Journal of Bacteriology

Seventeen Sxy-Dependent Cyclic AMP Receptor Protein Site-Regulated Genes Are Needed for Natural Transformation in Haemophilus influenzae

Sunita Sinha, Joshua C. Mell and Rosemary J. Redfield
J. Bacteriol. **2012, 194(19):5245. DOI: 10.1128/JB.00671-12.**
Published Ahead of Print 20 July 2012.

JB
Journals.ASM.org

Seventeen Sxy-Dependent Cyclic AMP Receptor Protein Site-Regulated Genes Are Needed for Natural Transformation in *Haemophilus influenzae*

Sunita Sinha, Joshua C. Mell, and Rosemary J. Redfield

Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada

Natural competence is the ability of bacteria to actively take up extracellular DNA. This DNA can recombine with the host chromosome, transforming the host cell and altering its genotype. In *Haemophilus influenzae*, natural competence is induced by energy starvation and the depletion of nucleotide pools. This induces a 26-gene competence regulon (Sxy-dependent cyclic AMP receptor protein [CRP-S] regulon) whose expression is controlled by two regulators, CRP and Sxy. The role of most of the CRP-S genes in DNA uptake and transformation is not known. We have therefore created in-frame deletions of each CRP-S gene and studied their competence phenotypes. All but one gene (*ssb*) could be deleted. Although none of the remaining CRP-S genes were required for growth in rich medium or survival under starvation conditions, DNA uptake and transformation were abolished or reduced in most of the mutants. Seventeen genes were absolutely required for transformation, with 14 of these genes being specifically required for the assembly and function of the type IV pilus DNA uptake machinery. Only five genes were dispensable for both competence and transformation. This is the first competence regulon for which all genes have been mutationally characterized.

Many bacteria are naturally competent, able to bind and take up DNA from their extracellular environment (35, 55, 67). Most of this DNA is degraded inside the cell, and the nucleotides are reused, but if sequence homology permits, incoming DNA can recombine with the chromosome. When this recombination changes the genotype, the cell is said to be transformed. Natural transformation is a major mechanism of genetic exchange: it shapes bacterial genomes and allows pathogens to evade the host immune response by spreading antibiotic resistance genes, virulence determinants, and capsular serotype genes (26, 31, 36, 53, 54, 88).

In almost all Gram-negative bacteria, DNA uptake across the outer membrane requires either type IV pili (Tfp), long cell surface fibers made up of polymerized pilin subunits, or related pseudopili that do not protrude beyond the cell surface (22). Tfp are also important for interactions with host cells during colonization, biofilm formation, and intracellular invasion (76). The ability of Tfp to retract is thought to provide the force that pulls DNA into the periplasm through an oligomeric outer membrane secretin pore (15). Evidence from *Bacillus subtilis* also implicates Tfp-related pseudopili in the initial steps of DNA uptake by Gram-positive bacteria (35). All competent bacteria then transport DNA across the inner membrane into the cytoplasm through a separate channel. Once inside the cytoplasm, any homologous DNA that escapes degradation by exonucleases can integrate into the host chromosome using the cell's RecA-dependent recombination pathway (67).

Although the big picture presented above is clear, our knowledge of the proteins responsible for DNA uptake and transformation is piecemeal (1, 3–5, 8–11, 14, 19, 23, 32–34, 43, 45, 48, 51, 56, 57, 59, 69, 72, 80, 84, 97, 98, 100, 101, 106, 110, 112), and the complete set is not known for any bacterium. Early studies of mutants with transformation defects led to the identification of competence regulons in some species, but only some of the new candidate genes that these regulons contain have been examined, and not all of these genes contribute to DNA uptake and transformation (12, 78, 85). The problem is worst for the Gram-positive model species *B. subtilis* and *Streptococcus pneumoniae*, as their competence regulons are very large (165 and 124 genes, respectively) and contain many genes with no obvious connection to competence (12, 78), and for *Neisseria*, where competence is not regulated at all (103).

The human pathogen *Haemophilus influenzae* may be the best system with which to address this problem, since its competence is controlled by a small and well-defined regulon (85). *H. influenzae* competence genes are induced by nutrient starvation, when a set of 13 operons (26 genes) is strongly upregulated (44, 61, 62, 67). These operons are united by a common promoter motif (Sxy-dependent cyclic AMP receptor protein [CRP-S] site) induced by two transcriptional regulators, CRP and the competence-specific activator Sxy (16, 17, 85). For some of these genes, transposon insertions were previously characterized, demonstrating roles in natural competence (10, 20, 32, 42, 48, 100, 101, 107, 112). However, most of the *H. influenzae* CRP-S genes are part of operons, so their mutant phenotypes may be due to polar effects on downstream genes. Clean deletion mutations in genes of two of these operons were recently shown to prevent transformation (20), but their effects on DNA uptake were not measured. We have now used recombineering to create nonpolar in-frame deletions of all of the CRP-S genes and have determined their effects on growth, DNA uptake, and transformation phenotypes.

Received 24 April 2012 **Accepted** 17 July 2012
Published ahead of print 20 July 2012
Address correspondence to Sunita Sinha, sinha@zoology.ubc.ca.

doi:10.1128/JB.00671-12

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* cells were cultured at 30°C or 37°C in Luria-Bertani (LB) medium, with ampicillin (1,000 μg/ml) and spectinomycin (50 μg/ml) when required. Routine cloning was done using strain DH5α [F80*lacZ* Δ(*lacIZYA-argF*) *endA1*], and recombineering was done using strain SW102 {F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7649 rpsL nupG* [λcI857 (*cro-bioA*) <> *tet*] Δ*galK*} (108).

H. influenzae cells were cultured at 37°C in brain heart infusion (BHI) medium supplemented with NAD (2 mg/ml) and hemin (10 mg/ml) (sBHI medium). When required, the following antibiotics were used: novobiocin at 2.5 μg/ml, spectinomycin at 20 μg/ml, streptomycin at 100 μg/ml, or kanamycin at 7 μg/ml. All mutants were made in an Str^r version of standard laboratory strain Rd KW20, created by transformation with MAP7 chromosomal DNA (see below) and selection on streptomycin.

Creation of marked *H. influenzae* chromosomal mutations. The sequences of all primers used are available upon request. For each CRP-S gene to be mutated, a 2- to 5-kb region including the target gene was PCR amplified, with flanking homology of 400 to 1,500 bp on either side of the gene of interest. The purified PCR product was then TA cloned into pGEMT-Easy (Promega) or pCR2.1-TOPO (Invitrogen). *E. coli* DH5α cells were made chemically competent with rubidium chloride following protocol 2 of the QIAexpressionist manual (Qiagen) and transformed with plasmid ligations by heat shock. Positive clones were confirmed by PCR and restriction digestion.

A Spec^r cassette was amplified from pRSM2832 by using gene-specific mutagenic primers as described previously by Tracy et al. (104). This cassette contains both a gene encoding spectinomycin resistance and a wild-type *rpsL* allele designed to confer streptomycin sensitivity to a Str^r strain (although it did not do so in standard *H. influenzae* laboratory strain Rd KW20 [see Results]). For each CRP-S gene, primers were designed to delete sequences between the start codon and the last 7 codons.

To prepare *E. coli* cells for recombineering, a culture of strain SW102 grown overnight was diluted and grown at 30°C. The expression of the lambda Red recombinase was induced by heat shock at an optical density at 600 nm (OD_{600}) of 0.6 for 15 min at 42°C, after which the cells were cooled on ice for 5 min. The cells were then pelleted, washed three times with ice-cold distilled water (dH_2O), and resuspended in ice-cold dH_2O to a 100× concentration. The plasmid containing the CRP-S insert (Amp^r) (50 ng) and the gene-specific mutagenic cassette (Spec^r) (200 ng) were coelectroporated into 40 μl of cells using the following settings: 2.5 kV, 200 Ω, and 25 μF. Cells were then allowed to recover in 1 ml LB medium for 1 h at 30°C before plating onto medium containing ampicillin and spectinomycin. After overnight growth at 30°C, mutagenized clones were confirmed by PCR and restriction digestion.

CRP-S–Spec^r constructs were either released from the mutated plasmid by restriction digestion or PCR amplified by using gene-specific primers. The DNA was then used to transform MIV-competent *H. influenzae* cells as described previously (79). Mutant genotypes were confirmed by PCR. The transformation defects of the *HI0659*, *comN*, *comO*, and *comP* mutants were confirmed by backcrosses in which wild-type cells that had been transformed to Spec^r by DNA from mutant cells were tested for a loss of transformability.

Conversion to unmarked nonpolar mutations. Electrocompetent cells of each marked *H. influenzae* mutant were prepared and transformed with FLP-encoding plasmid pRSM2947, as described previously by Tracy et al. (104). Cells containing pRSM2947 were then grown in sBHI medium containing kanamycin, and FLP expression was induced at an OD_{600} of 0.3 with 200 ng/ml anhydrotetracycline for 2 h at 30°C. Cells were then diluted, plated onto plain sBHI medium, and grown overnight at 37°C. The next day, colonies were patched onto plates with plain sBHI medium and sBHI medium containing spectinomycin. Clones that had lost Spec^r were saved, and their genotypes were confirmed by PCR and sequencing. The loss of pRSM2947 was confirmed by sensitivity to kanamycin.

Growth analysis. Growth was measured by using a BioScreen C instrument (BioScreen Instruments Pvt. Ltd.). A small single colony of each strain was dispersed into 10 ml of sBHI medium, and 300 μl was placed into 8 to 12 replicate wells of a BioScreen 100-well plate. Wells along the edges of each plate contained sBHI medium alone, as these wells were previously found to have anomalously slow growth, likely due to a lower temperature at the edges. Plates were incubated in the BioScreen plate at 37°C with agitation, and the OD_{600} was measured every 10 min for 12 to 16 h. The growth curve of each well was baseline corrected to its minimum value (these values were always within the range of values of control wells), and the average OD_{600} of the replicates at each time point was calculated.

Transformation assays. *H. influenzae* cells were made competent by the transfer of sBHI medium-grown cells into MIV medium at an OD_{600} of 0.2 to 0.25 and incubation for 100 min at 37°C, as described previously (79). These cells were incubated with 1 μg of MAP7 chromosomal DNA per 1 ml of culture for 15 min at 37°C, after which the free DNA was degraded by incubation with DNase I (10 μg/ml) for 5 min. Cells were then diluted and plated onto sBHI agar with and without novobiocin (79). Transformation frequencies were calculated as the number of novobiocin-resistant (Nov^r) transformants per cell.

DNA uptake assays. A 222-bp PCR fragment containing a single uptake signal sequence was used as the donor DNA (66). This was radiolabeled by primer extension with [^{33}P]dATP as described elsewhere previously (66). MIV-competent *H. influenzae* cells were incubated with 50 ng of labeled USS1 DNA per 1 ml of culture for 25 min at 37°C, after which the free DNA was degraded by incubation with DNase I (10 μg/ml) for 5 min. Cells were then pelleted and washed twice with cold MIV medium and finally resuspended in MIV medium for scintillation counting. The percentage of uptake was calculated as the ratio of pellet-associated radioactive counts to total input counts. The amount of uptake (in ng DNA/ml cells) was then calculated by dividing the total ng of input DNA per ml of cells by the percentage of uptake.

RESULTS

Construction of the mutant collection. CRP-S genes and operons of *H. influenzae* are diagrammed in Fig. 3B. Details about their homologs in other species and their predicted functions and cellular localizations are given in Table 1. To test the contribution of each gene to competence, we used a method described previously by Tracy et al. (104) to create Spec^r-marked insertion-deletion mutations in 25 of the 26 *H. influenzae* CRP-S genes (as described below, a knockout of *ssb* was not viable). We also created mutations in *comI* and *comJ*, which are adjacent to *comABCDEF* but not regulated by CRP-S (see Fig. 4), because transposon insertions in *comI* were found previously to reduce competence, and *comJ* had never been investigated (100, 101). To eliminate potentially confounding effects of polarity on downstream genes, we also created an in-frame unmarked deletion mutant from each Spec^r mutant. We chose as a background standard laboratory strain Rd KW20, whose competence is highly induced upon transfer to MIV starvation medium (79).

To create the mutants, the central codons in the coding sequence were deleted, leaving only the start and last 7 codons of each gene. In the marked mutants, the deleted residues were replaced with a cassette comprised of a spectinomycin resistance gene and the counterselectable *rpsL* gene of *Neisseria gonorrhoeae*. The unmarked mutants were created by the excision of this cassette, leaving behind a 27-codon cassette-derived scar sequence, which kept the last 7 codons in frame, avoiding polar effects on downstream genes (104).

An important feature of this multistep mutagenesis system failed to work as intended. It was developed for *H. influenzae* strain 2019, where the *rpsL* gene in the cassette acts as a counter-

TABLE 1 Genes and proteins of the CRP-S regulon of *H. influenzae*[a]

Gene	Locus tag	Reported *H. influenzae* mutant phenotype (reference[s])	*H. influenzae* protein sequence prediction[c]	Homolog(s)	Function(s) of homolog(s) (reference[s])
pilA	HI0299	Uptake$^-$, TF$^-$ (32, 104)	16 kDa, pI 9.3; prepilin peptidase signal; periplasmic and extracellular locations	*pilE* (*Neisseria*), *pilA* (*Pseudomonas*)	Major pilin subunit (2, 58, 73, 77)
pilB	HI0298	Uptake$^-$, TF$^-$ (32)	53 kDa, pI 5.7; no signal detected; cytoplasmic location	*pilB* (*Neisseria*, *Pseudomonas*)	ATP-dependent extension of pilus subunits (39, 74)
pilC	HI0297	Uptake$^-$, TF$^-$ (32)	47 kDa, pI 9.4; no signal detected; IM location, 3 transmembrane helices	*pilC* (*Neisseria*, *Pseudomonas*)	Pilus assembly (39, 74, 102)
pilD	HI0296	None available	27 kDa, pI 9.3; no signal detected; IM location, 7 transmembrane helices	*pilD* (*Neisseria*, *Pseudomonas*)	Prepilin peptidase (25, 39, 74, 75)
comA	HI0439	Uptake$^-$, TF$^-$ (101)	32 kDa, pI 5.7; no signal detected; IM location	*pilM* (*Neisseria*, *Pseudomonas*)	Pilus assembly (7, 18, 65)
comB	HI0438	None available	20 kDa, pI 8.3; noncleavable signal peptide; location unknown	*pilN* (*Neisseria*, *Pseudomonas*)	Pilus assembly (7, 18, 65, 86, 98)
comC	HI0437	Uptake$^-$, TF$^-$ (101)	20 kDa, pI 9.3; signal peptidase I signal; location unknown	*pilO* (*Neisseria*, *Pseudomonas*)	Pilus assembly (7, 18, 65, 86, 98)
comD	HI0436	None available	16 kDa, pI 4.6; signal peptidase II signal; location unknown	*pilP* (*Neisseria*, *Pseudomonas*)	Pilus assembly, IM lipoprotein; interacts with secretin (7, 8, 18, 65, 98)
comE	HI0435	Uptake$^-$, TF$^-$ (32, 101)	49 kDa, pI 7.4; signal peptidase I signal; OM location	*pilQ* (*Neisseria*, *Pseudomonas*)	OM secretin, binds T4P and DNA (5, 28, 65)
comF	HI0434	WT uptake, TF$^-$ (100, 101)	27 kDa, pI 10.3; no signal detected; cytoplasmic location	*comF* (*Neisseria*, *Pseudomonas*)	Function unknown
comN	HI0938	None available	20 kDa, pI 9.6; prepilin peptidase signal; location unknown	No homolog found	Function unknown
comO	HI0939	TF$^-$ (107)	27 kDa, pI 9.6; signal peptidase I signal; location unknown	No homolog found	Function unknown
comP	HI0940	None available	25 kDa, pI 9; signal peptidase I signal; location unknown	No homolog found	Function unknown
comQ	HI0941	None available	12 kDa, pI 9.9; signal peptidase I signal; location unknown	No homolog found	Function unknown
comE1	HI008	None available	12 kDa, pI 8.3; signal peptidase I signal; location unknown	*comE* (*Neisseria*, *Pseudomonas*), *comEA* (*Bacillus*)	DNA binding protein (23, 45, 72, 82)
HI0365	HI0365	WT uptake and TF (107)	44 kDa, pI 6.7; no signal detected; cytoplasmic location; rRNA large subunit methyltransferase N; Fe-S cluster redox enzyme	Hypothetical protein (*Neisseria*, *Pseudomonas*)	Function unknown
pilF2	HI0366	Uptake$^-$, TF$^-$ (107)	21 kDa, pI 8.9; signal peptidase I signal; location unknown; tetratricopeptide repeats	*pilW* (*Neisseria*), *pilF* (*Pseudomonas*)	Pilotin, stabilizes secretin multimers (3, 19, 50, 51, 95, 105)
rec2	HI0061	WT uptake, TF$^-$ (68, 90)	90 kDa, pI 9.7; no signal detected; IM location, 13 transmembrane helices	*comA* (*Neisseria*), *rec2* (*Pseudomonas*), *comEC* (*Bacillus*)	Putative DNA membrane channel (34)
ligA	HI1182, HI1183[b]	WT uptake, TF down (107)	31 kDa, pI 9.2; signal peptidase I signal; location unknown	*adl* (*Neisseria*)	Periplasmic ATP-dependent DNA ligase (63)
HI0659	HI0659	None available	11 kDa, pI 9.2; no signal detected; cytoplasmic location; HTH motif	No homolog found	Function unknown
HI0660	HI0660	None available	14 kDa, pI 9.46; no signal detected; cytoplasmic location; putative Holliday junction resolvase	No homolog found	Function unknown
HI1631	HI1631	None available	22 kDa, pI 9.5; no signal detected; location unknown; restriction endonuclease-like superfamily; putative Holliday junction resolvase	No homolog found	Function unknown
comM	HI1117	WT uptake, TF down (42)	56 kDa, pI 9.2; no signal detected; cytoplasmic location; putative Mg chelatase subunit	*comM* (*Neisseria*, *Pseudomonas*)	Function unknown
dprA	HI0985	TF$^-$ (48, 100)	42 kDa, pI 6.3; no signal detected; cytoplasmic location	*dprA* (*Neisseria*), *smf* (*Pseudomonas*)	Protects DNA from degradation in cytoplasm (4)
radC	HI0952	None available	25 kDa, pI 6.2; no signal detected; cytoplasmic location	*radC* (*Pseudomonas*)	Replication fork stabilization and repair (6, 87)
ssb	HI0250	None available	19 kDa, pI 5.3; no signal detected; cytoplasmic location	*ssb* (*Neisseria*, *Pseudomonas*)	Ubiquitous single-stranded-DNA binding protein (83)

[a] TF, transformation frequency; WT, wild type; IM, inner membrane; OM, outer membrane; T4P, type IV pilus.
[b] Incorrectly annotated as two open reading frames due to a translational frameshift.
[c] Predictions run with LipoP, PsortB, TargetP, SignalP, Pilfind, and InterProScan.

selectable marker, converting a streptomycin-resistant strain to streptomycin sensitivity and thus allowing mutants that excise the cassette to be selected by their restored streptomycin resistance. Although this counterselection step also works with another *H. influenzae* strain, 86-028NP (20), we and Tracy et al. (104) found that it did not work with streptomycin-resistant derivatives of strain Rd, where clones that had integrated the Spec^r cassette remained Str^r. For each marked mutant, clones that had excised the cassette were instead identified by manual screening for the loss of the resistance marker after the induction of the FLP recombinase. We observed extensive experiment-to-experiment variation in the frequency of marker excision (0 to 20%); the reason for this is not understood.

Of the 28 genes which we tested, only *ssb* could not be deleted: repeated attempts at transforming *H. influenzae* with an *ssb*::Spec deletion construct failed to give any transformants. This is consis-

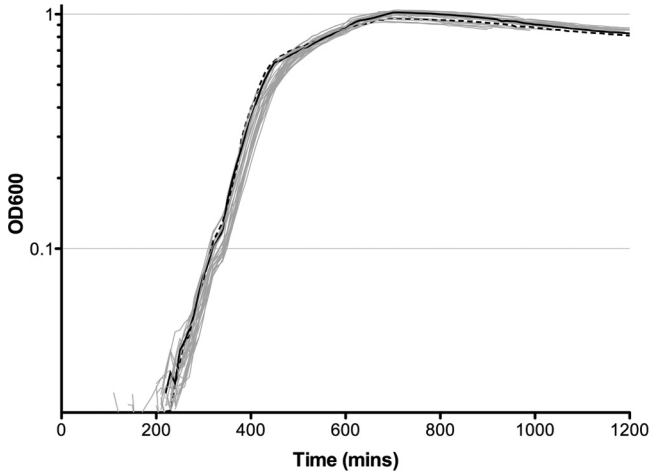

FIG 1 Growth of unmarked *H. influenzae* CRP-S mutants in rich medium. Cells were grown in sBHI broth in a BioScreen incubator with the OD_{600} recorded every 10 min. Each line shows the mean of 8 to 12 replicates for one strain. Optical densities lower than 0.02 are not shown. To account for differences in inoculum sizes, the lines have been shifted along the *x* axis to superimpose the growth curves of all mutants. Positive and negative controls are shown as black lines (full lines, wild type; dashed lines, *sxy* mutant).

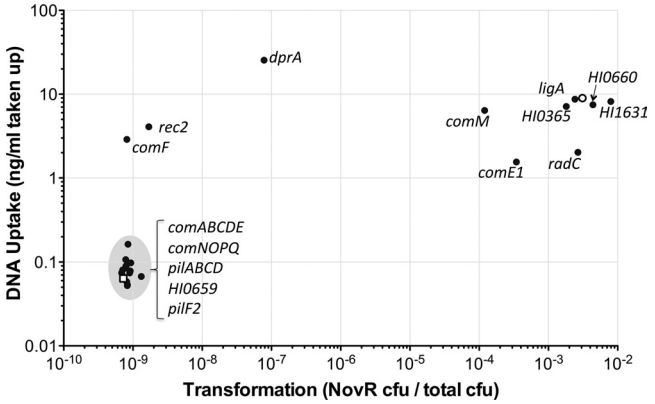

FIG 2 Competence phenotypes of unmarked *H. influenzae* CRP-S mutants. Each point represents the mean of at least three biological replicates. Mean values for positive (wild-type) and negative (*sxy* mutant) controls are shown as an open circle and an open square, respectively. Mutants in which competence is abolished are shaded.

tent with previous studies of other organisms, which relied on point mutations or partial deletions because the deletion of *ssb* is lethal (40, 41, 64, 70, 71, 81, 84, 91, 109).

Although previously described *H. influenzae* competence gene knockouts have not been reported to impair growth and viability, the presence of *ssb* in the CRP-S regulon emphasizes the possibility that other genes in the regulon could contribute to growth or other housekeeping functions. The level of expression of CRP-S genes is relatively low during exponential growth, but all genes are partially induced as cells approach the stationary phase (85). To test for contributions to growth, we used a BioScreen incubator to compare the growths of all mutants in rich broth (sBHI medium). Figure 1 shows that all mutants had growth curves indistinguishable from those of their wild-type parent and an *sxy* knockout mutant, whose CRP-S regulon is not inducible (112). We also found no significant differences in cell viability after 2 h of incubation in MIV starvation medium, which was used to fully induce competence (data not shown).

Competence phenotypes of the mutants. The competence phenotypes of all mutants were assessed by DNA uptake and transformation assays. Uptake assays use short radiolabeled DNA fragments with an *H. influenzae* uptake sequence but no other chromosomal homology and show the extent of DNA uptake into the periplasm and cytoplasm by measuring cell-associated radioactivity after DNase I treatment to degrade any DNA not taken up. Transformation assays use chromosomal DNA carrying a point mutation conferring antibiotic resistance and show the combined effects of DNA uptake and recombination by measuring the fraction of transformed cells in a culture (transformation frequency [TF]). Provided that postuptake steps are intact, transformation assays provide a much more sensitive measure of DNA uptake (detection limit of $\sim 10^{-6}$ wild-type CFU, rather than $\sim 10^{-2}$ wild-type CFU in DNA uptake assays). Figure 2 summarizes the transformation frequencies of all unmarked mutants as a function of their DNA uptake levels. Individual uptake and transformation phenotypes are shown in Fig. 3A, C, and D and are discussed in detail below. The transformation phenotypes of the unmarked mutants that we created (Fig. 3C) were identical to those of the marked mutants (Fig. 3D) and to those of previously characterized transposon mutants.

Mutants in which natural competence is abolished. As shown in Fig. 2 and 3, mutations in 15 CRP-S genes eliminated detectable DNA uptake and transformation. Mutations in some of these genes (*pilA*, *pilB*, *pilC*, *comA*, *comC*, *comE*, *comO*, and *pilF2*) were previously described (32, 100, 101, 104, 107). Our results agree with previously reported phenotypes, and the new results that we provide show that all genes in the *comABCDE*, *comNOPQ*, and *pilABCD* operons play essential roles in competence. Unmarked deletions of the *comABCDEF* and *pilABCD* genes were also recently reported to abolish transformation in a different strain (86-028NP) (20, 104). Because an independent corroboration of the transformation defects was lacking for knockouts of *HI0659*, *comN*, *comO*, and *comP*, backcrosses were created to rule out effects of unlinked mutation. All four backcrossed strains had undetectable levels of transformation, confirming that their phenotypes resulted from the mutations that we created.

In bacteria with long type IV pili, homologs of *pilABCD* and *comABCDE* are required for Tfp biogenesis and function (3, 7, 15, 27, 30, 33–35, 38, 60, 73, 76, 106, 111). In addition, mutations of these genes in strain 86-028NP were shown previously to have defects in epithelial cell adherence and biofilm formation, phenotypes which require functional Tfp (20). The sequence of *H. influenzae* PilF2 contains the canonical lipobox found in pilotin proteins (with a lipidation site at the Cys20 residue) and the TPR residues typical of protein-protein interactions (Table 1). These features and its homology to the PilF/PilW pilotins of *Pseudomonas aeruginosa* and *Neisseria meningitidis* suggest that it likely promotes the export and assembly of the secretin complex of the type IV pilus machinery (3, 19, 95, 105).

The finding that the deletion of HI0659 eliminated DNA uptake and transformation was unexpected, since it lacks homology to known Tfp proteins (unlike all other proteins required for DNA uptake), and its N-terminal signals predict a cytoplasmic location

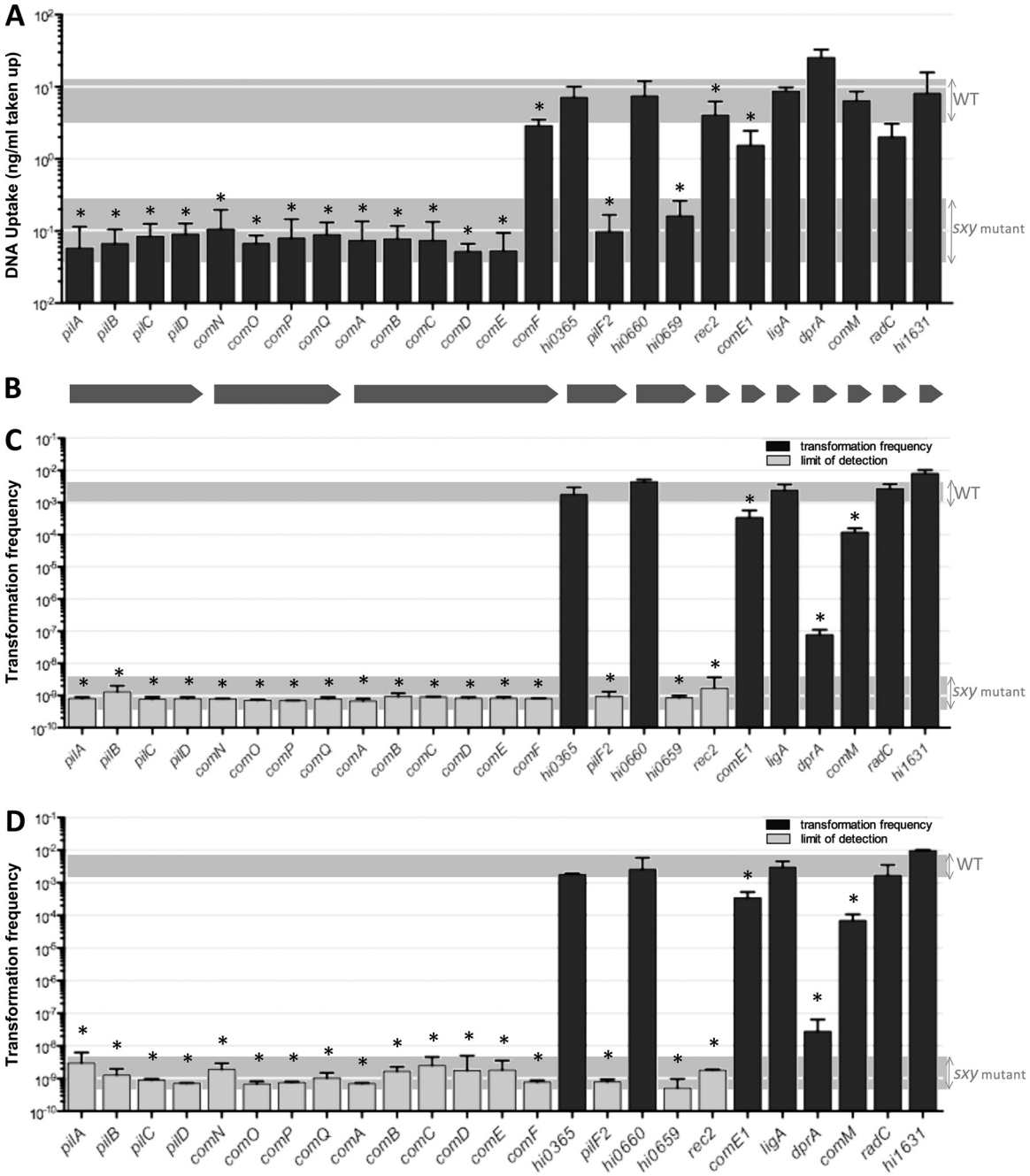

FIG 3 DNA uptake and transformation by *H. influenzae* CRP-S mutants. (A) DNA uptake by unmarked mutants. (B) Operon structure of the CRP-S genes of *H. influenzae*. (C) Transformation frequencies of unmarked mutants. (D) Transformation frequencies of marked mutants. In panels A, C, and D, vertical bars represent the means of at least three biological replicates ± standard deviations. The range of wild-type and *sxy* mutant control values are shown as gray horizontal bars. Statistically significant differences from the wild type (P value of <0.05, determined by a two-tailed t test) are indicated with asterisks.

(Table 1). This small protein had never been investigated, and its function is unknown, although its sequence contains a helix-turn-helix (HTH) motif, typical of DNA binding proteins. This suggests that HI0659 either acts directly in DNA uptake by binding transforming DNA or acts indirectly as a transcriptional regulator of one or more competence genes (although the HI0659 sequence lacks other features characteristic of transcriptional regulators). We are currently analyzing this mutant in transcriptional assays of CRP-S genes to help distinguish between these possibilities. Competence regulation is not likely to be disrupted in the other uptake-defective mutants, since their homologs in other species are known to act in DNA uptake.

Mutants that take up DNA but do not transform. Two mutations, *rec2* and *comF*, were not transformable despite taking up near-normal amounts of DNA (Fig. 2 and 3), a phenotype consistent with transposon insertion mutations of both genes (10, 100,

101). Rec2 homologs are needed for the transport of a single DNA strand across the cytoplasmic membrane in all species where this has been examined (DNA remains trapped in the periplasm in the absence of *rec2* in *H. influenzae*) (68, 89), and its sequence predicts that it is located in the inner membrane (Table 1). The function of ComF is not known, and its homologs in other bacteria have not been studied. Carruthers et al. found previously that the 86-028NP *comF* mutant had the same adherence and biofilm defects as mutations in other genes of its operon, suggesting a role in Tfp-associated phenotypes (20). This is, however, difficult to reconcile with the dispensability of *comF* for DNA uptake. In addition, although *comF* homologs are found throughout the gammaproteobacteria, *H. influenzae* is the only species in which *comF* is cotranscribed with known Tfp genes. ComF is predicted to be cytoplasmic, with no transmembrane helices found (Table 1), so it might form the inner part of the DNA translocation machinery. Its apparent role in Tfp functions suggests that uptake and translocation may be coupled. Both *rec2* and *comF* mutants showed slight but significant decreases in DNA uptake (these would not have been detected in previous studies), suggesting that transport across the inner membrane might sometimes enhance outer membrane transport.

Our *dprA* deletion mutant had the same transformation defect and normal DNA uptake as a previously described transposon mutant (Fig. 2 and 3) (48, 100). Polarity effects on *dprB* and *dprC* were suggested previously by another group (47), but our previous microarray analysis of competent cells found that *dprB* and *dprC* were not substantially coinduced with *dprA* (85), and the similarity of the phenotypes of our marked and unmarked mutants confirmed that the transformation defect observed is due to the loss of *dprA* (Fig. 3B and C). In other bacteria, DprA is known to protect incoming single-stranded DNA (ssDNA) in the cytoplasm before recombination, acting as a mediator protein that passes ssDNA to RecA (11, 94, 96, 97). Because DprA is also found in noncompetent species, it is likely to have more general functions than transformation (93).

Other mutants. The deletion of *comE1* reduced DNA uptake and transformation 6- and 10-fold, respectively (Fig. 2 and 3). ComE1 encodes a putative periplasmic DNA binding protein that is essential for transformation in other species; its *Bacillus subtilis* homolog (ComEA) has been shown to accept DNA from the pseudopilus and to pass it to the inner membrane translocation complex (45). ComE1 proteins from *H. influenzae* and its relatives have been shown to bind DNA *in vitro* through a domain that also binds fibronectin (72). *Neisseria gonorrhoeae* ComE1 also binds DNA, and a knockout of all four *comE* copies reduces transformation over 10,000-fold without affecting piliation, confirming that it does not contribute to Tfp (23). The modest effect of the *H. influenzae* knockout suggested that a paralog might be present elsewhere in the genome, but none were identified by PSI-BLAST searches of all available *H. influenzae* genome sequences using sequences of ComE1 homologs from *H. influenzae*, *N. gonorrhoeae*, *Streptococcus pneumoniae*, and *B. subtilis*.

The *ligA* gene product is the only other CRP-S protein thought to act in the periplasm, and the fact that its deletion had no effect was also unexpected. *ligA* is predicted to encode a periplasmic ATP-dependent ligase, which is in itself surprising, both because the periplasm is not thought to contain any ATP and because a periplasmic ligase is not expected to make any contribution to DNA uptake or transformation. VanWagoner et al. previously found 5-fold-reduced transformation in a *ligA* knockout mutant (with normal uptake) (107), but we did not detect any transformation defect either in our mutants (Fig. 3C and D) or when we re-created the previously reported strain by using chromosomal DNA from their laboratory (data not shown). The ligase function and its ATP dependence have been confirmed for both the *H. influenzae* LigA protein and its *Neisseria* homolog (24, 63). The distribution of LigA homologs in other bacteria is sporadic, and most but not all have the N-terminal hydrophobic signals that predict export beyond the cytoplasm. In the process of investigating this distribution, we found that the terminology used is not always consistent and that both ATP- and NAD-dependent ligases are often annotated as LigA.

The deletion of *comM* had an unexpectedly small effect. As previously reported for a *comM* transposon mutant, DNA uptake was normal, but transformation by our mutant was reduced only 6-fold rather than the 300-fold previously reported (Fig. 3) (42). Although the specific function of ComM is not known, studies of the transposon mutant showed that it does not affect translocation or DNA degradation (42). The ComM sequence contains motifs typical of Mg chelatase subunits (Table 1); these are part of a broader group of force-exerting proteins but are not known to contribute to transformation in other species.

Finally, transformation was normal in deletion mutants of several other genes whose products are predicted to be cytoplasmic: *HI0365*, *HI0660*, *HI1631*, and *radC* (Fig. 2 and 3). *HI0365* is upstream of the pilotin gene *pilF2*, and its sequence is related to Fe-S cluster redox enzymes (Table 1). It is sporadically distributed in transformable and nontransformable bacteria, often but not always upstream of a pilotin homolog. *HI0660* is upstream of and cotranscribed with *HI0659* (85), whose essential contribution to DNA uptake in *H. influenzae* remains unknown. Its sequence contains a motif typical of Holliday junction resolvases (Table 1), suggesting that it may interact with DNA. In other *H. influenzae* strains, alleles of *HI0660* often contain large deletions, and homologs are absent from most members of the *Pasteurellaceae*, reinforcing its dispensability for competence. The sequence of *HI1631* contains a DNA cleavage motif found in restriction enzymes and archaeal resolvases (Table 1), but it is absent from almost all other sequenced *H. influenzae* strains and from other members of the *Pasteurellaceae* (17, 66). The function of RadC in *H. influenzae* has not previously been investigated, but in other bacteria, it contributes to the repair of DNA strand breaks and the restart of stalled replication forks (29, 37, 49, 87). The *radC* gene is also competence induced but dispensable for natural transformation in *Streptococcus pneumoniae* (6). The deletion of *radC* resulted in a small but significant reduction in DNA uptake without affecting transformation (Fig. 3); the relevance of this small change is uncertain.

We also created mutations in two genes that are not part of the CRP-S regulon, *comI* and *comJ*, which are transcribed divergently from the *comABCDEF* operon (Fig. 4). The *comI* gene is also known as *ponA* and encodes a penicillin binding protein (92), while the function of *comJ* is not known. The deletion of either gene had no effect on competence (Fig. 4), so the phenotypes previously observed were likely to be an experimental artifact from the transposon insertion, possibly due to secondary effects on *comA*.

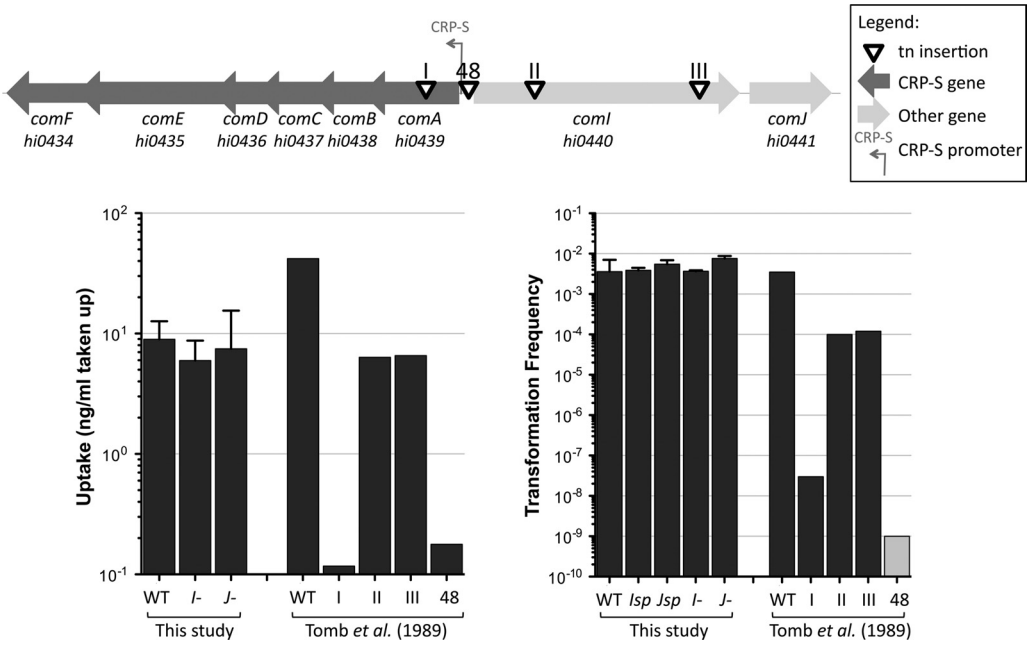

FIG 4 Competence phenotypes of the *H. influenzae comI* and *comJ* mutants. The operon structure is shown at the top: the *comI* and *comJ* genes are divergently transcribed from the *comABCDEF* operon and its CRP-S promoter. Triangles show the locations of previously described transposon insertions (100, 101). The uptake and transformation phenotypes of the *comI* and *comJ* mutants that we created (*I*-, unmarked *comI*; *J*-, unmarked *comJ*; *Isp*, marked *comI*::Spec; *Jsp*, marked *comJ*::Spec) and of previously described transposon mutants (100, 101) are shown at the bottom. Each bar represents the mean of at least three biological replicates ± the standard deviation.

DISCUSSION

This is the first complete mutational analysis of a competence regulon. It shows that 17 of the 26 genes in the CRP-S regulon of *H. influenzae* are absolutely required for natural transformation, with 14 of these being required for DNA uptake by the Tfp machinery. Only five genes appear to play no role in DNA uptake or transformation. These genes are strongly competence induced (80-fold for *ligA*, 10-fold for *HI0365*, 20-fold for *HI0660* and *HI1631*, and >100-fold for *radC*) (85), so they may make unrecognized contributions to fitness under competence-inducing conditions. The identification of these contributions could clarify how natural selection has acted on competence genes.

Although other genes required for competence could exist outside the competence regulon, the only non-CRP-S genes found by multiple mutant hunts have important housekeeping functions: *recA* (recombinational repair), *topA* (topoisomerase I), and *por* (disulfide oxidoreductase) (21, 52, 99). Thus, we think that we have now identified all of the genes required for DNA uptake and transformation in *H. influenzae*, the model system for natural competence in the gammaproteobacteria.

This study and the materials that it describes pave the way for a more detailed functional characterization of these genes. In the process of creating our knockout collection, we have cloned each CRP-S gene and operon on plasmids, so these can be used in mutagenesis and complementation experiments. In addition, because natural transformation is a valuable tool for strain construction and genetic manipulation, this work now allows the manipulation of competence in the many *H. influenzae* strains and *Pasteurellaceae* species that are not transformable, presumably due to the absence of functional copies of one or more competence genes (13, 66). Similar sporadic distributions of competence have been reported for other model systems, and the identification of the full complement of required genes makes it possible to identify the proximate and ultimate causes of these patterns.

ACKNOWLEDGMENTS

We thank Robert Munson and Erin Tracy for providing the materials required for the construction of our mutant collection and for helpful insights and troubleshooting with this. We also thank the laboratory of Charles Thompson for use of their BioScreen instrument and Timothy VanWagoner for providing Δ*ligA* Sp^r DNA.

This work was supported by an operating grant from the Canadian Institutes of Health Research to R.J.R.

REFERENCES

1. **Aas FE, et al.** 2002. Competence for natural transformation in Neisseria gonorrhoeae: components of DNA binding and uptake linked to type IV pilus expression. Mol. Microbiol. **46**:749–760.
2. **Albano M, Dubnau DA.** 1989. Cloning and characterization of a cluster of linked Bacillus subtilis late competence mutations. J. Bacteriol. **171**: 5376–5385.
3. **Alm RA, Mattick JS.** 1997. Genes involved in the biogenesis and function of type-4 fimbriae in Pseudomonas aeruginosa. Gene **192**: 89–98.
4. **Ando T, Israel DA, Kusugami K, Blaser MJ.** 1999. HP0333, a member of the *dprA* family, is involved in natural transformation in *Helicobacter pylori*. J. Bacteriol. **181**:5572–5580.
5. **Assalkhou R, et al.** 2007. The outer membrane secretin PilQ from *Neisseria meningitidis* binds DNA. Microbiology **153**:1593–1603.
6. **Attaiech L, Granadel C, Claverys JP, Martin B.** 2008. RadC, a misleading name? J. Bacteriol. **190**:5729–5732.
7. **Ayers M, et al.** 2009. PilM/N/O/P proteins form an inner membrane complex that affects the stability of the Pseudomonas aeruginosa type IV pilus secretin. J. Mol. Biol. **394**:128–142.

8. **Balasingham SV, et al.** 2007. Interactions between the lipoprotein PilP and the secretin PilQ in Neisseria meningitidis. J. Bacteriol. **189**:5716–5727.
9. **Barouki R, Smith HO.** 1986. Initial steps in *Haemophilus influenzae* transformation. Donor DNA binding in the *com10* mutant. J. Biol. Chem. **261**:8617–8623.
10. **Barouki R, Smith HO.** 1985. Reexamination of phenotypic defects in *rec-1* and *rec-2* mutants of *Haemophilus influenzae* Rd. J. Bacteriol. **163**: 629–634.
11. **Berge M, Mortier-Barriere I, Martin B, Claverys JP.** 2003. Transformation of Streptococcus pneumoniae relies on DprA- and RecA-dependent protection of incoming DNA single strands. Mol. Microbiol. **50**:527–536.
12. **Berka RM, et al.** 2002. Microarray analysis of the Bacillus subtilis K-state: genome-wide expression changes dependent on ComK. Mol. Microbiol. **43**:1331–1345.
13. **Bosse JT, et al.** 2009. Natural competence in strains of Actinobacillus pleuropneumoniae. FEMS Microbiol. Lett. **298**:124–130.
14. **Briley K, Jr, et al.** 2011. The secretion ATPase ComGA is required for the binding and transport of transforming DNA. Mol. Microbiol. **81**:818–830.
15. **Burrows LL.** 2005. Weapons of mass retraction. Mol. Microbiol. **57**: 878–888.
16. **Cameron AD, Redfield RJ.** 2008. CRP binding and transcription activation at CRP-S sites. J. Mol. Biol. **383**:313–323.
17. **Cameron AD, Redfield RJ.** 2006. Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. Nucleic Acids Res. **34**:6001–6014.
18. **Carbonnelle E, Helaine S, Nassif X, Pelicic V.** 2006. A systematic genetic analysis in Neisseria meningitidis defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. Mol. Microbiol. **61**:1510–1522.
19. **Carbonnelle E, Helaine S, Prouvensier L, Nassif X, Pelicic V.** 2005. Type IV pilus biogenesis in Neisseria meningitidis: PilW is involved in a step occurring after pilus assembly, essential for fibre stability and function. Mol. Microbiol. **55**:54–64.
20. **Carruthers MD, et al.** 2012. Biological roles of nontypeable Haemophilus influenzae type IV pilus proteins encoded by the pil and com operons. J. Bacteriol. **194**:1927–1933.
21. **Chandler MS, Smith RA.** 1996. Characterization of the Haemophilus influenzae topA locus: DNA topoisomerase I is required for genetic competence. Gene **169**:25–31.
22. **Chen I, Dubnau D.** 2004. DNA uptake during bacterial transformation. Nat. Rev. Microbiol. **2**:241–249.
23. **Chen I, Gotschlich EC.** 2001. ComE, a competence protein from Neisseria gonorrhoeae with DNA-binding activity. J. Bacteriol. **183**:3160–3168.
24. **Cheng C, Shuman S.** 1997. Characterization of an ATP-dependent DNA ligase encoded by Haemophilus influenzae. Nucleic Acids Res. **25**:1369–1374.
25. **Chung YS, Dubnau D.** 1995. ComC is required for the processing and translocation of comGC, a pilin-like competence protein of Bacillus subtilis. Mol. Microbiol. **15**:543–551.
26. **Cody AJ, et al.** 2003. High rates of recombination in otitis media isolates of non-typeable Haemophilus influenzae. Infect. Genet. Evol. **3**:57–66.
27. **Collins RF, Davidsen L, Derrick JP, Ford RC, Tonjum T.** 2001. Analysis of the PilQ secretin from Neisseria meningitidis by transmission electron microscopy reveals a dodecameric quaternary structure. J. Bacteriol. **183**:3825–3832.
28. **Collins RF, et al.** 2005. Interaction with type IV pili induces structural changes in the bacterial outer membrane secretin PilQ. J. Biol. Chem. **280**:18923–18930.
29. **Cox MM, et al.** 2000. The importance of repairing stalled replication forks. Nature **404**:37–41.
30. **Craig L, Pique ME, Tainer JA.** 2004. Type IV pilus structure and bacterial pathogenicity. Nat. Rev. Microbiol. **2**:363–378.
31. **Didelot X, Maiden MC.** 2010. Impact of recombination on bacterial evolution. Trends Microbiol. **18**:315–322.
32. **Dougherty BA, Smith HO.** 1999. Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. Microbiology **145**(Pt 2):401–409.
33. **Doughty SW, Ruffolo CG, Adler B.** 2000. The type 4 fimbrial subunit gene of *Pasteurella multocida*. Vet. Microbiol. **72**:79–90.
34. **Draskovic I, Dubnau D.** 2005. Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds. Mol. Microbiol. **55**:881–896.
35. **Dubnau D.** 1999. DNA uptake in bacteria. Annu. Rev. Microbiol. **53**: 217–244.
36. **Feil EJ, Maiden MC, Achtman M, Spratt BG.** 1999. The relative contributions of recombination and mutation to the divergence of clones of Neisseria meningitidis. Mol. Biol. Evol. **16**:1496–1502.
37. **Felzenszwalb I, Sargentini NJ, Smith KC.** 1986. Escherichia coli radC is deficient in the recA-dependent repair of X-ray-induced DNA strand breaks. Radiat. Res. **106**:166–170.
38. **Forest KT, Tainer JA.** 1997. Type-4 pilus-structure: outside to inside and top to bottom—a minireview. Gene **192**:165–169.
39. **Freitag NE, Seifert HS, Koomey M.** 1995. Characterization of the pilF-pilD pilus-assembly locus of Neisseria gonorrhoeae. Mol. Microbiol. **16**: 575–586.
40. **Glassberg J, Meyer RR, Kornberg A.** 1979. Mutant single-strand binding protein of Escherichia coli: genetic and physiological characterization. J. Bacteriol. **140**:14–19.
41. **Golub EI, Low KB.** 1985. Conjugative plasmids of enteric bacteria from many different incompatibility groups have similar genes for single-stranded DNA-binding proteins. J. Bacteriol. **162**:235–241.
42. **Gwinn ML, Ramanathan R, Smith HO, Tomb JF.** 1998. A new transformation-deficient mutant of *Haemophilus influenzae* Rd with normal DNA uptake. J. Bacteriol. **180**:746–748.
43. **Hamilton HL, Dillard JP.** 2006. Natural transformation of Neisseria gonorrhoeae: from DNA donation to homologous recombination. Mol. Microbiol. **59**:376–385.
44. **Herriott RM, Meyer EM, Vogt M.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101**:517–524.
45. **Inamine GS, Dubnau D.** 1995. ComEA, a Bacillus subtilis integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. J. Bacteriol. **177**:3045–3051.
46. Reference deleted.
47. **Karudapuram S, Barcak GJ.** 1997. The *Haemophilus influenzae dprABC* genes constitute a competence-inducible operon that requires the product of the *tfoX* (*sxy*) gene for transcriptional activation. J. Bacteriol. **179**: 4815–4820.
48. **Karudapuram S, Zhao X, Barcak GJ.** 1995. DNA sequence and characterization of *Haemophilus influenzae dprA*+, a gene required for chromosomal but not plasmid DNA transformation. J. Bacteriol. **177**:3235–3240.
49. **Katsiou E, Nickel CM, Garcia AF, Tadros MH.** 1999. Molecular analysis and identification of the radC gene from the phototrophic bacterium Rhodobacter capsulatus B10. Microbiol. Res. **154**:233–239.
50. **Koo J, Burrows LL, Howell PL.** 2012. Decoding the roles of pilotins and accessory proteins in secretin escort services. FEMS Microbiol. Lett. **328**: 1–12.
51. **Koo J, et al.** 2008. PilF is an outer membrane lipoprotein required for multimerization and localization of the Pseudomonas aeruginosa type IV pilus secretin. J. Bacteriol. **190**:6961–6969.
52. **Kooistra J, Venema G.** 1980. Properties of Haemophilus influenzae mutants that are slightly recombination deficient and carry a mutation in the rec-1 gene region. J. Bacteriol. **142**:829–835.
53. **Kroll JS, Moxon ER.** 1990. Capsulation in distantly related strains of *Haemophilus influenzae* type b: genetic drift and gene transfer at the capsulation locus. J. Bacteriol. **172**:1374–1379.
54. **Kroll JS, Wilks KE, Farrant JL, Langford PR.** 1998. Natural genetic exchange between *Haemophilus* and *Neisseria*: intergeneric transfer of chromosomal genes between major human pathogens. Proc. Natl. Acad. Sci. U. S. A. **95**:12381–12385.
55. **Kruger NJ, Stingl K.** 2011. Two steps away from novelty—principles of bacterial DNA uptake. Mol. Microbiol. **80**:860–867.
56. **Lang E, et al.** 2009. Identification of neisserial DNA binding components. Microbiology **155**:852–862.
57. **Lauer P, Albertson NH, Koomey M.** 1993. Conservation of genes encoding components of a type IV pilus assembly/two-step protein export pathway in Neisseria gonorrhoeae. Mol. Microbiol. **8**:357–368.
58. **Lee KK, Doig P, Irvin RT, Paranchych W, Hodges RS.** 1989. Mapping the surface regions of Pseudomonas aeruginosa PAK pilin: the impor-

tance of the C-terminal region for adherence to human buccal epithelial cells. Mol. Microbiol. **3**:1493–1499.
59. **Long CD, Tobiason DM, Lazio MP, Kline KA, Seifert HS.** 2003. Low-level pilin expression allows for substantial DNA transformation competence in Neisseria gonorrhoeae. Infect. Immun. **71**:6279–6291.
60. **Lu HM, Motley ST, Lory S.** 1997. Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin subunits in complex formation and extracellular protein secretion. Mol. Microbiol. **25**:247–259.
61. **Macfadyen LP.** 2000. Regulation of competence development in *Haemophilus influenzae*. J. Theor. Biol. **207**:349–359.
62. **Macfadyen LP, et al.** 2001. Competence development by *Haemophilus influenzae* is regulated by the availability of nucleic acid precursors. Mol. Microbiol. **40**:700–707.
63. **Magnet S, Blanchard JS.** 2004. Mechanistic and kinetic study of the ATP-dependent DNA ligase of Neisseria meningitidis. Biochemistry **43**:710–717.
64. **Marceau AH, et al.** 2011. Structure of the SSB-DNA polymerase III interface and its role in DNA replication. EMBO J. **30**:4236–4247.
65. **Martin PR, Watson AA, McCaul TF, Mattick JS.** 1995. Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in Pseudomonas aeruginosa. Mol. Microbiol. **16**:497–508.
66. **Maughan H, Redfield RJ.** 2009. Extensive variation in natural competence in Haemophilus influenzae. Evolution **63**:1852–1866.
67. **Maughan H, Sinha S, Wilson L, Redfield RJ.** 2008. *Pasteurellaceae*: biology, genomics and molecular aspects. Caister Academic Press, Norfolk, United Kingdom.
68. **McCarthy D.** 1989. Cloning of the rec-2 locus of Haemophilus influenzae. Gene **75**:135–143.
69. **Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK.** 2005. Chitin induces natural competence in *Vibrio cholerae*. Science **310**:1824–1827.
70. **Meyer RR, Glassberg J, Kornberg A.** 1979. An Escherichia coli mutant defective in single-strand binding protein is defective in DNA replication. Proc. Natl. Acad. Sci. U. S. A. **76**:1702–1705.
71. **Meyer RR, Voegele DW, Ruben SM, Rein DC, Trela JM.** 1982. Influence of single-stranded DNA-binding protein on recA induction in Escherichia coli. Mutat. Res. **94**:299–313.
72. **Mullen LM, et al.** 2008. Pasteurellaceae ComE1 proteins combine the properties of fibronectin adhesins and DNA binding competence proteins. PLoS One **3**:e3991. doi:10.1371/journal.pone.0003991.
73. **Nguyen Y, Jackson SG, Aidoo F, Junop M, Burrows LL.** 2010. Structural characterization of novel Pseudomonas aeruginosa type IV pilins. J. Mol. Biol. **395**:491–503.
74. **Nunn D, Bergman S, Lory S.** 1990. Products of three accessory genes, pilB, pilC, and pilD, are required for biogenesis of Pseudomonas aeruginosa pili. J. Bacteriol. **172**:2911–2919.
75. **Nunn DN, Lory S.** 1991. Product of the Pseudomonas aeruginosa gene pilD is a prepilin leader peptidase. Proc. Natl. Acad. Sci. U. S. A. **88**:3281–3285.
76. **Pelicic V.** 2008. Type IV pili: e pluribus unum? Mol. Microbiol. **68**:827–837.
77. **Perry AC, Hart CA, Nicolson IJ, Heckels JE, Saunders JR.** 1987. Inter-strain homology of pilin gene sequences in Neisseria meningitidis isolates that express markedly different antigenic pilus types. J. Gen. Microbiol. **133**:1409–1418.
78. **Peterson S, Cline RT, Tettelin H, Sharov V, Morrison DA.** 2000. Gene expression analysis of the Streptococcus pneumoniae competence regulons by use of DNA microarrays. J. Bacteriol. **182**:6192–6202.
79. **Poje G, Redfield RJ.** 2003. Transformation of *Haemophilus influenzae*. Methods Mol. Med. **71**:57–70.
80. **Pollack-Berti A, Wollenberg MS, Ruby EG.** 2010. Natural transformation of Vibrio fischeri requires tfoX and tfoY. Environ. Microbiol. **12**:2302–2311.
81. **Porter RD, Black S.** 1991. The single-stranded-DNA-binding protein encoded by the Escherichia coli F factor can complement a deletion of the chromosomal ssb gene. J. Bacteriol. **173**:2720–2723.
82. **Provvedi R, Dubnau D.** 1999. ComEA is a DNA receptor for transformation of competent Bacillus subtilis. Mol. Microbiol. **31**:271–280.
83. **Raghunathan S, Kozlov AG, Lohman TM, Waksman G.** 2000. Structure of the DNA binding domain of E. coli SSB bound to ssDNA. Nat. Struct. Biol. **7**:648–652.
84. **Reddy MS, Guhan N, Muniyappa K.** 2001. Characterization of single-stranded DNA-binding proteins from mycobacteria. The carboxyl-terminal of domain of SSB is essential for stable association with its cognate RecA protein. J. Biol. Chem. **276**:45959–45968.
85. **Redfield RJ, et al.** 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. J. Mol. Biol. **347**:735–747.
86. **Sampaleanu LM, et al.** 2009. Periplasmic domains of Pseudomonas aeruginosa PilN and PilO form a stable heterodimeric complex. J. Mol. Biol. **394**:143–159.
87. **Saveson CJ, Lovett ST.** 1999. Tandem repeat recombination induced by replication fork defects in Escherichia coli requires a novel factor, RadC. Genetics **152**:5–13.
88. **Seifert HS, Ajioka RS, Marchal C, Sparling PF, So M.** 1988. DNA transformation leads to pilin antigenic variation in Neisseria gonorrhoeae. Nature **336**:392–395.
89. **Setlow JK, Beattie KL, Boling ME.** 1972. Expression of a recombination gene on transforming DNA in a recombination-defective Haemophilus influenzae recipient cell. J. Mol. Biol. **68**:379–381.
90. **Setlow JK, Boling ME, Beattie KL, Kimball RF.** 1972. A complex of recombination and repair genes in Haemophilus influenzae. J. Mol. Biol. **68**:361–378.
91. **Sharma R, Rao DN.** 2009. Orchestration of Haemophilus influenzae RecJ exonuclease by interaction with single-stranded DNA-binding protein. J. Mol. Biol. **385**:1375–1396.
92. **Sharma UK, Dwarakanath P, Banerjee T, Town C, Balganesh TS.** 1995. Expression and characterization of the *ponA* (ORF I) gene of *Haemophilus influenzae*: functional complementation in a heterologous system. J. Bacteriol. **177**:6745–6750.
93. **Smeets LC, et al.** 2006. Functional characterization of the competence protein DprA/Smf in *Escherichia coli*. FEMS Microbiol. Lett. **263**:223–228.
94. **Smeets LC, Bijlsma JJ, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG.** 2000. The dprA gene is required for natural transformation of Helicobacter pylori. FEMS Immunol. Med. Microbiol. **27**:99–102.
95. **Szeto TH, Dessen A, Pelicic V.** 2011. Structure/function analysis of Neisseria meningitidis PilW, a conserved protein that plays multiple roles in type IV pilus biology. Infect. Immun. **79**:3028–3035.
96. **Tadesse S, Graumann PL.** 2007. DprA/Smf protein localizes at the DNA uptake machinery in competent Bacillus subtilis cells. BMC Microbiol. **7**:105. doi:10.1186/1471-2180-7-105.
97. **Takata T, Ando T, Israel DA, Wassenaar TM, Blaser MJ.** 2005. Role of dprA in transformation of Campylobacter jejuni. FEMS Microbiol. Lett. **252**:161–168.
98. **Tammam S, et al.** 2011. Characterization of the PilN, PilO and PilP type IVa pilus subcomplex. Mol. Microbiol. **82**:1496–1514.
99. **Tomb JF.** 1992. A periplasmic protein disulfide oxidoreductase is required for transformation of *Haemophilus influenzae* Rd. Proc. Natl. Acad. Sci. U. S. A. **89**:10252–10256.
100. **Tomb JF, Barcak GJ, Chandler MS, Redfield RJ, Smith HO.** 1989. Transposon mutagenesis, characterization, and cloning of transformation genes of Haemophilus influenzae Rd. J. Bacteriol. **171**:3796–3802.
101. **Tomb JF, el-Hajj H, Smith HO.** 1991. Nucleotide sequence of a cluster of genes involved in the transformation of Haemophilus influenzae Rd. Gene **104**:1–10.
102. **Tonjum T, Freitag NE, Namork E, Koomey M.** 1995. Identification and characterization of pilG, a highly conserved pilus-assembly gene in pathogenic Neisseria. Mol. Microbiol. **16**:451–464.
103. **Tonjum T, Koomey M.** 1997. The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships—a review. Gene **192**:155–163.
104. **Tracy E, Ye F, Baker BD, Munson RS, Jr.** 2008. Construction of non-polar mutants in Haemophilus influenzae using FLP recombinase technology. BMC Mol. Biol. **9**:101. doi:10.1186/1471-2180-11-208.
105. **Trindade MB, Job V, Contreras-Martel C, Pelicic V, Dessen A.** 2008. Structure of a widely conserved type IV pilus biogenesis factor that affects the stability of secretin multimers. J. Mol. Biol. **378**:1031–1039.
106. **van Schaik EJ, et al.** 2005. DNA binding: a novel function of *Pseudomonas aeruginosa* type IV pili. J. Bacteriol. **187**:1455–1464.
107. **VanWagoner TM, Whitby PW, Morton DJ, Seale TW, Stull TL.** 2004.

Characterization of three new competence-regulated operons in Haemophilus influenzae. J. Bacteriol. **186**:6409–6421.
108. **Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG.** 2005. Simple and highly efficient BAC recombineering using *galK* selection. Nucleic Acids Res. **33**:e36. doi:10.1093/nar/gni035.
109. **Whittier RF, Chase JW.** 1981. DNA repair in E. coli strains deficient in single-strand DNA binding protein. Mol. Gen. Genet. **183**:341–347.
110. **Wolfgang M, et al.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae. Mol. Microbiol. **29**:321–330.
111. **Zhang Y, et al.** 2000. Identification of type 4 fimbriae in *Actinobacillus pleuropneumoniae*. FEMS Microbiol. Lett. **189**:15–18.
112. **Zulty JJ, Barcak GJ.** 1995. Identification of a DNA transformation gene required for *com101A*+ expression and supertransformer phenotype in *Haemophilus influenzae*. Proc. Natl. Acad. Sci. U. S. A. **92**:3616–3620.