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# A New Transformation-Deficient Mutant of *Haemophilus influenzae* Rd with Normal DNA Uptake

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***Haemophilus influenzae*** **Rd is a gram-negative natural transformer. A mutant strain, RJ248, that has normal DNA uptake and translocation but whose transformation frequency is 300 times lower than that of wild-type *H. influenzae* and whose phage recombination is 8 times lower was isolated. The affected gene, *comM*, is induced during competence development in wild-type *H. influenzae* but not in RJ248.**

*Haemophilus influenzae* Rd is a gram-negative bacterium capable of natural DNA transformation. It achieves low-level competence in late log phase and can be induced to high levels of competence by a nutritional downshift into MIV medium (12) or by transient anaerobic growth (10).

Transformation involves several steps. The first is the uptake of naked DNA into a membrane-protected compartment (13). Uptake of DNA is facilitated by an uptake signal sequence (20). Following uptake, the DNA translocates slowly into the cytoplasm, where the 5′ strand is completely degraded and the 3′ strand is partially degraded (2). If homology exists between the incoming DNA and the chromosome of the cell, the entering 3′ strand will invade and replace the chromosomal DNA by homologous recombination (2).

The *comABCDEF* operon is essential for transformation and is induced during competence development but not during exponential growth (21, 22). A 26-bp palindromic competence-regulatory element (CRE) (also known as the dyad symmetry element) is required for induction of the operon (9, 14, 22). This element has been found upstream of three other known competence genes (14, 23) (*pilABCD* [8, 23], *rec2* [7], and *dprA* [14]), all of which have been shown to be induced during competence development (14, 23). In all cases, the element is positioned about one turn of the helix upstream of either a known or a putative promoter (14, 22). It is likely that this sequence is the binding site for a competence-specific, positively acting transcriptional regulator. A known regulator of competence which could fill this role is the product of the gene *sxy* (also known as *tfoX*). This gene is required for competence development and for the induction of both *dprA* and *comF* (14, 24, 26). In addition, certain point mutations of this gene which allow cells to be competent all of the time exist (18). It is not known whether the effect of *sxy* on expression of competence genes is direct or indirect (14).

Strains with mutations in *rec2*, *dprA*, and *comF* have normal DNA uptake but are transformation deficient (7, 14, 23, 26). In *rec2* mutants, the DNA remains trapped in the membrane compartment and never translocates into the cytoplasm (4). The *H. influenzae* homolog of the *Escherichia coli recA* gene, *rec1*, is required for homologous recombination of donor DNA into the chromosome (16).

Here we report on RJ248, a new transformation mutant of *H. influenzae* Rd that has a transformation frequency 300-fold lower than that of wild-type *H. influenzae* and a deficiency in the competence-induced increase in phage recombination. However, it has normal DNA uptake and translocation. The affected gene has been identified and named *comM*. It has a CRE site upstream of its putative promoter. It is induced during competence development in wild-type cells but not in RJ248.

**Growth, transformation, and β-galactosidase activity.** *H. influenzae* was grown as described by Barcak et al. (3). *E. coli* was grown as described by Sambrook et al. (19). The MIV nutritional-downshift procedure was used to measure transformation frequencies (3, 12). DNA uptake was measured as described by Gwinn (11). β-Galactosidase activity was measured with a permeabilized-cell assay (17).

**Mutagenesis and isolation of RJ248.** MIV-competent wild-type cells were transformed with a plasmid carrying Tn*916* (6, 15). Transposition events were selected for by tetracycline resistance, which is encoded by Tn*916*. Tetracycline-resistant colonies were screened by the cyclic AMP transformation plate assay (25). One strain, RJ24, was consistently transformation deficient. Genomic DNA from RJ24 was transformed into wild-type cells to isolate a pure strain with only one Tn*916* insertion, resulting in RJ248. Southern and MIV transformation analysis confirmed that RJ248 carries one Tn*916* insertion responsible for the transformation phenotype and that the insertion was not in a known transformation gene.

**UV sensitivity.** RJ248, KW20 (wild type), and DB117 (*rec1* mutant; negative control) were grown and exposed to a GE 615T8 germicidal lamp for 0, 3, or 6 s at a distance of 37 cm. Plates were incubated at 37°C for 24 h, and survivors were counted.

**Phage recombination.** Exponential-phase cells and competent cells were separately coinfected with *ts*1 and *ts*3, temperature-sensitive point mutants of the *H. influenzae* phage Hp1c1. Recombinant progeny were identified by their ability to grow at the nonpermissive temperature (40°C) (5).

**Translocation.** A plasmid containing an *H. influenzae* genomic *Pst*I fragment was cut with *Pst*I and end labeled with [$^{32}$P]dCTP by using T4 polymerase. Cells which had been made competent by the MIV method were incubated with labeled plasmid. At 5, 10, 15, 30, and 60 min, samples of the cells were removed and washed with MIV medium by centrifugation.

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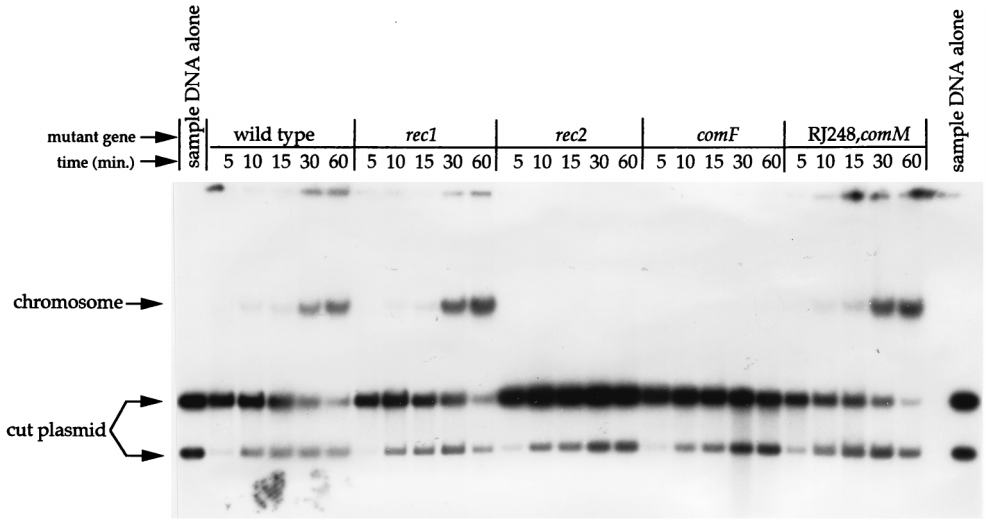


FIG. 1. Results of a translocation experiment. Translocation is indicated by the appearance in the chromosome of labeled plasmid DNA.

Total DNA was isolated. DNA samples were electrophoresed on a 0.8% agarose gel and autoradiographed (4).

**RNA isolation and Northern analysis.** All buffers used in RNA isolation were made with diethyl pyrocarbonate-treated water. KW20 (wild type) and RJ248 were made competent by the MIV method. At 0, 50, and 100 min in MIV medium, 35 ml of cells was removed. Cells were lysed, and RNA was isolated by hot-phenol extraction. Northern analysis was performed with the Gene Images CDP-Start labeling and detection kits (Amersham, Indianapolis, Ind.). Probes were gene-internal fragments of HI1118 and HI1117 DNA.

**Phenotype of RJ248.** RJ248 has a transformation frequency that is 300-fold lower than that of the wild type. It has a wild-type DNA uptake level and grows at the wild-type rate. Its resistance to UV light is the same as that seen in the wild type (data not shown).

To test whether the mutation in RJ248 affected regulation, we transformed RJ248 with genomic DNA from MGH100 (a strain containing *lacZ kan* in an operon fusion with *comA*), which resulted in a strain with the RJ248 mutation and a β-galactosidase transcription indicator in *comA*. We found that induction of *comA*, as measured by β-galactosidase activity, is unaffected in the RJ248 background (data not shown).

Competent cells exhibit a higher rate of phage recombination than cells in exponential phase (5). We found that with wild-type cells there is about a 40-fold increase in the recombination rate in competent cells over that in cells in exponential phase, while with RJ248 the increase is only 5-fold.

To test whether DNA translocation is normal in RJ248, we compared the strain to several other mutant strains. Samples of competent cells were given radioactively labeled, digested plasmid DNA (two fragments). In wild-type cells, labeled donor DNA was taken up and became associated with the chromosome over time (Fig. 1). As expected, *rec2* mutants did not translocate DNA into the cytoplasm, as indicated by the absence of accumulation of label in the chromosome. Cells with a mutation in *rec1* take DNA into the cytoplasm; however, since the DNA cannot undergo homologous recombination, it is degraded in the cytoplasm and the labeled nucleotides are incorporated into the chromosome through subsequent reuse (4). We found that the accumulations of label in the chromosome in the wild type and RJ248 were indistinguishable, indicating that translocation in RJ248 is normal (Fig. 1). It is theoretically possible that in RJ248 the DNA becomes trapped in the membrane compartment, where it undergoes degradation and subsequent entry via another mechanism. However, we feel this to be unlikely, since there is substantial evidence indicating that large-scale degradation of donor DNA does not occur until it reaches the cytoplasm. In addition, although slow DNA degradation has been observed in the membrane compartment, this has not been shown to allow the DNA to then enter the cytoplasm (2, 4).

**Identification of the affected gene.** Sequence analysis of clones carrying the region of insertion revealed that Tn*916* was inserted between the HI1117 and HI1118 genes (9), which are in the same orientation (Fig. 2). The predicted products of both open reading frames have significant homology to hypothetical proteins in *E. coli* (see the *H. influenzae* database at www.tigr.org). In addition, HI1117 has homology to a magnesium chelatase gene of *Rhodobacter capsulatus*, *bchI*, involved in bacteriochlorophyll biosynthesis (1) and to related genes from other photosynthetic organisms. The putative promoter region of HI1117 contains a CRE sequence about one turn of the helix upstream of a putative promoter (Fig. 2). The insertion point of Tn*916* is in the −10 hexamer region of the

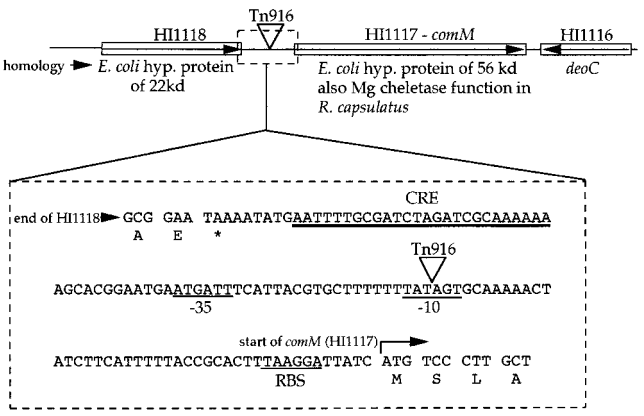


FIG. 2. Sequence around the point of insertion of Tn*916* with the putative *comM* promoter indicated. hyp., hypothetical. The asterisk denotes a stop codon. RBS, ribosomal binding site.

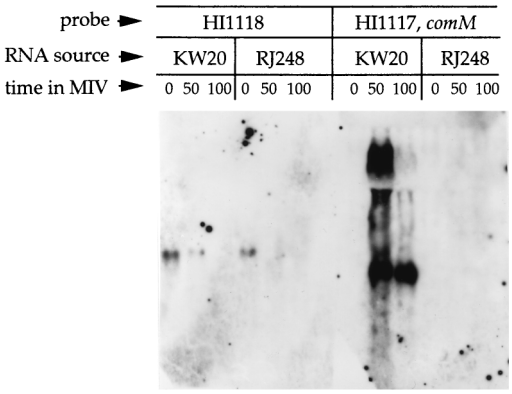


FIG. 3. Northern analysis of RJ248. Times are given in minutes.

possible promoter. This insertion is beyond the stop codon of HI1118. We conclude that HI1117 is affected by the disruption of its promoter and have named it *comM*.

To test whether *comM* is induced during competence development, as would be expected based on the presence of the CRE, and whether induction is prevented by the presence of Tn*916* in the mutant, total RNA from both KW20 (wild type) and RJ248 before and after competence induction was probed with a DNA fragment from within *comM* and with a DNA fragment from within HI1118. We found that in the wild type, *comM* is strongly induced during competence development; however, in RJ248, there is no detectable induction of *comM* (Fig. 3). Expression of HI1118 diminishes in both backgrounds and is unaffected by the presence of Tn*916* (Fig. 3).

The *comM* gene is clearly not induced in RJ248 during competence development. This is due to the presence of Tn*916*, which disrupts the putative promoter. In addition to its transformation deficiency, the only competence-associated defect we have found in RJ248 is a reduction in competence-dependent phage recombination. This phenotype and the ability of RJ248 to translocate DNA normally imply that *comM* acts very late in transformation, presumably at a step in the recombination of the donor DNA into the chromosome.

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