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sxy-1, a *Haemophilus influenzae* Mutation Causing Greatly Enhanced Spontaneous Competence

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A *Haemophilus influenzae* strain carrying a competence-enhancing mutation (*sxy-1*) was selected by transformation of a mutagenized culture in exponential growth at low cell density, where spontaneous competence is very rare. Under these conditions, *sxy-1* cells spontaneously transformed 100 to 1,000 times more efficiently than wild-type cells. Moreover, *sxy-1* cells responded to all known competence-inducing treatments with further increases in transformation frequency. At high cell densities, *sxy-1* cells spontaneously developed the level of competence reached by wild-type cells only after maximal induction by transfer to starvation medium. The *sxy-1* mutation appears to act early in the sequence of events leading to competence; it increased the competence of cells carrying the early-acting transformation-defective (Tfo$^-$) mutation *tfo-98* by as large a factor as it did the competence of wild-type cells, but it had no effect when combined with another early-acting Tfo$^-$ mutation (*tfo-87*) or with the late-acting Tfo$^-$ mutation *rec-2*.

Natural competence in *Haemophilus influenzae* is tightly regulated. The mechanisms controlling this regulation and the reasons for it are not understood. Natural transformation may serve any of several functions, including acquisition of nucleotides (20), DNA repair (26), elimination of deleterious mutations (15), and generation of genetic variation, and there is as yet no particular reason to favor any single explanation. Under optimal inducing conditions, all of the cells in a culture become competent and are then able to take up 50 to 100 kb of homologous chromosomal DNA and recombine it into their chromosomes, giving transformation frequencies of 1 to 2%. In contrast, during exponential growth at low cell densities (early-log-phase growth), the transformation frequency is usually no higher than 10^{-8}. Competence development requires protein synthesis (14) and in addition is known to involve induction of both DNA uptake and the ability to recombine phage DNA. These processes are undetectable in early-log-phase cells and are increased at least 100-fold in fully competent cells. Low levels of competence (transformation frequencies about 10^{-4}) develop spontaneously in late-log-phase cultures and can be induced in early-log-phase cultures by cyclic AMP (cAMP) (25). Higher transformation frequencies are induced by temporary anaerobiosis (7) and, most efficiently, by transfer to a starvation medium (8).

Apart from cAMP, we know neither the identity nor the mode of action of the molecular signals that induce competence. A number of mutations are known to interfere with competence or transformation. Some appear to act late, permitting DNA uptake but not further processing (e.g., *rec-1* and *rec-2* mutations [18]); others show no DNA uptake or inducible recombination and may be required for the initial induction steps (22) (unpublished data). Because all of these mutations prevent transformation, the phenotypes of double mutants are not informative, and it has not been possible to order the genes into a regulatory hierarchy.

To augment the existing competence-negative mutations, I have begun to isolate new mutations that cause competence to develop under conditions that normally prevent it. Because *H. influenzae* competence is tightly regulated, such mutations can be isolated by incubating an early-log-phase culture with marked DNA and selecting clones that have been able to take up this DNA and thus become transformed. Analysis of the phenotypes of these highly competent mutants, both alone and in combination with other mutations, will increase our understanding of the control of competence and of its consequences for the cell.

MATERIALS AND METHODS

Strains, plasmids, and DNA. Strains and plasmids used are listed in Table 1. All strains are descendants of the original *H. influenzae* Rd of Alexander and Leidy (1). Strains carrying single antibiotic resistance genes were constructed by transforming KW20 with MAP7 DNA. Plasmids were isolated by alkaline lysis, and chromosomal DNAs by the rapid-prep method described elsewhere (2). No special precautions were taken to prevent fragmentation of chromosomal DNAs; even after vortexing, most fragments were larger than 50 kb. Plasmids were introduced into *H. influenzae* by glycerol-enhanced transformation (21). pRRNov1 was constructed by ligating the Nov1 *Pvu*II fragment of pNov1 (19) into *Sma*I-cut pSU2718 (12), which replicates efficiently in *H. influenzae* (5).

Culture conditions. Cells were grown at 37°C in supplemented brain heart infusion (sBHI) as described elsewhere (2). BHI medium from Difco and Merck gave equivalent growth and competence, but phage HP1 formed plaques only on Difco Bacto Agar. Cells were spread on plates containing 1 to 1.5% agar, with novobiocin (2.5 μg/ml), kanamycin (7 μg/ml), streptomycin (250 μg/ml), or chloramphenicol (2 μg/ml) when required. sBHI plates more than 48 h old were spread with additional hemin (0.3 ml of 1-mg/ml stock) before use.

Competence induction. (i) Spontaneous competence. Cells were grown in flasks with shaking (20 ml culture in a 250-ml flask or 35 ml in a 500-ml flask, shaking at 150 to 200 rpm).

(ii) Induction by MIV starvation medium. Cells at approximately 10^9 CFU/ml were pelletted twice, resuspended in an equal volume of MIV medium (8) at room temperature, and shaken (in MIV) at 37°C for 100 min.

(iii) Induction by cAMP. cAMP (1.0 mM) was added to sBHI cultures at 2×10^8 CFU/ml, and cells were shaken for 30 min.

TABLE 1. Strains and plasmids used in this work

Strain	Relevant phenotype or genotype	MIV transformation frequency	Source or reference
KW20	Wild-type *H. influenzae* Rd	1×10^{-2}–2×10^{-2}	1
RR521	Uncharacterized *sxy* mutation	1×10^{-2}–2×10^{-2}	EMS-mutagenized KW20
RR528	*sxy-1 str*	1×10^{-2}–2×10^{-2}	EMS-mutagenized KW20
RR563	*sxy-1 str*	1×10^{-2}–2×10^{-2}	KW20 transformed by RR528 DNA
JG87	*crp*::mini-Tn*10kan*	8×10^{-9}	22
RR587	*sxy-1 crp*::mini-Tn*10kan*	1×10^{-8}	RR563 transformed by JG87 DNA
RR588	*sxy-1 crp*::mini-Tn*10kan*	1×10^{-8}	RR563 transformed by JG87 DNA
JG98	*tfo-98*::mini-Tn*10kan*	2×10^{-7}	22
RR589	*sxy-1 tfo-98*::mini-Tn*10kan*	5×10^{-5}	RR563 transformed by JG98 DNA
RR590	*sxy-1 tfo-98*::mini-Tn*10kan*	7×10^{-5}	RR563 transformed by JG98 DNA
RR622	*rec-2*::mini-Tn*10kan*	2×10^{-8}	13
RR620	*sxy-1 rec-2*::mini-Tn*10kan*	2×10^{-8}	RR563 transformed by pDM79
RR621	*sxy-1 rec-2*::mini-Tn*10kan*	9×10^{-9}	RR563 transformed by pDM79
MAP7	*str kan nov nal spc vio stv*		J. Setlow
RR514	*str*		KW20 transformed by MAP7 DNA
RR518	*nov*		KW20 transformed by MAP7 DNA
RR520	*kan*		KW20 transformed by MAP7 DNA
pNov1	Nov^r gene in pRSF0885		19
pSU2718	Cm^r		12
pRRNov1	pSU2718::Nov^r		This work; Nov^r from pNov1
pDM79	*rec-2*::mini-Tn*10kan*		13

(iv) Induction by "anaerobic-aerobic" shift. Cultures (12 ml) were grown in 500-ml sidearm flasks to 1×10^8 to 2×10^8 CFU/ml. The cultures were then tipped into the side arm, left without shaking at 37°C for 60 min, tipped back into the bottom of the flask, and shaken for 30 min (7).

Mutagenesis and screening. EMS (methanesulfonic acid ethyl ester; Sigma) was added to cells growing in sBHI at about 10^9 CFU/ml, to give concentrations of 0.05 and 0.08 M. Cultures were shaken at 37°C for 45 or 30 min (for 0.05 and 0.08 M EMS, respectively), washed twice in BHI, resuspended in fresh sBHI, and shaken for an additional 6 h at 25°C before being frozen at −70°C. About 10% of cells survived mutagenesis, and these underwent two cell doublings in the interval before freezing. The effectiveness of mutagenesis was estimated by the frequency of mutations giving resistance to 1.0 μg of novobiocin per ml (Nov_1^r); the spontaneous frequency of Nov_1^r was 5×10^{-9}, and 0.05 and 0.08 M EMS induced Nov_1^r mutations at 3.4×10^{-6} and 3.5×10^{-6}, respectively.

For screening, frozen mutagenized cells (1 ml) were thawed, pelletted, and resuspended in 20 ml of sBHI. Cells were shaken at 37°C to a density of 2×10^8 CFU/ml and incubated for 30 min with 1 μg of pRRNov1 which had been cut with *Kpn*I-*Xba*I to free the Nov1 insert. DNase I (1 μg/ml) was added for 15 min, and cells were plated with 10 μg of novobiocin per ml.

Transformation assay for the *sxy-1* mutation in genomic DNA. A transformation assay was used to show that a Tfo^- double mutant retained the *sxy-1* mutation in its genome, by showing that some cells transformed with its DNA acquired the Sxy phenotype of early-log-phase competence. Maximally competent KW20 cells were incubated with 2 μg of the DNA to be tested per ml and were diluted and plated on sBHI agar. Unselected colonies were pooled (500 to 5,000 colonies per pool), diluted to 2×10^7 CFU/ml, grown for 90 min to early log phase, and transformed to Nov^r with RR518 DNA. Pools of cells originally transformed with *sxy-1* DNA contained 1 to 5% Sxy colonies and thus gave Nov^r transformation frequencies higher than 2×10^{-7}, whereas control Nov^r transformation frequencies (pools from transformations with nonmutant DNA) were less than 2×10^{-8}.

Routine transformation assays. Cells (1 ml in MIV or sBHI) were incubated for 15 min with 1 μg of MAP7 DNA on a roller wheel at 37°C. DNase I (1 μg) was then added, and incubation was continued for a further 5 min. If Kan^r or Nov^r transformants were to be selected, cells were diluted and plated immediately; for resistance to other antibiotics, incubation was continued for a further 60 min to allow expression of the resistant phenotype. If cells were in MIV or at high density in sBHI, the culture was diluted 3:1 in sBHI before this incubation to allow growth and gene expression. To determine the transformation frequencies of the very dilute cultures described in Fig. 1, larger volumes of culture were incubated with 1 μg of DNA per ml and with DNase I and were concentrated by centrifugation before plating. The transformation frequency is the ratio of transformed to total CFU per milliliter. Where no transformed colonies were detected, an upper limit for the transformation frequency was calculated as the frequency that would be calculated if one colony had been observed.

DNA uptake. MAP7 DNA was ^{32}P labeled in vitro by nick translation. To maximize fragment length, after labeling, the nicks were sealed by incubation with T4 ligase (16). Cells (approximately 2×10^9 in 0.5 to 1.0 ml of sBHI or MIV) were incubated with 600,000 cpm of this DNA at 37°C for 15 min. DNase I was added (1 μg/ml) for a further 5-min incubation. NaCl was added to 1.0 M, and the cells were chilled on ice. The cells were washed twice in 1.0 ml of cold 1.0 M NaCl–1 μg of DNase I per ml and finally resuspended in 100 μl of 1.0 M NaCl for counting. The number of viable cells was simultaneously determined, and DNA uptake was then expressed as counts per minute per CFU.

Phage recombination. Cells were coinfected with phage HP1c1 temperature-sensitive mutants *ts2* and *ts3* at multiplicities of two to five each (4). After 15 min of adsorption at 33°C, cells were twice pelletted and resuspended in BHI, diluted 100-fold in sBHI, and shaken at 33°C for 90 min. Lysates were shaken with a small volume of chloroform for

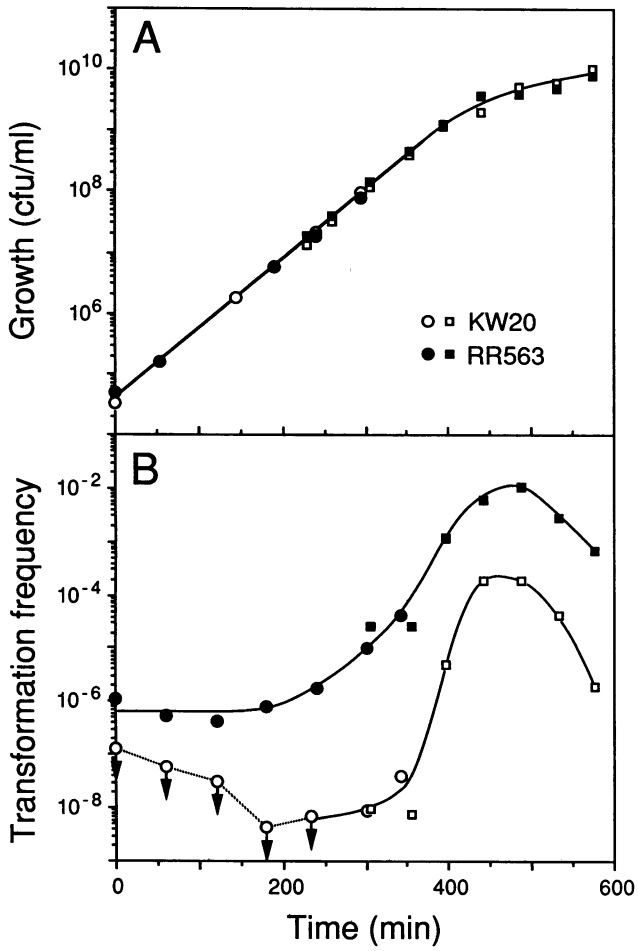

FIG. 1. Growth and competence of cells in sBHI. Squares, experiment 1; circles, experiment 2. (A) Growth in sBHI. CFU-per-milliliter values in experiment 1 are those at the time DNA was added, not after completion of transformation. (B) Frequency of transformation to Nov^r by MAP7 DNA. The KW20 transformation frequencies before 300 min are upper limits because no transformants were detected. Volumes of culture transformed in experiment 1: 0 min, 100 ml; 60 min, 50 ml; 120, 180, and 240 min, 25 ml; 300 min, 20 ml; 340 min, 10 ml.

5 min, titers were determined on KW20 lawns, and titered stocks were stored at 4°C. Recombination frequency is the ratio of the titer at 41°C to that at 33°C.

RESULTS

Spontaneous competence in *H. influenzae*. Most previous studies of competence development in *H. influenzae* have sought conditions that induced maximal competence. However, to select mutants with high spontaneous competence from a background of wild-type cells, first we had to identify conditions that minimized spontaneous competence. Figure 1 shows growth and competence development by the standard *H. influenzae* Rd strain KW20 and by the *sxy-1* mutant described in the sections below. In well-aerated sBHI at low cell densities, KW20 grows with a doubling time of 26 to 30 min (Fig. 1A, open symbols). Growth begins to slow when cell density reaches about 2×10^9 CFU/ml, and cultures reach a maximum density of about 10^{10} CFU/ml. Transformation (Fig. 1B, open symbols) is undetectable in early log phase (less than one transformant per 25 ml for cultures below 10^7 CFU/ml), is usually about 10^{-8} for cultures between 1×10^7 and 5×10^8 CFU/ml, and rises abruptly once cell density passes 5×10^8 CFU/ml. The cells become increasingly competent as growth slows, reaching a maximum transformation frequency of 1×10^{-4} to 5×10^{-4} when cell density reaches about 5×10^9. Competence falls slowly as cells enter stationary phase and is completely lost within about 90 min if cells are transferred to fresh medium at low cell density (not shown).

Selection of mutants with increased early-log-phase competence. By incubating an early-log-phase culture of EMS-mutagenized cells (2×10^8 CFU/ml) with a cloned DNA fragment encoding resistance to novobiocin, rare spontaneously competent cells were selected as novobiocin-resistant transformants. Individual transformed colonies were then tested for elevated early-log-phase competence by growth to early log phase, incubation with Kan^r chromosomal DNA, and plating on sBHI-kanamycin plates. Second-round Kan^r transformants were then tested for competence throughout their growth cycle, using DNA carrying a Str^r marker. Because *str*, *kan*, and *nov* are closely linked on the *H. influenzae* genome (10, 17, 23), many of the Str^r transformants were Nov^s Kan^s. We tested 20 Nov^r colonies and found two independent isolates showing greatly enhanced competence in early log phase; these are strains RR521 and RR528. The growth rates and spontaneous competence of these were similar, and only RR528 has been backcrossed and analyzed in detail.

Before further analysis, the putative mutation in RR528 was transferred into a nonmutagenized genetic background. Because cells with elevated competence cannot be directly selected on plates, this transfer used an indirect selection similar to that used for the original mutant isolation. KW20 was transformed with RR528 DNA, unselected cells were pooled, and cells competent in early log phase were selected from the pool by a second round of transformation (to Nov^r with RR518 DNA). Pools from cells originally transformed with RR528 DNA transformed 20 to 65 times more efficiently than did control pools from cells originally transformed with KW20 DNA. Seventeen of the Nov^r colonies derived from the original pooled transformants were tested for early-log-phase competence by transformation to Kan^r with RR520 DNA; all but two were highly competent. One of these was transformed to Str^r Nov^s Kan^s with DNA from RR514 and frozen as strain RR563. The backcross has been repeated by transforming KW20 with RR563 DNA, again giving pooled cells (whose early-log-phase transformation frequencies were 50 times that of the control cells) and clones as spontaneously competent as RR563. The success of the backcross suggests that the hypercompetence phenotype is caused by a single mutant gene. This mutation has been named *sxy-1* (mnemonic "sexy one"), because the mutant cells are always in the mood.

Phenotype of the *sxy-1* mutant. The solid symbols in Fig. 1A show that RR563 grew as rapidly as KW20 in sBHI, with doubling times of 26 to 30 min at low cell densities, and grew to similar final densities. The mutant cells were no more sensitive to UV light or to mitomycin than their parent (data not shown).

(i) Spontaneous competence. Figure 1B shows the transformation frequencies measured in the same experiments as Fig. 1A, and Fig. 2 summarizes the transformation frequencies seen with KW20 and RR563 in sBHI and after the various competence-inducing treatments discussed below.

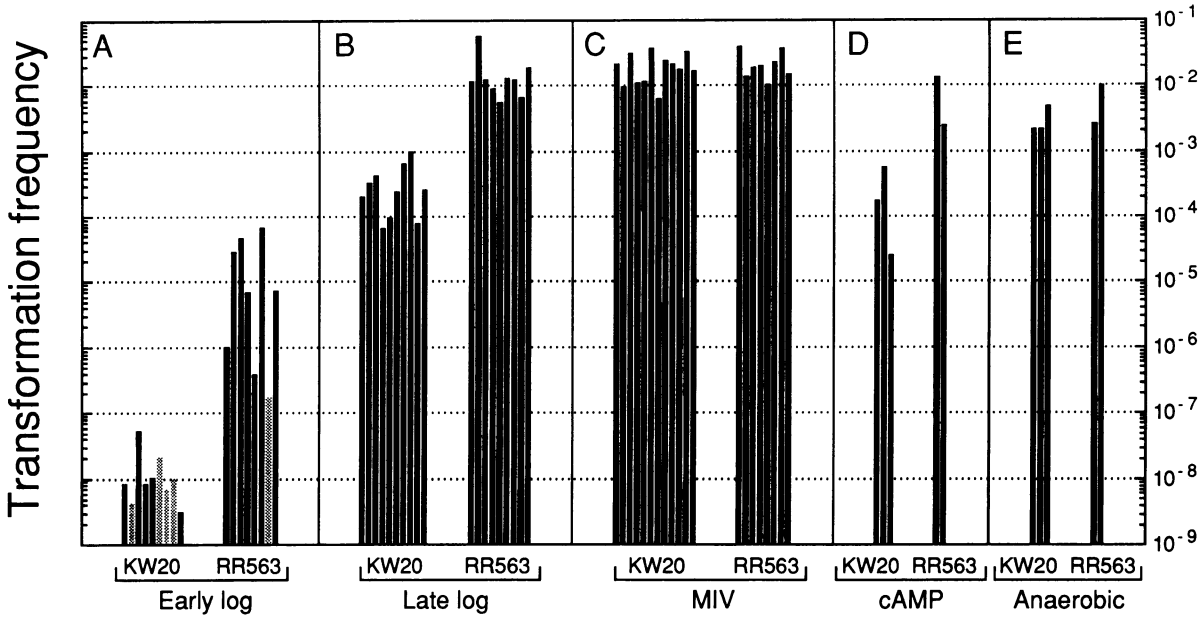

FIG. 2. Cumulated transformation frequencies. Cultures were treated as indicated and transformed to Nov^r or Kan^r with 1 μg of MAP7 DNA per ml. Within each panel, each bar is from a separate culture. (A) Minimum transformation frequencies in early log phase in sBHI. Grey bars are upper limit values, from experiments where no transformants were detected. (B) Maximum transformation frequencies in late log phase in sBHI. (C) Transformation frequencies after 100 min in MIV. (D) Transformation frequencies after 30-min incubation with 1.0 mM cAMP. (E) Transformation frequencies 30 min after return to aerobic culture.

At very low cell densities (5×10^4 to 2×10^7 CFU/ml) about 10^{-6} of *sxy-1* cells became transformed (solid symbols in Fig. 1B). RR563 transformation frequencies began to increase at cell densities of 5×10^7 CFU/ml and continued to increase with increasing cell density. Cultures in sBHI spontaneously reached transformation frequencies of 10^{-2} or higher, similar to the transformation frequencies of maximally competent (MIV-induced) wild-type cells (compare the right columns in Fig. 2B with the left columns in Fig. 2C). As the culture entered stationary phase, the transformation frequency of *sxy-1* cells decreased, as did that of wild-type cells.

(ii) Induction by MIV. In wild-type *H. influenzae* the highest levels of competence are induced by transferring rapidly growing cells from sBHI to a starvation medium (MIV) that contains salts and amino acids but lacks the carbon source, cofactors, and nucleotide precursors needed for growth (8). After 100 min in MIV medium, DNA synthesis and cell division have ceased and the transformation frequency for single chromosomal markers is 1×10^{-2} to 2×10^{-2} (Fig. 2C, left). On longer incubation in MIV, both competence and cell viability decline. Under standard MIV induction conditions (transfer to MIV at 10^9 CFU/ml), RR563 did not increase its transformation frequency above the spontaneous level of 1×10^{-2} to 2×10^{-2}. To find out whether *sxy-1* cells can respond to MIV, cells were transferred to MIV at 5×10^7 CFU/ml, while spontaneous competence was relatively low. Under these conditions both KW20 and RR563 responded to MIV, with induced transformation frequencies of 1×10^{-3} and 1×10^{-2}, respectively, whereas the uninduced controls (cells in sBHI) transformed at 3×10^{-8} (KW20) and 2×10^{-5} (RR563). Thus we conclude that *sxy-1* cells do become more competent after MIV treatment.

(iii) Induction by cAMP. Wise and coworkers showed that addition of 1 mM cAMP to the medium induces competence in early-log-phase cells (25). The transformation frequencies induced are similar to those seen spontaneously in late log growth (about 2×10^{-4}; compare left columns of Fig. 2B and D), and addition of cAMP to late-log-phase cells does not increase their competence above this level. The columns to the right of Fig. 2D show that the *sxy-1* mutant also became more competent in response to cAMP, with transformation frequencies increasing to near 10^{-2}. As with wild-type cells, the competence of RR563 induced with cAMP in early log phase resembled that of spontaneously competent cells in late log phase (compare right columns of Fig. 2B and D).

(iv) Induction by anaerobic-aerobic shift. Wild-type cells also become more competent if aeration is stopped for an hour and then restored (7). The standard protocol is to cease aeration at a cell density of 5×10^8. This gives transformation frequencies of 3×10^{-3} to 5×10^{-3}, 10- to 20-fold higher than those seen in continuously aerated cultures (Fig. 2E, left). When RR563 was treated similarly, its transformation frequency was about the same as that of KW20; however, this represented a slight reduction compared with its high spontaneous competence (Fig. 2E right). When the anaerobic-aerobic treatment was applied to cells in early log phase, competence increased in both wild-type and *sxy-1* cultures, to about 3×10^{-5} for KW20 and about 8×10^{-3} in RR563, showing that *sxy-1* cells do develop competence in response to temporary anaerobiosis.

Both DNA uptake and recombination are induced. Transformation requires both DNA uptake and recombination; both processes are greatly enhanced in competent cells. Uptake of radioactively labeled DNA is measured after removal of externally bound DNA by DNase I and washing, recombination by coinfecting cells with phage carrying two different temperature-sensitive mutations, and scoring production of wild-type recombinant phage (4). The sensitivities

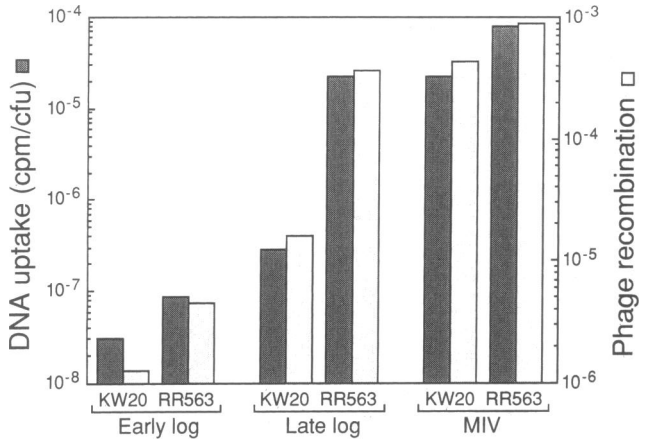

FIG. 3. DNA uptake and phage recombination by wild-type and *sxy-1* mutant cells. Cells in early and late log phases and after 100 min in MIV medium were incubated with ^{32}P-labeled MAP7 DNA to measure DNA uptake and with HP1 phages *ts2* and *ts3* to measure production of wild-type phage by recombination.

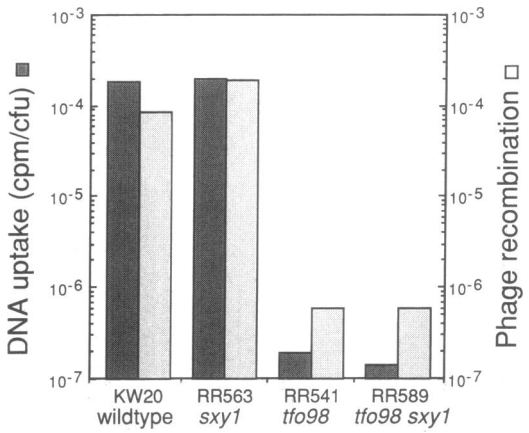

FIG. 4. DNA uptake and phage recombination by cells carrying *sxy-1* and *tfo-98* mutations. After a 100-min incubation in MIV medium, cells were incubated with ^{32}P-labeled MAP7 DNA to measure DNA uptake and with HP1 phages *ts2* and *ts3* to measure production of wild-type phage by recombination. The phage recombination values for strains RR541 and RR589 are upper limits; no recombinant plaques were detected.

of both of these assays are limited (by background counts per minute and phage revertants, respectively), and with wild-type cells, neither DNA uptake nor phage recombination is detectably above background in early log phase. However, both are increased to 100- to 500-fold above background by MIV treatment.

One possible explanation for the *sxy-1* phenotype of enhanced but still regulated competence might be that the mutation causes constitutive expression of one component of competence (either DNA uptake or recombination) but does not interfere with regulation of the other. For example, *sxy-1* cells might take up DNA under all conditions but recombine it efficiently only when competence was induced. To test this, phage recombination and uptake of labeled DNA were measured in RR563 in early and late log phases and after MIV induction. The results (Fig. 3) show that neither uptake nor recombination proficiency is constitutive in *sxy-1* cells. Instead both are increased at all growth stages but are still regulated; for each treatment, both DNA uptake and phage recombination were comparable to the levels seen in wild-type cells at the same level of competence.

Interactions with other mutations affecting competence. A major goal of this research is to determine how the genes regulating competence interact. Accordingly, *sxy-1* was tested with each of three mutations that block competence or transformation (*rec-2* [13], *crp* [6] [previously called *tfo-87*], and *tfo-98* [22]). Each of these mutations is caused by an insertion of mini-Tn*10kan* (24). Double mutants were isolated by transforming RR563 to Kan^r with limiting (100 pg/ml) mutant DNA. To confirm that the Kan^r transformants of RR563 had not lost the *sxy-1* mutation, DNA was prepared from two Kan^r strains of each double-mutant genotype and assayed for the presence of *sxy-1* as described in Materials and Methods. The cells transformed with the putative double-mutant DNAs gave transformation frequencies 15- to 23-fold higher than the cells transformed with KW20 DNA, confirming that the mutant DNAs carried the *sxy-1* mutation.

Each confirmed double mutant was then tested for MIV-inducible competence. The transformation frequencies given in Table 1 show that the presence of *sxy-1* did not increase the ability of cells carrying either *crp* or *rec-2* mutations to be transformed. The *rec-2* mutation does not interfere with DNA uptake (13), and in late-log-phase growth the *rec-2 sxy-1* double mutants showed the same elevated DNA uptake as their *sxy-1* parent, although they did not transform or carry out phage recombination (data not shown).

The *sxy-1 tfo-98* double mutants showed partial restoration of competence (Table 1), with MIV-induced transformation frequencies of 5×10^{-5} and 7×10^{-5}, more than 100-fold higher than their *tfo-98* parent but still more than 100-fold lower than the wild type. This increase was not due to a large increase in either DNA uptake or phage recombination; both were still not significantly above background in MIV-treated cultures (Fig. 4). Transformation frequencies in late log phase in sBHI were also increased 30- to 40-fold over those of their *tfo-98* parent. Cells carrying *tfo-98* grow slowly (doubling time, ~45 min) and are moderately sensitive to UV irradiation (22), and the presence of the *sxy-1* mutation does not alleviate either of these defects (not shown). However these assays, like DNA uptake measurements, may lack the sensitivity needed to detect small increases.

DISCUSSION

Mutations causing enhanced competence (Sxy mutations) have not previously been reported in *Haemophilus* spp. However, the potential for Sxy mutations should exist if repressors regulate competence or if nonlethal mutations can cause competence-inducing intracellular signals to be generated. Because Sxy mutants can be selected by the transformation they permit, they should be much easier to isolate than nontransformable (Tfo$^-$) mutants, which can be identified only by laborious screening. The actual power of the selection for Sxy mutants will depend on their viability and level of competence and on the efficiency of transformation by the DNA used for selection. In these experiments this was maximized by using a cloned *nov* gene rather than chromosomal DNA. Use of a pure fragment also eliminated the possibility that a *sxy* mutation might be lost by recombination with unselected DNA that had been taken up along with the selectable marker.

Lacks and Greenberg (9) isolated a *Streptococcus pneu-*

moniae mutant (*trt*) with a Sxy phenotype. The selection strategy was similar to that described above; nitrosoguanidine mutagenesis followed by two rounds of transformation to select cells that had become competent in the presence of trypsin (which prevents accumulation of competence factor, the natural inducer). The *trt* mutant is highly competent at all cell densities. At low densities and in the presence of trypsin or other proteases, *trt* cultures give 1,000-fold more transformants than wild-type cultures. Unfortunately the *trt* mutation has not been studied in a wild-type genetic background, and its molecular basis is not known.

What do our experiments tell us about *sxy-1*? Several simple explanations can probably be eliminated. The mutation is unlikely to be interfering with essential metabolic pathways, because cells carrying it have a normal growth rate. Pathways of DNA repair are probably also intact, because *sxy-1* cells are not sensitive to DNA-damaging agents. The high competence of *sxy-1* cells cannot be due to constant saturating levels of intracellular cAMP, because exogenous cAMP increases transformation to the frequency seen spontaneously in late log phase, similar to its effect on wild-type cells. Response is also normal to the other previously characterized inducers (high cell density, temporary anaerobiosis, transfer to starvation medium). The increased transformation frequencies of *sxy-1* cultures are not due to constitutively induced DNA uptake or recombination. Both are higher in *sxy-1* cultures than in wild-type cultures, but both remain proportional to the corresponding transformation frequencies.

All of this suggests that the *sxy-1* mutation acts early in competence development. Because *sxy-1* increases transformation, whereas all previously described transformation mutants decrease or eliminate it, we were able to confirm its early action by constructing and characterizing double-mutant strains. When cells carrying the *rec-2* insertion from pDM79 are transferred to MIV medium, they become able to take up labeled DNA but not to translocate the DNA into the cytoplasm (13). This suggests that the *rec-2* defect is in the mechanics of transformation rather than in the regulation of competence. However, *rec-2* cells are also defective in competence-associated phage recombination (3) (unpublished results), so their defect may be more fundamental. *sxy-1 rec-2* cells showed the elevated DNA uptake characteristic of *sxy-1* cells but were unable to transform or carry out phage recombination, so the *sxy-1* mutation probably acts before *rec-2*.

The nature of the defect in the *tfo-98* mutant is unknown. Cells grow slowly and are moderately sensitive to UV light, suggesting that the gene product is needed for processes other than competence and is probably involved in DNA metabolism (22). Introduction of *sxy-1* partially restored MIV-inducible competence to *tfo-98* mutants but did not increase their growth rate or UV resistance, indicating that the *sxy-1* mutation does not correct the primary *tfo-98* defect. The development of competence showed the same dependence on cell density and MIV as does competence development in wild-type cells.

The gene identified by the *tfo-87* insertion has been found to encode a protein highly homologous to CRP, the *Escherichia coli* catabolite regulator protein (6), and accordingly has been named *crp*. *H. influenzae* cells carrying the *tfo-87* insertion do not transform under any conditions and do not take up DNA or carry out phage recombination after incubation in MIV (22) (unpublished data). This is consistent with the ability of cAMP to induce competence in normal cells and suggests that binding of cAMP plus CRP to one or more promoters is essential for the induction of competence. This binding may not, however, be sufficient for full competence induction, since exogenous cAMP gives transformation frequencies 100-fold lower than those induced by MIV medium, even at cAMP concentrations that derepress synthesis of other cAMP-regulated enzymes (27).

Taken together, these observations support the hypothesis put forward by Zoon and coworkers (27) that multiple regulatory events are required for induction of competence. One of the events regulating competence would be controlled by CRP plus cAMP, and the genes identified by the *sxy-1* and *tfo-98* mutations may be involved in generating the second. This model is also consistent with the action of CRP in other systems, where it usually is only one component of complex regulatory circuits (11).

In an alternative model, cAMP concentration could be the primary determinant of competence under all conditions, with MIV-induced transformation frequencies being due to intracellular concentrations of cAMP sufficient to saturate CRP, whereas the lower transformation frequencies seen in late log phase would result from lower cAMP concentrations causing only partial competence induction. Because exogenous cAMP raises transformation frequencies to only the late-log-phase level, we would have to assume that exogenous cAMP enters cells poorly and can raise intracellular concentrations to only a partially inducing level.

Under this model, we might postulate that the *sxy-1* mutation acts by causing cAMP concentrations to be higher at all stages of growth (i.e., partially inducing in early log phase and fully inducing in late log phase). This could be caused by a decrease in phosphodiesterase activity or by an increase of adenylate cyclase activity. If the latter is correct, to accommodate the observation that addition of cAMP to *sxy-1* cells fully induces competence, we would also have to assume that *sxy-1* enhances uptake of extracellular cAMP. Another possibility consistent with this second model would be that the *sxy-1* mutation increases sensitivity to normal concentrations of cAMP. We know *sxy-1* is not a mutation in or closely linked to *crp*, because *sxy-1* cells retained the *sxy-1* mutation after transformation with chromosomal DNA carrying the *crp*::mini-Tn*10* insertion. However, *sxy-1* might be an alteration in a cAMP-regulated competence-specific promoter.

Our immediate priority is to map and clone the *sxy-1* gene, so that it can be sequenced and checked for homology to known genes in other bacteria. Cloning will also allow construction and analysis of *sxy-1* null mutants and reveal whether *sxy-1* is dominant or recessive to the wild-type gene. It will also facilitate construction of double mutants and testing for allelism with other Sxy mutations we have isolated.

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