Transformation of *Haemophilus influenzae*

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1. Introduction

Conjugation: Although *H. influenzae* genes may be transferred by plasmid-mediated conjugation, this process is not discussed further below as it has received little attention and is rarely used for strain construction. Conjugative plasmids are common in *H. influenzae* as in other bacteria [1, 2, 3]. The F plasmid of *Escherichia coli* can conjugate into *H. influenzae* cells [4] (and into many other cell types), and can then transfer by conjugation from one *H. influenzae* strain to another. As in *E. coli*, efficient transfer of chromosomal genes requires a region of homology between the plasmid and the chromosome. A method for F-mediated conjugation into *H. influenzae* is described by Deich and Green[4].

Transformation: *H. influenzae* has an efficient natural competence system, which also serves as the primary method of strain construction. Cells are described as competent when they are able to take up DNA from the environment, and transformation occurs when this DNA uptake leads to a changed genotype, either by recombination or by establishment of a plasmid. In the laboratory, transformation is used to transfer chromosomal alleles into different backgrounds, to introduce plasmids into cells, and to create new mutants by introducing into the chromosome mutations created in *E. coli* or *in vitro*.

Natural competence and transformation are also areas of ongoing research in their own right. There is extensive interest in mechanisms by which DNA is transferred across the cell envelope, in the regulatory processes that control competence, and in the evolutionary function and consequences of DNA uptake and recombination. *H. influenzae* cells develop competence spontaneously when appropriate physiological conditions induce expression of the genes responsible for DNA uptake, and they then efficiently take up and recombine linear DNA fragments. In this they differ from *E. coli* and many other bacteria, which become competent only after artificial cell-permeablizing treatments and which can only be transformed with self-replicating plasmids. Before describing the laboratory methods used to transform *H. influenzae*, we will consider how competence is induced and how DNA is taken up and recombined.

There are no recent reviews of competence or transformation in *Haemophilus*; however, several old reviews contain useful information [5, 6, 7]. Recent reviews on DNA uptake and on the regulation of competence in diverse bacteria are those by Dubnau [8] and by Solomon [9]; both include discussions of *H. influenzae*. For a discussion of the evolution of competence, see Redfield [10 and 10a].

Not all strains of *H. influenzae* can be easily transformed with all genes. Derivatives of the completely-sequenced laboratory strain Rd (also called KW20) are readily transformed, as are the standard serotype b strains. However, some clinical strains do not develop competence at all, at least under laboratory conditions. In such cases electroporation can be used to introduce plasmids [11]. Efficiency of transformation can also be limited by sequence differences between donor and recipient DNAs.

Regulation of competence: Many bacteria can develop natural competence, but regulation of competence appears to be quite idiosyncratic, with different regulatory systems controlling competence development in different bacteria. In *H. influenzae*, competence development requires expression of the regulatory genes *sxy* (also called *tfoX*) [12] and *crp*[13], and an elevated intracellular concentration of cyclic AMP (cAMP) [14]. These regulators act to induce expression of a number of genes, many under the transcriptional control of a promoter sequence called the competence regulatory element or CRE box [15].

The relationship between culture growth and development of competence is shown in Figure 1. When *H. influenzae* cells are growing exponentially in a rich medium such as sBHI, competence is undetectable until their density reaches about 10^9 cells/ml, at which time growth begins to slow and the first competent cells can be detected by transformation assays. Competence increases as growth slows and gradually decreases once growth stops. However under these "spontaneous competence" conditions only about 1% of the cells in the culture become competent, and transformation frequencies with chromosomal markers rarely exceed 10^{-4}. A similar level of competence is rapidly induced when 1mM cAMP is added to exponentially-growing cells[16]. Several mutants are known whose spontaneous competence is greatly elevated, [17, 18]; although their mode of action is not understood they may provide useful tools for certain experiments.

Somewhat higher competence can be induced by temporarily shifting a rapidly growing well-aerated culture to anaerobic growth conditions. However, the usual procedure for inducing competence is to transfer exponentially-growing cells to a synthetic medium called M-IV, which lacks a number of components necessary for sustained growth, including sugars, nucleotides and cofactors. In this medium cell division quickly ceases but synthesis of new proteins continues. Incubation in M-IV for 100 minutes causes most of the cells in the culture to develop competence, giving transformation frequencies which average about 5×10^{-3} (range 10^{-3}–2×10^{-2}).

DNA uptake and transformation: During DNA uptake in *H. influenzae* (Figure 2), DNA is transported across the outer membrane and into the periplasmic space as intact duplex molecules [7]. Once in the periplasm, DNA crosses the thin peptidoglycan layer and is then moved across the inner membrane into the cytoplasm. Only linear molecules can be transported into the cytoplasm; circular molecules remain in the periplasm, suggesting that that DNA must be threaded through a translocation complex in the inner membrane[19]. Transport of DNA across the inner membrane is thought to be concomitant with degradation of the strand whose 5' end first enters the cytoplasm.

Competent *H. influenzae* cells are very proficient at recombination and after DNA enters the cytoplasm homologous strands are often integrated into the recipient chromosome. If donor and recipient DNA sequences differ slightly, heteroduplex regions may be

corrected in either direction by mismatch repair enzymes, or may give rise to homoduplex chromosomes by DNA replication. However recombination is very inefficient if sequences differ by more than a few percent.

Practical issues:

1. Amount of DNA taken up: Each competent cell can take up about 200kb of DNA, of which about 20kb will recombine with the chromosome if homology permits. The rest is normally degraded before homologous sequences are found. Linkage studies indicate that single fragments as long as 100 kb may be taken up by competent cells. Usually cells take up multiple fragments (8-12 long fragments, or a larger number of shorter fragments). Uptake is usually saturated by a DNA concentration of 200ng/ml; adding additional DNA will not increase the transformation frequency. This is not a kinetic saturation of the rate of uptake but an absolute saturation: once cells have taken up a certain amount of DNA they do not take up any more. In experiments designed to measure competence an excess of chromosomal DNA (1 µg/ml) is standard. Because cells usually take up multiple fragments they may acquire two independent alterations if both are present in the DNA fragments provided. This can cause problems if the recipient gains an unwanted allele from donor DNA, but can also be exploited to create doubly-altered strains in a single transformation.

2. Measuring competence: The most sensitive measure of the competence of a culture is transformation frequency–the fraction of cells transformed when given a saturating amount of genetically-marked DNA. This provides a very sensitive assay of competence; frequencies as low as 10^{-8} can be detected. Sensitivity may be limited by "bald patches" on plates, caused by very high backgrounds of non-transformed cells (Note 1). A convenient DNA for competence assays is that of strain MAP7, which carries seven different chromosomal point mutations conferring antibiotic resistance. Competence may also be quantitated by uptake of radiolabelled DNA; this is less sensitive than transformation but provides a measure of competence that is independent of recombination. *In vivo* DNA labelling is inefficient, and it is usually easier to label DNA *in vitro*, either by nick-translation of chromosomal DNA or by end-labelling of short fragments. Labelling with ^{33}P provides a good compromise between high specific activity and long half-life. When the goal is to carry out a time-course analysis of the development of competence, DNA uptake should be stopped by addition of DNase I after 15 minutes; a shorter time may be used if greater precision is needed.

3. Transformation with linear DNA fragments: The issues to be addressed depend on the purpose of the transformation experiment. If the goal is to introduce a different allele into the chromosome, transformation may use either chromosomal DNA from a different strain, or a linear DNA fragment produced by cloning or PCR. Transformation with chromosomal DNA is usually trouble-free. The presence of insertions or deletions of 10kb or more does not normally reduce the transformation frequency by more than 10-fold, presumably because in chromosomal DNA these heterologies usually occur in large fragments with extensive flanking homologous DNA. However, cloned or PCR product fragments present several problems. Transformation with short fragments (smaller than 1kb) is usually quite inefficient, as these often are completely degraded before homology can be found. Short fragments carrying insertions or deletions will recombine poorly unless there is at least 1kb of homologous sequence on each side of the heterology.

Another potential problem arises when transforming with a saturating amount of a pure DNA fragment (a plasmid insert or a PCR product), where cells may take up multiple copies of the same fragment. This often leads to integration of artifactually-compound fragments and tandem repeats, presumably formed by an unidentified periplasmic ligase acting on the incoming fragments. To prevent this, it is best to use a limiting amount of transforming DNA in such experiments. The simplest procedure is to transform with several different DNA concentrations in parallel (e.g. 100ng/ml, 10ng/ml, 1ng/ml), and to retain only colonies from the lowest concentration that produces transformants. For important constructions it is wise to also check the transformants by Southern blotting, to ensure that they have the desired genotype.

4. Transformation by a plasmid: Different issues apply if the goal is to introduce an autonomously-replicating plasmid into a *H. influenzae* strain. The strategy used depends on whether the plasmid contains or lacks a segment homologous to the cell's chromosome, and on whether recombination with the chromosome is to be promoted or prevented. If the goal is to introduce a plasmid-borne allele into the chromosome, transform with the isolated insert or with plasmid linearized by cleavage in the vector, rather than with intact plasmid.

Plasmids with no chromosomal homology: Because the DNA uptake machinery cannot transport circular molecules across the inner membrane, the second step of DNA uptake must be circumvented by an osmotic shock using glycerol, which promotes passage of the intact plasmid into the cytoplasm. Plasmid molecules that have been linearized can be taken up but rarely yield plasmid-bearing transformants because of the exonucleolytic degradation that accompanies transport across the inner membrane. However, if a cell takes up two or more broken plasmid molecules, strands from these may sometimes recombine within the cell and regenerate an intact plasmid.

Transformation by plasmids with chromosomal homology: Homologous recombination between incoming DNA and the chromosome is of little consequence if the plasmid and chromosome carry the same allele. However, if they carry different alleles this recombination may either be exploited to transfer a chromosomal allele to the plasmid or a plasmid allele to the chromosome, or prevented to maintain the original alleles on the plasmid and chromosome. Plasmid-chromosome recombination can be promoted by cutting the plasmid in the region of DNA homology, by using no glycerol in the transformation, and by selecting for transformants expressing the antibiotic resistance encoded by the vector (not an antibiotic resistance in the insert). Although the transformation frequency will have been reduced by the cutting, a substantial fraction of the plasmids in the successful transformants will have acquired the chromosomal allele by recombinational repair. On the other hand, recombination between plasmid and host can be prevented by using the *rec-1* mutant strain DB117, which lacks the *H. influenzae* homolog of RecA and thus cannot carry out homologous recombination. If the *rec-1* mutation will interfere with subsequent manipulations, recombination can be instead limited by using only supercoiled DNA and glycerol-promoted transformation.

However the resulting transformants will need to be carefully screened to ensure the plasmid retains its original allele.

Plasmids not grown in *H. influenzae* will lack *H. influenzae*-specific modification and thus be sensitive to intracellular cleavage by *H. influenzae*'s restriction enzymes (*Hin*dII and *Hin*dIII in the Rd strain, others in other strains). This need not prevent successful transformation, but may reduce its efficiency. Cloned fragments and PCR products also lack appropriate modification but usually transform efficiently, presumably because linear DNAs enter the cytoplasm as single strands which are not substrates for restriction enzymes.

Transformation can also be used to integrate into the chromosome a plasmid that cannot replicate in *H. influenzae*. This is useful when constructing strains carrying null alleles and gene fusions, as it allows retention of a wild-type allele[20]. To promote recombination between chromosomal and insert sequences, the plasmid may be linearized by restriction cleavage somewhere within the insert. However, Southern blotting should be used to check the structure of the resulting transformants.

5. Sequence-specificity of DNA uptake (especially its relevance to transformation): Competent *H. influenzae* cells preferentially bind and take up DNA fragments containing the uptake signal sequence AAGTGCGGT (the USS, which is abundant in *H. influenzae* genomic DNA. The bias may be as strong as 100-fold for some fragments. However, the absence of USS from particular genes or cloned fragments does not normally limit strain-construction experiments, for two reasons. First, fragments of genomic DNA are large enough that they usually carry many USS. Second, cells will readily take up purified fragments and plasmids with no USS provided there are no competing fragments carrying it.

2. Materials

2.1 Preparation of competent *H. influenzae*.

1. Exponentially-growing culture of the cells to be transformed, at a density of $OD_{600} \leq 0.3$. A 35 ml culture in a 500 ml flask at an OD_{600} of 0.3 is traditional, but larger or smaller volumes may be used, and an OD_{600} of ≤ 0.25 is optimal. Keep the flask size large to promote aeration.
2. Vacuum filtration apparatus with sterile filter funnel (0.2µ pore size). (Note 2)
3. M-IV medium, usually prepared fresh before each use from the stock solutions outlined in Table I. To 50 ml of solution 21 (or 5 ml of 10X solution 21 and 45 ml H_2O), add 0.5 ml each of solutions 22, 23, 24 and 40).
4. 250 ml flask
5. Sterilized forceps (wiping with EtOH is sufficient)
6. Sterile 80% glycerol and sterile freezer tubes if cells are to be frozen.

2.2 Transformation.

1. Chromosomal or plasmid DNA (100 µg/ml stock in 10mM Tris 1mM EDTA pH 7.5) (a simple miniprep method for chromosomal DNA is given in Chapter 1). If the DNA or the competent cells have not been previously tested, include MAP7 or other genetically marked DNA of known quality, as a positive control.
2. 1 ml competent cells for each transformation, and 1 ml for the no-DNA control.
4. DNase I, 100µg/ml. Stock is prepared aseptically in sterile 50% glycerol and stored at -20°C.
5. Dilution solution (recipe in Chapter 1).
6. Sterile culture tubes for transformations and dilutions.
7. sBHI agar plates with and without appropriate antibiotics.
8. Sterile 3mm glass beads or spreader.

2.3 DNA uptake assays

Preparation of radiolabelled DNA:

<u>For end-labelling of short fragments:</u>

1. DNA equivalent to 1-50 pmol of 5' termini.
2. T4 polynucleotide kinase.
3. T4 polynucleotide kinase buffer (10X).
4. 50 pmol gamma-^{33}P ATP, 2500 Ci/mmol.
5 0.5 M EDTA.
6. Phenol-chloroform for extraction.
7. Microspin G-50 column (Amersham).

<u>For nick-translation of long fragments:</u>

1. DNA, 0.25 µg.
2. DNase I, 10 ng/ml (10^{-4} dilution of the DNase I stock used for transformations).
3. Mix of 3 dNTPs (0.5 mM each of dCTP, dGTP and dTTP).
4. Alpha-^{33}P dATP (or other alpha-labeled deoxynucleotide – change mix accordingly).

5. *E. coli* DNA polymerase I (not Klenow fragment).
6. *E. coli* DNA polymerase I buffer (10X).
7. EDTA, phenol-chloroform and G-50 column as above.

DNA uptake assay:

1. 200 ng of ^{33}P-labelled DNA for each sample of competent cells. Specific activity should be about 10^5 cpm/µg. DNAs with higher specific activity may be diluted with unlabelled DNA.
2. Competent cells
3. DNase I, 1 mg/ml in 50% glycerol (note that this is tenfold more concentrated than the stock used for transformations).
4. Cold 5 M NaCl.
5. Cold M-IV supplemented with 1 M NaCl (20 ml M-IV + 5 ml 5 M NaCl).
6. Room temperature M-IV
7. Scintillation vials, fluid and counter.

3. Methods

3.1 Induction of competence

1. Place 10 ml room temperature M-IV into a 250 ml flask and set aside.
2. When culture to be made competent reaches an OD_{600} of 0.20-0.25 remove 10 ml to the filter funnel and collect cells by filtering under gentle vacuum. (Note 3)
3. As soon as the sBHI has been drawn through the filter, replace it with 10 ml M-IV and continue filtration.
4. Release vacuum and remove top of filter housing. Lift the filter from the apparatus with sterilized forceps and place it in the flask of M-IV. The cells will quickly resuspend from the filter, which may remain in the flask.
5. Shake culture at 100 rpm 37°C for 100 minutes. At this time the culture will have achieved the maximum level of competence and will maintain it for at least 1 hour at 37°C.
6. If cells are to be frozen, add 1 ml 80% glycerol for each 4 ml of cells and store at -80°C. Aliquots of 1.0 ml are convenient.

3.2 Transformation.

1. If using previously-frozen competent cells, first pellet the cells (5000g, 2 min at room temp.) and remove the M-IV and glycerol by aspiration. Resuspend the pellet in an equal volume of M-IV. (Note 4)
2. To 1 ml of competent cells add 1 µg of chromosomal DNA or a smaller amount of a cloned DNA or a PCR product and mix gently. Incubate cells with DNA at 37°C for 15 minutes.

   Modifications:

   i. If transforming with a closed-circular plasmid, extend the incubation to 30 minutes. Then add sterile 80 % glycerol to a final concentration of 30-32%, mix, and leave at room temperature for 10 min. Dilute and plate as usual.

   ii. If carrying out a time course of competence, terminate DNA uptake by adding 10µl of DNase (100µg/ml) and incubating for an additional 5 minutes at 37°C before plating.

   iii. Resistance to certain antibiotics requires expression time. Cells can be plated immediately onto kanamycin or novobiocin plates. For other antibiotics add two volumes of sBHI to the transformation mixture and incubate at 37°C for the time indicated in Table 2 before plating.

3. Plating: When transforming with chromosomal DNA, expect a transformation frequency of about 10^{-3}-10^{-2}. Cloned and PCR-product DNAs may give higher or lower transformation frequencies, depending on the molecule and the concentration used. If using chromosomal DNA and standard conditions, plate 100µl of 10^{-5} and 10^{-6} dilutions on sBHI plates without antibiotic, and 100µl of 10^{-2}, 10^{-3} and 10^{-4} dilutions on antibiotic plates.

7. Controls: Transform cells with 1µg of MAP7 DNA and select for resistance to the same antibiotic, or to novobiocin. If using a non-Rd strain, transform the DNA into M-IV-competent Rd cells (strain KW20). As a negative control, plate cells that have not been incubated with DNA on the antibiotic plates.

9. Incubate plates at 37°C for 16-24 hr (48 hr if selecting for chloramphenicol resistance).

10. The degree of competence of the cells is usually measured as the transformation frequency obtained with an excess of chromosomal DNA. This is calculated by dividing the number of transformants per ml (calculated from colony numbers on antibiotic plates) by the total number of cells per ml (calculated from colony numbers on plain plates). If the transforming ability of the DNA is in question, a non-saturating concentration should be used, ideally less than one molecule per cell if a pure fragment is being evaluated. The DNA's transformation efficiency

can then be calculated by dividing the number of transformants per ml (calculated from colony numbers on antibiotic plates) by the amount of DNA used to transform this volume of cells.

3.3 DNA uptake measurements.

End-labelling short DNA fragments:

1. Combine DNA (1-50 pmol of 5' ends), 20 units T4 polynucleotide kinase, 50 pmol gamma ^{33}P ATP, an appropriate volume of the supplier's kinase buffer and H_2O to a final volume of 50 µl.
2. Incubate at 37°C for 30 minutes.
3. Stop reaction by adding 1 µl of 0.5M EDTA and 50 µl of H_2O. Extract once with phenol-chloroform.
4. To remove unincorporated nucleotides, load onto G-50 column and collect flow-through.

Nick-translation:

1. Combine 0.25 µg DNA, 1 µl DNase I, 2.5 µl dNTP mix, an appropriate volume of the supplier's polymerase buffer, 1 µl of DNA polymerase (5-15 units), and H_2O to a final volume of 25 µl.
2. Incubate at 12-14°C for 15-45 minutes.
3. Stop reaction and remove unincorporated nucleotides as above.

Measuring DNA uptake:

1. Place 200 ng of labeled DNA and 1 ml of cells to be tested in a microfuge tube (assays should be done in duplicate). Include as negative controls non-competent cells and tubes with no DNA.
2. Roll or shake gently for 10 minutes at 37°C.
3. Stop uptake by adding 50 µl of DNase I and placing on ice for 5 minutes.
4. Add 100 µl of 5 M NaCl, vortex, and pellet cells at 13,000 for 1 min.
5. Discard supernatant and resuspend cells in M-IV + NaCl
6. Pellet cells again.
7. Discard supernatant and resuspend cells in 200 µl of room temperature M-IV
8. Transfer cells to a scintillation vial and add 1 ml scintillation fluid.
9. Calculate DNA uptake: First correct uptake for background counts in the negative control tubes, then multiply corrected counts taken up by the specific activity of the DNA.

4. Notes.

1. Bald patches can interfere with competence assays. These are areas of small or missing colonies, often centrally-located on sBHI agar plates containing antibiotic. They are commonly seen when large numbers of antibiotic-sensitive cells have been plated to detect a small number of resistant cells. In some cases no colonies at all are seen on a plate that should contain thousands of colonies. Bald patches are most troublesome when using kanamycin selection. The biological cause of these patches is unknown, but they interfere with colony counts and thus can limit the ability to measure transformation frequencies of moderately-competent cultures. Use of MBI brand agar appears to reduce the problem.
2. The disposable filter funnels from Nalgene (illustrated in Figure 3) are very convenient, especially if several cultures are to be made competent. A non-disposable filtration apparatus may also be used; this is less convenient as it must be presterilized with the filter in place. Use only filters with 0.2µ pores because *H. influenzae* cells will pass through 0.45µ pores. If the volume to be filtered is too large or the culture is too dense, the cells will clog the filter. If 0.2µ filters are not available, or if large or very small volumes of competent cells are needed, exponentially growing cells can instead be collected and washed by centrifugation at 5000 rpm for 5 minutes at room temperature.
3. Cells must be in exponential growth when they are filtered. Do not let the culture sit for even a few minutes before filtering; if the cells are ready but you are not, dilute the culture with additional sBHI to keep it in exponential growth below OD_{600}=0.3. A larger volume of cells at a lower density may be used if more convenient.
4. The transformation frequency of competent cells may decrease slightly on freezing. This does not usually cause problems, but freshly prepared cells should be used if highly competent cells are needed.

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TABLE I M-IV competence medium

Solution 21

L-Aspartic acid	4.0 g
L-Glutamic acid	0.2 g
Furmaric Acid	1.0 g
NaCl	4.7 g
K_2HPO_4	0.87 g
KH_2PO_4	0.67 g
Tween 80	0.2 ml
Distilled water	850 ml

Adjust pH to 7.4 with 4N NaOH. Add distilled water to 1 liter. Dispense 100 ml per bottle; autoclave. Solution may appear cloudy after autoclaving but clears upon cooling.

Solution 22

L-Cystine	0.04 g
L-Tyrosine	0.1 g

Dissolve in 10 ml of 1N HCl at 37°C. Bring to 100 ml with distilled water and add

L-Citruline	0.06 g
L-Phenylalanine	0.2 g
L-Serine	0.3 g
L-Alanine	0.2 g

Filter sterilize

Solution 23

$CaCl_2$ 0.1 M solution, *autoclave.*

Solution 24

$MgSO_4$ 0.1 M solution, *autoclave.*

Solution 40

5% (w/v) vitamin-free casamino acids (Difco) in distilled water, *autoclave.*

Table II. Expression times needed for development of antibiotic resistances

Antibiotic	Concentration	Expression time (min)
Kanamycin	7 µg/ml	0
Novobiocin	2.5 µg/ml	0
Spectinomycin	20 µg/ml	70
Naladixic Acid	3 µg/ml	80
Chloramphenicol	2 µg/ml	90
Tetracycline	5 µg/ml	90
Streptomycin	2.5 µg/ml	100