulate transcription of many genes involved in *E. coli's* global carbon-energy regulon. These effectors also control utilization of various sugars in *H. influenzae* (6), showing that their roles in *H. influenzae* are not specific to competence. The *cya* and *crp* mutations thus give little insight into competence-specific regulation, beyond adding to the evidence that competence is triggered at least in part by nutritional limitation and suggesting that induction of competence in *H. influenzae* may use a pathway similar to catabolite regulation in *E. coli.*

We have previously isolated a mutation (*sxy-1*) in a new gene affecting competence (13). Rather than preventing competence as do the *crp* and *cya* mutations, this mutation enhances competence 100- to 1,000-fold: *sxy-1* cultures exhibit moderate levels of competence under noninducing conditions and develop full competence under what are otherwise partially inducing conditions. We have hypothesized that *sxy* might be a competence-specific regulatory gene, because the *sxy-1* mutation increases both the DNA uptake and recombination components of competence and because cells carrying *sxy-1* show no alterations in growth or DNA repair.

We have now mapped the *sxy-1* mutation to an open reading frame adjacent to the *rec-1* gene in a small DNA fragment previously shown to increase competence and have determined the DNA sequence alteration in the *sxy-1* mutant allele. We have also found that a transposon insertion in *sxy* entirely prevents competence and that the plasmid-associated constitutive competence previously attributed to overexpression of the *rec-1* gene is more likely caused by *sxy.*

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Strain KW20 is the original *H. influenzae* Rd (1). *H. influenzae* cells were grown in brain heart infusion broth (Difco) supplemented with hemin at 10 μg/ml and NAD at 2 μg/ml (sBHI) and solidified with 1.2% Bacto Agar (Difco) where appropriate. Novobiocin at 2.5 μg/ml, kanamycin at 7 μg/ml, nalidixic acid at 3 μg/ml, and cAMP at 1.0 mM were used.

Competence time courses. The general procedure for competence time courses has been described elsewhere (13). Briefly, at regular intervals, 1-ml samples were taken from cultures growing in sBHI and were incubated for 15 min with 1 μg of novobiocin-resistant chromosomal DNA from strain MAP7 (3) and for 5 min with 1 μg of DNase I. Samples were then diluted and plated on selective and nonselective sBHI plates.

Colony competence assay. Each colony to be tested was transferred from an agar plate (using a Pipetman tip) into 5 ml of sBHI containing MAP7 DNA at 0.1 μg/ml. After 15 min at 37°C, 0.1 ml of the cell suspension was plated directly onto a novobiocin-containing sBHI plate, and 0.1 ml of the cell suspension was diluted 10^{-4} and plated onto a plain sBHI plate. Novobiocin-resistant transformants do not require expression time before plating. Fresh colonies 1 to 2 mm in diameter gave the best results.

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Sequencing and PCR. Oligonucleotide primers for PCR amplification and for DNA sequencing were designed by using sequences determined by Zulty and Barcak (19) and Barcak (2). DNA sequences of plasmid inserts were determined by using double-strand sequencing with T7 sequencing kits from Pharmacia. PCR products were sequenced directly with a Circumvent kit from New England Biolabs.

Site-directed mutagenesis. The basic site-directed mutagenesis method of Kunkel was used (9) with the modifications described by Lorimer and coworkers (10). Mutagenesis was carried out on plasmid pDJM90, using a primer carrying the *sxy-1* mutation. Candidate mutant plasmids were screened by sequencing the region between the left *Sca*I site and the *Cla*I site (see Fig. 2B) to confirm that only the desired change was present. The checked mutagenized *Sca*I-*Cla*I fragment was then subcloned into nonmutagenized pDJM90, replacing the corresponding nonmutagenized segment and generating plasmid pRRS6.

RESULTS

Constitutive competence caused by pHKrec. Stuy reported that a plasmid containing the *H. influenzae rec-1* gene enhances the competence of wild-type cells (17). Because this phenotype resembles the hypercompetence caused by the *sxy-1* mutation, we characterized it in detail. However, Stuy's plasmid prec2255 had been exposed to methyl methane sulfonate and UV irradiation during selection, so we used the independently cloned plasmid pHKrec (4), which carries the same 3.1-kb *Eco*RI fragment as prec2255 (see Fig. 2) but has not been exposed to a mutagen. Preliminary experiments had shown that pHKrec, like prec2255, increased the spontaneous competence of wild-type cells.

Figure 1 compares the spontaneous competence developed by wild-type cells carrying various plasmids. During exponential growth, plasmid-free cells and those carrying only the vector plasmid pHK (4) had transformation frequencies of about 10^{-8}/CFU, which rose to about 10^{-4}/CFU at the onset of stationary phase. These frequencies are typical for wild-type cells (13). In contrast, cells carrying pHKrec exhibited constitutive competence, with transformation frequencies near 10^{-2}/CFU even during early exponential growth. Uptake of radiolabelled DNA by these cells was increased proportionately (results not presented), showing that this is true competence and not simply more efficient recombination caused by overproduction of Rec-1 protein, the *H. influenzae* homolog of *E. coli's* RecA.

We used the related plasmid pHKfec to show that this constitutive competence is not caused by the *rec-1* gene. This plasmid contains the *H. influenzae rec-1* gene but carries, in place of the sequences upstream of *rec-1*, unrelated DNA unintentionally substituted during cloning (4, 19) (see Fig. 2B). Intact Rec-1 protein is expressed from pHKrec and pHKfec, both of which complement the Fec phenotype of an *E. coli recA* mutant and the transformation, phage induction, and UV resistance defects of a *H. influenzae rec-1* mutant (4). However, their effects on competence were very different. Spontaneous competence developed identically in cells with and without pHKfec (Fig. 1, solid triangles and solid circles, respectively), demonstrating that Rec-1 is not responsible for the constitutive competence observed in cells carrying pHKrec.

Mapping *sxy-1*. Zulty and Barcak (19) have sequenced the entire 3.1-kb insert of pHKrec, shown in Fig. 2. Between the *rec-1* gene and the 5′ end of the *rrnA* operon, they found a small open reading frame which they called *tfoX* (19); most of this open reading frame is deleted in pHKfec. (The sequence

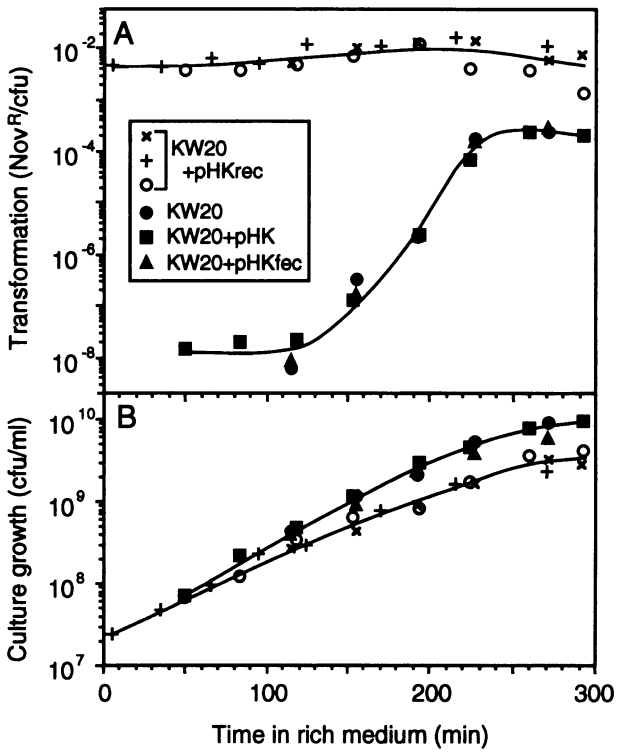

FIG. 1. Effects of plasmids pHK, pHKrec, and pHKfec on growth and spontaneous transformation of *H. influenzae*. (A) Frequency of transformation to novobiocin resistance by MAP7 DNA. (B) Growth in sBHI. The results are from three independent experiments.

of this open reading frame has not yet been published but has been placed in GenBank under accession number U13205.) Because both pHKrec and the *sxy-1* mutation caused greatly elevated competence and because we had observed that the *sxy-1* mutation, like *rec-1*, was closely linked to a streptomycin resistance marker in transformation experiments, we considered whether the same gene might be responsible for both phenotypes, i.e., that the new open reading frame encoded the *sxy* gene. We could not use plasmid complementation to test this hypothesis, because the plasmid carrying the putative *sxy*$^+$ gene, pHKrec, itself caused higher competence than the chromosomal *sxy-1* mutant gene it might be expected to complement. Instead we used the marker rescue experiments described below to show that pHKrec carried the *sxy* gene.

We first developed a colony competence screen to identify colonies containing the *sxy-1* mutation. In this assay, individual colonies were resuspended directly in medium containing genetically marked transforming DNA and were plated on selective plates after a brief incubation. Figure 3A and B show that, by this assay, most wild-type colonies (strain KW20) had transformation frequencies between 10^{-6} and 10^{-5}/CFU, whereas in most *sxy-1* colonies (strain RR563) between 10^{-4} and 10^{-3}/CFU of cells became transformed, i.e., about 100-fold higher than wild-type cells. Because there was some overlap between the ranges of the two genotypes, rigorous establishment of the *sxy* genotype of any specific colony still required a competence time course.

We initially used the colony competence test to ask whether the wild-type *Eco*RI fragment of pHKrec could transform *sxy-1* cells back to the wild type (i.e., whether it contained the wild-type homolog of the *sxy-1* allele). Strain RR563 (*sxy-1*)

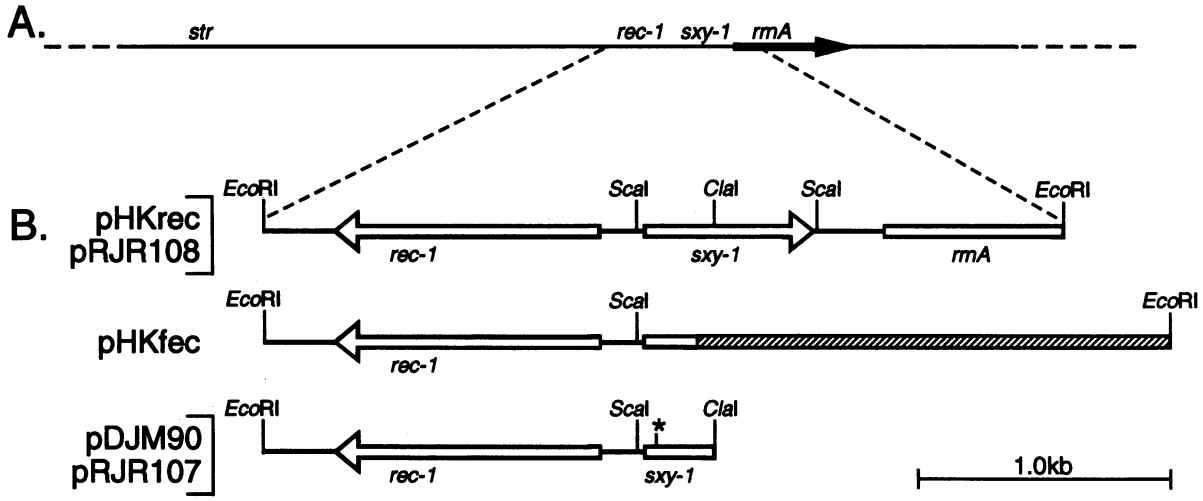

FIG. 2. Maps of *sxy* chromosomal and plasmid DNAs. (A) Chromosomal region around the *sxy* gene. (B) Inserts of *sxy* plasmids used in these experiments. The hatched part of pHKfec is derived from an unrelated part of the *H. influenzae* chromosome. The asterisk indicates the site of the *sxy-1* mutation in pRJR107.

was made maximally competent and incubated with *Eco*RI-digested pHKrec DNA. The culture was diluted and plated on nonselective medium, and the next day colonies were tested for competence. One-fifth of the colonies (25 of 120) were no longer hypercompetent by this assay; the wild-type phenotypes of four of these colonies were confirmed by detailed time courses of competence development (data not shown). This result suggested that the wild-type *sxy* allele was indeed contained in the 3.1-kb insert of pHKrec.

We have been unable to obtain a stable clone carrying the 3.1-kb *Eco*RI fragment from *sxy-1* cells. Stuy described extensively his difficulties in obtaining clones of the same wild-type fragment (17). Part of the problem may be the presence of the *rrnA* promoter in this fragment (19), but we have observed frequent rearrangements, substitutions, and deletions even when the *sxy-1* mutant fragment was placed in a vector containing a strong terminator. However, we did clone a 1.8-kb *Eco*RI-*Cla*I subfragment containing *rec-1* and about 500 bp of upstream sequences, including about 350 bp of the putative *sxy-1* gene. This plasmid, pRJR107 (Fig. 2B), was generated by ligation of RR563 chromosomal DNA into pGEM7$^+$ (both plasmids precut with *Eco*RI and *Cla*I) and identified by colony hybridization with a pHKrec probe. The experiments below show that this 1.8-kb insert was sufficient for chromosomal transformation to hypercompetence and thus, that it contained the *sxy-1* mutation.

We expected transformation by this short fragment to be very inefficient because of the exonucleolytic DNA degradation that accompanies DNA uptake during transformation (11). Accordingly, to enrich for potential hypercompetent transformants, we modified the above marker rescue procedure by including a second transformation step before screening by the colony transformation assay. We first incubated maximally competent KW20 cells with pRJR107 DNA (precut with *Eco*RI and *Cla*I), using as negative controls precut pHKrec and pDJM90 (a plasmid like pRJR107 but carrying a wild-type fragment subcloned from pHKrec [Fig. 2B]) and as a positive control RR563 chromosomal DNA. Cells from this first transformation step were plated without selection, and the resulting colonies were pooled (10^3 to 10^4 colonies per pool), grown to mid-exponential stage in sBHI, and enriched for spontaneously competent cells by a second transformation, this time to nalidixic acid resistance with limiting DNA from a Nal^r Nov^s strain (constructed by transforming KW20 with MAP7 DNA). We used the Nal^r gene because this gene is unlinked to *sxy*, and we used limiting DNA to prevent unwanted cotransformation by the *sxy*$^+$ allele in the donor DNA. Forty Nal^r colonies were picked from each of the original transformations and tested for spontaneous competence by the colony assay described above. The results, presented in Fig. 3C, D, and E, show that most of the colonies enriched from the pRJR107 transformation were highly competent (transformation frequencies of $>10^{-4}$), whereas none of the colonies from the control pHKrec transformation were. These results demonstrate that the *sxy-1* mutation is indeed contained within the 1.8-kb fragment of pRJR107.

Sequence analysis of p107 and controls. To identify the *sxy-1* mutation, we sequenced the inserts of pRJR107 (*sxy-1*) and pDJM90 (*sxy*$^+$). Barcak and Zulty kindly provided us with their pHKrec sequence which we used to design primers for our sequencing. We also sequenced a region of pHKrec extending 150 bp on either side of the postulated 5′ end of the *sxy* transcript and PCR-amplified fragments of the corresponding region from chromosomal DNA of strains KW20 and RR563 (19). Comparison of these sequences revealed a single *sxy-1*-specific G→A transition (at base 298) which would substitute an isoleucine for a valine at amino acid 19 of the protein specified by the new open reading frame. This mutation was present in both pRJR107 and the RR563 chromosome and was absent in pDJM90, pHKrec, and the KW20 chromosome. Direct sequencing of PCR products amplified from chromosomal DNA confirmed that eight hypercompetent (putative *sxy-1*) isolates from the experiment shown in Fig. 3C had all acquired the G→A mutation. (Our sequencing also revealed an apparent sequencing error. All of our sequences showed a T at base 181 [strengthening the presumptive *sxy* promoter and weakening the postulated CRP-binding site] rather than the G reported by Zulty and Barcak [19].)

Because substitution of isoleucine for valine is a very conservative change and would not be expected to dramatically alter a protein's properties, we were still concerned that we might have overlooked another nearby mutation. To com-

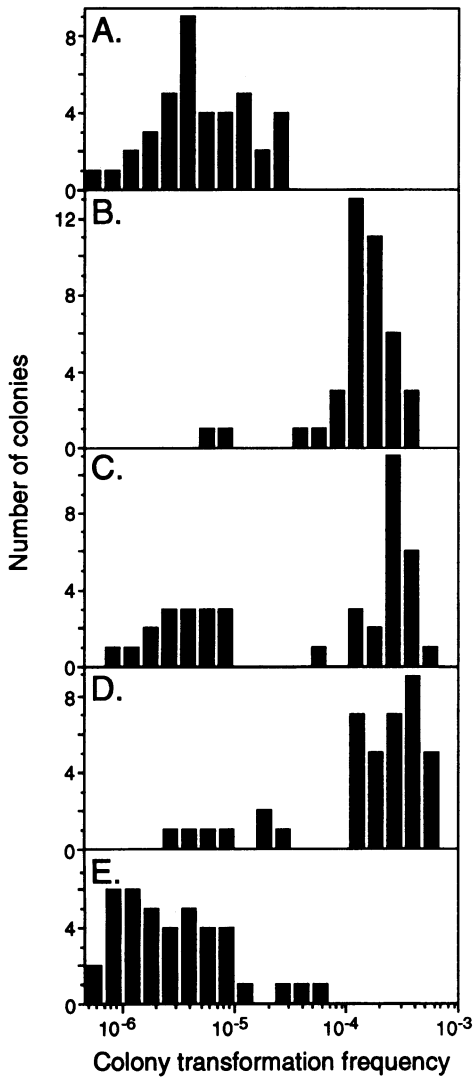

FIG. 3. Results of colony transformation assays. Each panel is the results of testing 40 colonies. (A) Colonies of KW20; (B) colonies of RR563; (C) colonies of KW20 that had been transformed with RR563 DNA; (D) colonies of KW20 that had been transformed with *Eco*RI-and-*Cla*I-digested pRJR107 DNA; (E) colonies of KW20 that had been transformed with *Eco*RI-digested pHKrec DNA. The *x* axis gives the transformation frequency exhibited by the cells in each tested colony. The *y* axis indicates the number of colonies showing that transformation frequency.

pletely rule this out, we used site-directed mutagenesis to place the *sxy-1* G→A substitution into the wild-type insert of pDJM90, generating the new mutant plasmid pRRS6, and repeated the transformation assay. Again, cultures transformed with pRRS6 gave hypercompetent colonies, and those transformed with pDJM90 gave none, confirming that the G→A substitution is responsible for the 100-fold increase in competence.

***sxy* is an essential gene for competence.** We generated a mutation inactivating the *sxy* gene by transposition of mini-Tn*10kan* into pRJR108 (6, 8). This plasmid carries the same 3.1-kb *sxy Eco*RI fragment as pHKrec, subcloned into the *E. coli* vector pKK223-3 (5). The resulting plasmid, pPW2, carried an insertion interrupting the *sxy* open reading frame very close to its N terminus. This insertion mutation was then introduced into the *H. influenzae* chromosome by transformation of KW20 with *Eco*RI-cut pPW2 followed by selection for the kanamycin resistance carried by the transposon. We used Southern blot analysis to confirm that the resulting strain (RR648) carried the expected simple insertion and then examined its ability to develop competence.

The data summarized in Table 1 show that this insertion completely prevented transformation both during growth in sBHI and after transfer to MIV competence-inducing medium. We also measured phage recombination, DNA uptake, and UV sensitivity by previously described procedures (13). Like all other competence-defective mutants, *sxy*::Tn cells were unable to carry out phage recombination under standard competence-inducing conditions [recombination frequencies were $(1.1 \pm 0.3) \times 10^{-3}$ for KW20 (*sxy*$^+$) and $(8.4 \pm 1.9) \times 10^{-6}$ for RR648 (*sxy*::Tn)]. This insertion could cause these defects by interfering with expression of the adjacent *rec-1* gene rather than with the induction of competence. However, under competence-inducing conditions, strain RR648 also failed to take up detectable amounts of labelled DNA (126 ± 13 and 0.6 ± 0.1 kb of DNA taken up per CFU for KW20 [*sxy*$^+$] and RR648 [*sxy*::Tn], respectively), showing that the cells were not only nontransformable but noncompetent. Furthermore, cells of strain RR648 were as UV resistant as wild-type cells, so *rec-1* expression must be within normal limits.

Another possible explanation for the noncompetence of strain RR648 (*sxy*::Tn) might be that the Sxy protein is essential for production of the elevated cAMP levels required for competence induction. This possibility seemed unlikely, as we have previously shown that strain RR648 ferments sugars normally (6). We have confirmed this by showing that the competence defect of strain RR648 is not corrected by the addition of cAMP, either in MIV medium or in sBHI (Table 1). However, strain RR648 did grow slowly, with a doubling time in sBHI of about 40 min compared with 30 min for KW20, suggesting that the Sxy protein does have some function in noncompetent cells.

DISCUSSION

In order to understand how naturally transformable cells benefit from the DNA they take up, we are investigating how cells make the decision to become competent. The most direct and immediate benefit of DNA uptake is likely to be nutritional, in the form of the nucleotides released when the DNA is degraded or integrated into the chromosome. However, if the DNA is homologous, it could also provide both templates for DNA repair and opportunities to benefit from genetic exchange (15).

The requirement for catabolite regulator protein CRP and its cofactor cAMP provides clear evidence for nutritional regulation. In *E. coli*, the CRP-cAMP complex provides a general signal of carbon-energy limitation to many transcription units, but expression of each transcription unit usually requires an additional operon-specific signal. Thus, it is not surprising that induction of competence in *H. influenzae* also appears to require more than just an increase in cAMP. Exogenous cAMP causes only about 1% of cells in an exponentially growing culture to become competent (6) and fails to increase the similar partial competence seen at the onset of stationary phase in rich medium (18). We know that entry of cAMP into the cell is not limiting the response, because exogenous cAMP restores full competence to a *cya* culture in the competence-inducing starvation medium MIV (6) and to *cya sxy-1* cells in rich medium (12). We have proposed a simple

TABLE 1. Transformation frequencies of three strains of *H. influenzae* in the presence and absence of exogenous cAMP

Strain	Transformation frequency[a] for cells					
	Early log phase in sBHI		Late log phase in sBHI		Induced in MIV	
	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP
KW20 (wild type)	1.4×10^{-8}	1.6×10^{-4}	1.3×10^{-4}	2.2×10^{-4}	7.5×10^{-3}	1.1×10^{-2}
RR668 (*cya*::Tn)	1.1×10^{-8}	8.1×10^{-5}	5.0×10^{-9}	3.9×10^{-5}	2.2×10^{-8}	3.7×10^{-3}
RR648 (*sxy*::Tn)	$<1.5 \times 10^{-8}$	$<3.7 \times 10^{-8}$	2.6×10^{-9}	$<8.5 \times 10^{-9}$	$<2.8 \times 10^{-9}$	$<3.8 \times 10^{-9}$

[a] Transformation frequencies (per CFU) of wild type, *cya*::Tn, and *sxy*::Tn strains of *H. influenzae* in the presence (+) and absence (−) of exogenous cAMP. Each value is the mean of results of several independent experiments.

model in which the *sxy* gene product is a component of a second regulatory mechanism controlling competence. We further proposed that the *sxy-1* mutant phenotype results from constitutive activation of this component of regulation. Results here are consistent with this model and extend it by more precisely delimiting the role of *sxy*.

The *sxy*::Tn insertion mutation shows that *sxy* function is required not only for maximal competence in MIV medium but is a requirement for all competence. We know this mutation does not block competence by interfering with cAMP levels or CRP function, because exogenous cAMP does not restore competence and because sugar fermentation is unchanged. We have not yet ruled out the reverse possibility that *cya* and *crp* mutations block competence because they prevent *sxy* expression. The presence of CRP sites near the *sxy* promoter suggests that its transcription could be regulated by the cAMP-CRP complex (19). However, the constitutive competence of cells carrying pHKrec during exponential growth suggests either that pHKrec itself causes cAMP levels to increase or that *sxy* is expressed from pHKrec at levels sufficient to induce full competence even in the absence of elevated cAMP. We have not yet been able to test whether pHKrec can restore competence to a *cya* mutant.

The constitutive competence of cells carrying pHKrec is surprising. Full competence has previously been observed only under conditions preventing growth, usually after transfer to the starvation medium MIV (7). Because competent cells transferred from MIV medium back into growth medium synchronously resume DNA synthesis, transcription, and cell division, Scocca and Haberstat proposed that competence development involves the cessation of growth at a defined point in the cell cycle (16). However, cells carrying pHKrec show near-normal growth rates (Fig. 1B), implying that the abilities to take up and recombine DNA need not interfere with normal progress through the cell cycle.

The *sxy-1* mutation causes isoleucine to be substituted for valine in the predicted protein product. This change is normally a very minor change in protein structure, yet it causes spontaneous competence to increase 100-fold. We are confident we have identified the true mutation. Because a plasmid-borne wild-type *sxy* gene causes even more extreme hypercompetence, the *sxy-1* mutation may act by increasing the abundance or activity of a Sxy protein with essentially normal function. Unfortunately, Zulty and Barcak reported that the *sxy* gene and its predicted protein show little homology to any previously reported gene or protein (19), so we do not know what this function may be.

Stuy has proposed that the *rec-1* gene, a homolog of *E. coli's recA*, participates in the regulation of competence (and thus that it might be the second regulator) (17). However, although mutations inactivating *rec-1* block the recombination step of transformation, they do not interfere with the normal development of competence (defined as the ability to take up DNA) (19). Furthermore, although Rec-1 is activated by DNA damage, we have found that DNA damage itself does not induce competence (14). Although some *rec-1*-containing plasmids (prec2255 and pHKrec) do cause high spontaneous competence, the *rec-1*$^+$ Δ*sxy* plasmid pHKfec does not.

We have now begun to investigate several independently derived mutations causing hypercompetent phenotypes like that of *sxy-1*. New changes in *sxy* may shed light on how the *sxy-1* mutation causes hypercompetence. Mutations in other genes will, we hope, allow us to identify additional components of the signal transduction pathway controlling competence.

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