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Genetic transformation in bacteria

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Abstract. Certain species of bacteria can become competent to take up high molecular weight DNA from the surrounding medium. DNA homologous to resident chromosomal DNA is transported, processed and recombined with the resident DNA. There are some variations in steps leading to transformation between Gram-positive bacteria like *biplococcus pneumoniae* and Gram-negative bacteria represented by *Haemophilus influenzae* but the integration is by single-strand displacement in both cases. Plasmid (RSF0885) transformation is low in *Haemophilus influenzae* but this is increased significantly if (homologous) chromosomal DNA is spliced to plasmid DNA. In *Haemophilus influenzae, rec*1 function is required for peak transformation with chimeric plasmids. Chimeric plasmid fixed presumably extrachromosomally undergoes frequent recombination between homologous segments contained in resident chromosome and the plasmid.

Keywords. DNA-cell interaction; competence; specificity in DNA uptake; single-strand displacement; chimeric plasmid transformation; *rec*1 function; recombination.

Introduction

In the state of competence, certain species of bacteria, notably *Streptococcus pneumoniae, Bacillus subtilis* and *Haemophilus influenzae* can take up high molecular weight (M_r) DNA from the surrounding medium. The end result of this interaction depends on whether the input DNA is homologous or heterologous, that of a phage or of a plasmid. In the event of input DNA being homologous, the resident DNA abstracts the information contained in the input DNA by a physical recombinational mechanism. Thus, the recipient cell would be heritably altered or transformed for one or more incoming gene markers. Phage DNA introduced in a similar manner produces phage particles although the efficiency of transfection is much lower than that of infection. Finally, the input plasmid DNA is fixed extrachromosomally, thereby 'adding' markers to the cell. At least in certain cases, the efficiency of transformation of a plasmid marker is lower than that for a homologous DNA marker. Chimeric plasmids have certain additional requirements in some systems which will also be discussed. More detailed reviews have appeared earlier (Spizizen *et al.,* 1966; Tomasz, 1969; Notani and Setlow, 1974; Lacks, 1977; Smith *et al.,* 1981; Goodgal, 1982).

Abbreviations used: M_r, Molecular weight; CF, competence factor; BF, binding factor.

Introduction of competence

Methods to induce very high levels of competence in *S. pneumoniae* and *H. influenzae* have been developed. A pneumococcal culture becomes competent as it reaches a certain cell density (10^7–10^8 cells/ml) during exponential phase growth but this state lasts for about 15 min or so. Competence in pneumococcus is initiated by competence factor (CF) of 5–10 kdal. It is made by the cell and is excreted in the medium. Exogenous CF will also induce competence. Two phases have been discerned (Ziegler and Tomasz, 1970). In the first phase CF interacts with the cell receptor (protein) to form a complex. In the second phase incompetent cell (CF) requires new protein synthesis to yield competent cell culture (Tomasz, 1970). The new protein synthesis may include the induction of an autolysin whose limited action on the cell wall may unmask the DNA binding sites (Seto and Tomasz, 1974). A DNA binding factor (BF) has been selectively released which has the properties of a DNA receptor (Seto and Tomasz, 1975). Lacks and Neuberger (1975) have reported the membrane localization of a deoxyribonuclease which is involved in transformation (Lacks, 1962; Lacks *et al.,* 1974). Apparently, autolysin exposes both DNA BF and the DNase for DNA uptake and entry.

Morrison and Baker (1979) labelled proteins synthesized before and during competence. They noted a dramatic decrease in most of the precompetence proteins. During competence only 11 major species of proteins were labelled, out of which only a 19·5 kdal species appeared definitely new although there was a hint of 3 others. If CF was inactivated these bands did not appear. Surprisingly, the main competence-specific 19·5kdal protein band was found similar in its electrophoretic properties to pure eclipse complex protein (see later). In *S. sanguis,* Raina and Ravin (1980) also observed synthesis of a set of 10 proteins, 8 of which were early and 2 were late. One of the early proteins, El6, with M_r of 15500 binds specifically to donor DNA after uptake. It was presumed that this protein is involved in translocation of DNA from cell membrane to the chromosome.

In contrast to pneumococci, *B. subtilis* cultures develop a relatively low level of competence—upto 18 % of the cells become competent (Bott and Wilson, 1968). It requires almost 8 h of growth in the synthetic medium of which 2-3 h are after cessation of exponential growth. Singh and Pitale (1968) could enrich competent cell fraction by sedimenting through sucrose gradients. Competent cells sediment slower than non-competent cells. Similarly, Cahn and Fox (1968) could separate competent from noncompetent cells by the differences in their buoyant densities in a Renografin gradient. Lighter density cells were the competent fraction and these constituted upto 10 % of the total.

H. influenzae and *H. parainfluenzae* appear to become competent when the cell division is blocked but protein synthesis is allowed to take place. No CF appears to be involved (Herriott *et al.,* 1970). A number of *H.influenzae com*$^-$ (competence-defective) mutants have been isolated (Caster *et al.,* 1970; Postel and Goodgal, 1972). Kahn *et al.* (1979) reported DNA binding activity of vesicles produced by competence-deficient mutants of *Haemophilus.* A number of polypeptides, presumably involved in competence development or DNA uptake, were detected by Concino and Goodgal (1981) but none of these were specifically implicated in a competence function. On the other hand, Concino and Goodgal (1982) observed that *com* 51 released vesicles containing

DNA receptor proteins during competence development which had the capacity to bind homologous but not heterologous DNA. Kahn *et al.* (1982) observed morphological differences between competent and non-competent cells of both *H. influenzae* and *H. parainfluenzae.* When thin sections were examined by electron microscopy they noted that competent *H. parainfluenzae* had five times as many membranous extensions as non-competent cells. Moreover, upon exposure to transforming DNA, *H. parainfluenzae* cell extensions disappeared and were apparently internalized. The name 'transformasome' has been proposed for the membranous extensions developed during competence of *Haemophilus* cells which apparently are involved not only in the uptake but also for protecting DNA even when unmethylated from restriction nuclease attack (Kahn *et al.,* 1983). An odd observation is that *Haemophilus* transfection with phage DNA is efficient only with cells made competent by aerobic-anaerobic-aerobic rather than MIV method.

Neisseria gonorrhae is another Gram-negative bacterium that is transformable and surprisingly throughout its growth phase. Virulent colony types are transformable with a high frequency (upto 1 %) and non-virulent with very low frequency (10^{-7}). Virulent colony types are also piliated and non-virulent ones are not and thus are correlated with competence. There is no evidence for other competence factors (Biswas *et al.,* 1977).

Specificity in DNA for uptake

While competent pneumococcal cells can take up virtually any high M_r DNA, there seems to be some specificity involved in *H. influenzae.* Thus, Scocca *et al.* (1974) observed that *H. influenzae* can take up its own DNA with a remarkable efficiency but not that of *Escherichia coli* B or *Xenopus laevis.* Only *H. parainfluenzae* DNA could compete with *H. influenzae* DNA. The basis for this specificity has been shown to be an uptake site (recognition sequence) in DNA, (Sisco and Smith, 1979). Deich and Smith (1980) have inferred that regardless of the input DNA length (from 1–40 kb), the efficiency of uptake binding sites is the same. Danner *et al.* (1980) sequenced 4 small fragments of *H. parainfluenzae* to find the common sequence in the ones that are not taken up efficiently and its absence in the ones that are taken up poorly. They came up with an asymmetric 11 bp sequence 5′ AAGTGCGGTCA 3′ as the uptake sequence. They also showed that ethylation of phosphoryl groups in this sequence reduced uptake significantly. Danner *et al.* (1982) synthesized the 11 bp uptake sequence and constructed a series of plasmids incorporating this sequence. They concluded that this sequence is necessary and sufficient for uptake in *H. influenzae.* However, there could be as much as 48-fold difference in uptake activity and this they attributed to AT-richness of the DNA next to the sequence. Using sonicated DNA, Vogt and Goodgal (1984a) inferred that the number of uptake signals has been underestimated before and this discrepancy was accounted for by assuming multiple receptors which bind to a high M_r DNA containing multiple uptake signals. Vogt and Goodgal (1984b) also suggested that the uptake recognition signal may have its basis in 'structural capacity' which may be non-specifically dependant on a sequence.

The nature of irreversibly-bound intracellular input DNA

The initial binding to a competent cell may be reversible by external agents but very soon it is not removable by DNAase. The irreversibly-bound DNA in pneumococci is not recoverable in native form—part of it is degraded and part of it is denatured (single-stranded). Entry of DNA into pneumococcus appears to be coupled with its becoming single-stranded with concomitant degradation of the complementary strand (Lacks, 1962; Lacks *et al.,* 1974). Surprisingly, a double mutant with two deficient DNases was normal for entry and post-uptake events. However, further selections made for higher deficiencies of the nucleases and those deficient in transformation provided illuminating information. Two classes of transformation-deficient mutants were isolated. One which did not bind DNA (*ntr* mutants) and the other bound and accumulated DNA at the surface but the DNA did not enter. These latter cells were missing a DNase (*noz* mutants). A DNA translocase function has been postulated for this enzyme (Lacks *et al.,* 1974). *noz* mutants are probably additional mutation (s) in the *end* gene. Lacks and Neuberger (1975) have localized endonuclease I in the membrane which appears to be responsible for converting double-stranded DNA to single-strand inside and oligonucleotides outside. They also localized autolysin in the membrane. BF and *end* I may be exposed by the action of autolysin. *ntr* mutants may lack BF.

Intracellular single-stranded pneumococcal DNA is in an 'eclipse' *i.e.* is not biologically active. It is 'coated' by a 19·5 kdal protein. Recovery from eclipse occurs following integration. The role of eclipse protein may be to promote its entry into the cell, to protect it and to have it in an 'integrational' conformation (Morrison, 1977).

In *B. subtilis,* Bodmer and Ganesan (1964) found intracellular input DNA in the native, hybrid and denatured form. Eisenstadt *et al.* (1975) reported a denatured DNA-binding protein activity from *B. subtilis* competent cells. This protein protects denatured DNA from nuclease solubilization. This' activity may be analogous to pneumococcal eclipse protein or may have some other function.

Unlike in pneumococcal or *B. subtilis* transformation, no denatured input DNA is detected intracellularly in *H. influenzae* (Notani and Goodgal, 1966). As a matter of fact the bulk of unintegrated DNA appears to be in a native form and is biologically active (Notani, 1971). However, slower-sedimenting fragments with lower biological activity, designated species II molecules, are observed but these were considered not intermediates but byproducts of transformation (Notani, 1971). Two of the recombination-deficient mutants, *rec* 1 and *rec* 2, have been analyzed. With *rec* 2, DNA is irreversibly-bound but is reisolatable without appreciable change. DNA is neither degraded nor is it integrated. On the other hand, with *rec* 1 the radioactive label in the input DNA is transferred to resident DNA but genetic information is not transferred (Notani *et al.,* 1972). Transformation in *H. parainfluenzae* also yields intracellular DNA in a biologically active form and is not denatured (Notani and Setlow, 1972). *Haemophilus* phage 1 (HP 1) DNA introduced by transfection also undergoes fragmentation. However, only in recombination-proficient strain part of it is reassembled yielding some phage biological activity (Notani *et al.,* 1973). Double-stranded nature of unintegrated donor DNA is a bit surprising because the integration appears to be primarily single-stranded (Notani and Goodgal, 1966). Elsewhere it is discussed that *Haemophilus* DNA first enters into transformasome and from there linear DNA exits

into the cell more rapidly than the covalently-closed circular DNA. It may be conjectured that *rec* 2 mutant has its exit pore blocked or that some nuclease is missing so that the initial processing of DNA does not occur.

Genetic recombination and integration of chromosomal DNA

Using radioactivity and density labels, Fox and Allen (1964), Fox (1966) showed that newly introduced DNA is a hybrid and physically a heteroduplex. The size of insert is about 1-2 million daltons. The picture of integration from *H. influenzae* is somewhat similar, even though a free single-stranded intermediate is not observed. The kinetics of integration could be followed even without a density label in the donor DNA. Bulk separation of donor DNA from resident DNA + integrated DNA was achieved by zone sedimentation (Notani and Goodgal, 1965; Notani *et al.,* 1966). However, intact physical integration of donor DNA could not be shown from those experiments. By density-label experiments, it was observed that in *H. influenzae* transformation also single-stranded donor DNA segments are integrated into the genome (Notani and Goodgal, 1966). The average size of the integrated segments was estimated at 5–6 $\times 10^6$ M_r. Subsequently, it was also shown that either strand is effective in transformation (Goodgal and Notani, 1968). In *B. subtilis* transformation, donor density-label atoms were found in native recipient, hybrid and 'denatured' donor DNA (Bodmer and Ganesan, 1964). There is evidence for single-stranded integration (Bodmer, 1965). It has been shown also for *B. subtilis* that transforming activity resides in both complementary strands (Chilton, 1967).

Genetic recombination during transformation occurs apparently by a single-strand invasion and displacement (Fox, 1966). In *H. influenzae* also a similar model would be valid with the proviso that the initial interaction takes place between two double-stranded DNA molecules out of which one-strand is integrated and the other degraded (Notani and Goodgal, 1966) and the interaction would produce a byproduct of species II molecules (Notani, 1971). There is little information on the proteins that are involved except the circumstantial evidence that *H. influenzae rec* 1 gene may be a type of annealing protein (Notani *et al.,* 1973).

The primary product during transformation is a duplex which is heterozygous for the input and resident gene marker. A mismatch at the site of heterozygosity would thus be expected. In pneumococcus, it has been observed depending on their relative efficiency of transformation of single-site markers can be grouped into four categories *viz.* VHE, HE, IE and LE with relative efficiency ratios of 1:0·5:0·2:0·05. respectively (Lacks, 1966). LE markers are low in efficiency presumably because donor DNA strand is corrected out more often. In Hex^- mutant cells, transformation of LE marker can be raised to that of HE. For amylomaltase *mal* P gene, Lacks *et al.* (1982) observed that transition mismatches are highly corrected by repair but transversion mismatches are less susceptible. With LE markers the donor DNA is apparently recognized as 'foreign' and may be attracting restriction mechanisms. Claverys *et al.* (1983) looked at the mismatch repair events at *ami* A locus. They observed that LE markers came from transitional changes AT→ GC. IE markers arise from transversional changes AT → TA and HE from transversions GC → TA or GC → CG.

Transformation with plasmid and chimeric DNA

Plasmid RSF0885 a multicopy plasmid originally isolated from *H. influenzae* b has been extensively used in transformation and recombinant DNA work in *H. influenzae* Rd. It has a M_r of $3{\cdot}7 \times 10^6$, carries an *amp*^{r} marker and is cut once with *Pvu* II outside the marker. Since *H. influenzae* cells become almost 100 % competent, it was expected that RSF0885 should give high transformation. As a matter of fact, only about 10^4 transformants/μg of DNA were obtained which was 3–4 orders of magnitude lower than expected (Notani *et al.,* 1981). Transformation with RSF0885 did not require the expression of *rec* 1 or *rec* 2 genes. It was observed also that chromosomal DNA effectively competes with the plasmid DNA and essentially wipes out the plasmid transformation. These observations were interpreted in the light of reports of (i) Scoca *et al.* (1974) that *H. influenzae* binds its own DNA well but not that of *E. coli* B and suggested that there is some specificity in uptake of DNA by *H. influenzae* and (ii) Sisco and Smith (1979) and Chung and Goodgal (1979) that *H. influenzae* DNA contains recognition regions (uptake sites which are responsible for the specificity of uptake). Sisco and Smith (1979) noted that 600 copies of an 11 bp sequence are distributed in the *H. influenzae* genome. The low efficiency of transformation with RSF0885 was thus attributed to the plasmid DNA not containing any uptake sequence.

Consistent with this notion it was observed that when chromosomal DNA was spliced to plasmid DNA the transformation for the plasmid marker increased 2 to 3 orders of magnitude (Notani, 1981; Setlow *et al.,* 1981). Surprisingly for efficient chimeric plasmid transformation, both *rec* 1 and *rec* 2 gene expression was required. Both *rec* 1 and *rec* 2 are deficient in recombination but the exact role of recombination in chimeric plasmid DNA transformation is not established.

Setlow *et al.* (1981) cloned a *nov*^{r} (25 μg/ml level) marker. When this chimeric plasmid p*Nov* 1 was used in transformation, 100-fold more *nov*^{r} than *amp*^{r} transformants were observed. None of the novobiocin transformants (17 examined) contained the plasmid, only 5 out of 20 *amp*^{r} transformants contained *nov*^{r} marker but plasmid was present in all the cases and was of the same size. Joshi and Notani (1983) have constructed another vector pJ1-8 which was derived from pD7 (Notani, 1981) consisting of RSF0885 + unmarked chromosomal DNA insert. pJ1-8 has a M_r of only $2{\cdot}5 \times 10^6$ and is cut once with *Eco*RI. It gives virtually no transformation for *amp*^{r}. Splicing chromosomal DNA to pJl-8 increases the *amp*^{r} transformants significantly providing even a more facile assay than with RSF0885. Using this plasmid several *nov*^{r} (2·5 μm/ml level) clones were isolated. Three of these were characterized in detail. In general, observations are in agreeement with those of Setlow *et al.* (1981). Some additional observations have been made.

When three different clones containing *nov*^{r} (2·5) marker were used in transformation, transformation was differential and the ratios of *amp*^{r} : *amp*^{r} *nov*^{r} were somewhat different. Whereas pJ1 -8N2 and pJl-8N20 yielded *amp*^{r} : *amp*^{r} *nov*^{r} ratios of 2:1, pJ1-8N19 gave a ratio of upto 6:1 implying that more recombinants (*amp*^{r} *nov*^{s}) were generated with pJl-8N19 (Joshi, V. P. and Notani, N. K., unpublished observations). Balganesh and Setlow (1984) have also observed different recombination percentages with different clones but they have correlated this with the length of the insert.

A number of chimeric plasmids in *H. influenzae* have been found to be unstable.

Setlow *et al.* (1984) have observed that such plasmids are however stable in a strain which lacks or does not have an inducible phage.

Kahn *et al.* (1983) and Barany *et al.* (1983) have inferred that *H. influenzae* DNA following uptake enters into membranous extensions called transformasomes and DNA there is protected against external nucleases and internal restriction enzymes. Entry into the cell apparently is much faster if the DNA is linear but slower if circular. These experiments were done with plasmids. pCML6 or pPUP3, both of which utilize pBR322 replicator and the only *H. influenzae* DNA that pPUP3 has is the 11-bp uptake sequence. It is not known if pBR 322 can replicate in *H. influenzae* and whether that would contribute to any of the observed effects. Furthermore, there is a requirement of *rec* gene expression for transformation with chimeric plasmids which in the case of pPUP3 may have created a problem.

In reextraction experiments in which a chimeric plasmid pD7 (RSF0885 replicater + chromosomal DNA) was used and reisolated plasmid DNA analyzed by sucrose sedimentation, it was observed that not only the relative specific biological activity goes down by about 60 % in 60 min but that profile of the plasmid transforming activity also changes. In 60 min profiles although the activity is somewhat dispersed more, the peak biological activity is surprisingly obtained from fractions sedimenting faster than at 0 min time. This indicates that in 60 min time more than 60 % of the material is processed or inactivated. In *S. pneumoniae,* a multicopy plasmid pMV158 has been utilized to clone genes. A gene for maltose was successfully cloned and expressed in strains having a deletion of the locus (Stassi *et al.,* 1982). Lopez *et al.* (1982) have made a distinction between *plasmid transfer* and *plasmid transformation.* In the formercase, the recombinant plasmid is established by itself and in the latter case an endogenous homologous plasmid, is altered. Mal^+ transformant frequency was only slightly higher (5×10^4) by transformation (*mal* deletion in chromosome and pMV 158) than by transfer ($1{\cdot}3 \times 10^4$) *i.e.* with only *mal* deletion in the chromosome. Since entry of DNA in *S. pneumoniae* is by a denaturation and degradative process, authors have proposed models which allow interaction between DNA molecules or with itself to yield intact plasmids.

In *S. pneumoniae,* Barany and Tomasz (1980) reported transformation for drug resistance markers with several heterologous plasmid DNAs. The frequencies ranged from 5×10^{-1} to 10^{-5}. This transformation did not occur with an endonuclease I deficient strain. Unlike in *B. subtilis* in which competent cells are transformed mainly by oligomers, in *S. pneumoniae* monomers are quite effective.

In *B. subtilis,* Canosi *et al.* (1978) and Mottes *et al.* (1979) showed that transformation in *B. subtilis* of competent cells is mainly by oligomers. However, *B. subtilis* protoplasts can be transformed by monomers and with a very high efficiency. Approximately 4×10^7 transformants were obtained per μg of CCCDNA (Chang and Cohen, 1979). Recombinant plasmids could also transform although with a lower efficiency (one to three orders of magnitude lower). Canosi *et al.* (1981) reported that monomeric form of pC194 transforms efficiently if chromosomal DNA is spliced to it. These observations have been interpreted by Canosi *et al.* (1981) to suggest the operation of two pathways: a major one requiring *rec* gene expression and homology between recipient and donor DNAs and a minor one *rec*-independent and homology-independent. The processing of incoming plasmid DNA requires conversion of single-stranded, denatured form

observed by deVos *et al.* (1981) to a double-stranded circular active form through *rec* E4 gene expression. The exact physiological role of *rec* E4 gene is unclear at present.

Discussion

Genetic transformation in bacteria has revealed several unusual features which include interaction between cell (receptors etc.) and DNA, the transport of the latter across the cell envelope, protection and preparation of DNA for recombination, the interaction between two DNA molecules and finally the act of recombination itself. Gram-positive (namely pneumococci) and Gram-negative (namely haemophilus) bacteria seem to have developed somewhat different mechanisms although the end result is a homologous displacement of resident DNA segments by input (chromosomal) DNA. While pneumococci use the CF, autolysin, BF, an endonuclease and 'eclipse' protein to do the uptake, protection and transport of DNA, *Haemophilus* shows a specificity in DNA uptake. Sequestering of DNA irreversibly-bound in transformasomes presumed to protect DNA from restriction is also a novel feature of this system. Also, whereas intracellular transforming DNA in pneumococci is recoverable only as degraded or denatured (single-stranded) material, bulk of intracellular donor DNA in *Haemophilus* is recoverable in native form. Of course some amount of degradation and fragmentation is also observed.

The role of *rec* genes in promoting transformation with chimeric plasmids is not readily understood. It may be conceivable that after pairing, resident chromosome DNA may physically assist its entry into the cell. We have observed that recombination with certain chimeric plasmids takes place quite early. In addition, majority of the fixed plasmids are recombinant for the marked region. We have imagined that the fixation of a recombinant plasmid is preceded by resident DNA single-strand invasion of the plasmid, followed by mismatch correction in which the resident marker would be eliminated 50% or more than 50% of the time.

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