Electrotransformation and natural transformation of *Streptococcus pneumoniae*: requirement of DNA processing for recombination

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Electrotransformation has been used as a tool to introduce genes carried on replicative vectors in hundreds of bacterial species. In this study, the technique was used to try to obtain recombination of markers in the chromosome of the naturally transformable bacterium *Streptococcus pneumoniae*. Recombination was not observed even using naturally competent cultures. Both chromosomal and cloned DNA, denatured or native, were without effect. These results suggest that it is not sufficient to introduce DNA into the cell to obtain recombinants in this bacterium. The integration of markers into the chromosome in naturally competent cells must require DNA processing during entry. Electrotransformation of replicating plasmids is *recA*-independent but can be facilitated by a *recA*-dependent process. This facilitation required the induction of the natural competence machinery, probably involving partial homologous pairing.

Keywords: electrotransformation, recombination, plasmid facilitation

INTRODUCTION

Streptococcus pneumoniae, previously classified as *Diplococcus pneumoniae*, has preoccupied bacteriologists since the turn of the century when pneumonia was the leading cause of death. A major advance in attempts to protect individuals from infection by using antiserum was the discovery by Heidelberger & Avery (1922) that the specific antigenic material within the pneumococcus capsule was not a protein but a polysaccharide. As a result of intense investigation of this pathogen, genetic transformation of capsular types was reported by Griffith (1928). This was shown to result from the transfer of DNA by Avery *et al.* (1944) and the modern field of molecular genetics was born. Over 20 years later, the DNA mismatch repair system was discovered in this organism (Ephrussi-Taylor & Gray, 1966) and later found in other prokaryotes and eukaryotes. A homologous human system was more recently shown to protect against certain types of colon cancer (Fishel *et al.*, 1993; Leach *et al.*, 1993). Such long-term ramifications of the original discovery of the DNA-induced

transformation of *S. pneumoniae* could not have been foreseen. The discoveries resulted from continuous investigations of the mechanism of genetic transformation by just a few laboratories (for a review, see Claverys & Lacks, 1986).

DNA uptake consists of two steps: binding of the DNA to the outer membrane and entry into the cell where the DNA becomes insensitive to DNases (Lacks *et al.*, 1974). Binding causes single-strand breaks in the donor DNA. During entry one of the two strands of donor DNA is degraded to oligonucleotides and the other strand is introduced into the cell in an ss form (Lacks, 1962), probably by the action of endo A nuclease. Specific end-labelling of donor DNA strands has shown that the incoming strand enters in a 3' to 5' direction (Méjean & Claverys, 1988). The entering strand is coated by proteins produced by competent cells (Morrison, 1978). This ssDNA anneals with the complementary strand of the recipient chromosome and becomes physically integrated. During the period when the donor DNA is single-stranded it has no transforming activity (Fox, 1960). This 'eclipse' is due to the inability of ssDNA to efficiently penetrate the bacteria. The half-time for recovery from eclipse at 30 °C is less than 5 min for single-site markers (Ghei & Lacks, 1967). This complex

Abbreviations: Csp, competence-stimulating peptide; FIGE, field inversion gel electrophoresis; ds, double-stranded; ss, single-stranded.

processing of DNA requires the activity of several genes. A few of these have been described: *endoA* encodes an endonuclease (Lacks, 1970; Lacks *et al.*, 1974), *comC* encodes a peptide that is cleaved to yield a hormone-like 17-aa competence-stimulating peptide (Csp), *comD* encodes a Csp receptor (Havarstein *et al.*, 1995) and the *recA* operon is required for recombination (Martin *et al.*, 1995).

Is the process of DNA entry into the cell connected with recombination into the chromosome? In other words, is it sufficient to introduce DNA across the cellular envelopes to obtain transformants? To study this process it is possible to use high-intensity electric field pulses to make biomembranes reversibly permeable to DNA and to test for transformability. In a preliminary investigation we were unable to transform chromosomal markers in *S. pneumoniae* by electroporation (Bonnassie *et al.*, 1989), whereas replicating plasmids were efficiently transferred under the same conditions. There are a number of possible reasons for this failure to obtain chromosomal transformants: inability to detect transformants if they are very rare, absence of induced competence or absence of requirements of the complex process at the entry stages during natural competence to integrate the recipient chromosome. Using electroporation to introduce DNA into cells, we have shown that the latter hypothesis accounts for our results.

METHODS

Strains, markers and vectors. The strains of *S. pneumoniae* used in this study are derivatives of R36A (Avery *et al.*, 1944). One of the markers used in the recombination experiments was constructed by insertion of a gene with its own promoter yielding resistance to 4.5 µg chloramphenicol ml⁻¹ (Claverys *et al.*, 1995) at a central position in the *amiA* locus which confers resistance to 2 × 10⁻⁵ M amethopterin (Sicard, 1964). This construct, allowing insertion of the chloramphenicol marker by means of surrounding homology provided by the *amiA* sequence, was cloned in p-Alter1 (Promega) which does not replicate in pneumococci. This vector contains a viral replication origin allowing production of ss phagemid. The other chromosomal marker (*str41*) confers resistance to 2 mg streptomycin ml⁻¹.

Penetration experiments. The 800 bp ds fragment used in penetration experiments was obtained by PCR amplification of part of the *str* gene. The ss form of this fragment as well as the 480-base ss *str* fragment were prepared by the same technique, using unequal amounts of primers to produce a 99% pure ssDNA. PCR amplification of the whole *tetL* gene was performed on pLS1 DNA (Lopez *et al.*, 1982). For all penetration experiments, except that of chromosomal DNA, DNA was labelled by PCR, adding [α -³²P]dTTP to the dNTP mix, to avoid gaps in the molecules which result in natural uptake interruption (Méjean & Claverys, 1993).

The labelled chromosomal DNA used in electroporation and the pLS1 and M13 DNAs used in hybridization experiments were produced with the Megaprime kit (Amersham) which incorporates labelled dNTPs by elongating randomly hybridized degenerate primers. Electroporation was carried out as described below for electroporation. The cells were washed twice before measuring retained radioactivity. The operation was repeated without electric shock and the results

compared. Natural penetration experiments were conducted in the same way, replacing electric treatment by natural penetration.

Field inversion gel electrophoresis (FIGE). FIGE has been described in detail by Kauc *et al.* (1989) and Gasc *et al.* (1991). Southern blot hybridizations (Southern, 1975) were performed as described by Sambrook *et al.* (1989) at 55 °C.

Preparation of competent cells and transformation. Natural competence was obtained from exponentially growing cultures in CAT medium (Morrison *et al.*, 1983) supplemented with 0.2% bovine serum albumin and 10⁻³ M CaCl₂ (final concentrations). The cells were centrifuged and resuspended in the same medium supplemented with 3 × 10⁻³ M NaOH and 0.25 µg Csp ml⁻¹ (Havarstein *et al.*, 1995). The replicative plasmid used was pSP2 (Prats *et al.*, 1985a) which carries resistance markers for erythromycin and tetracycline. Transformants were selected after incubation for 2 h at 37 °C on selective plates (1.5 µg tetracycline ml⁻¹) to test for plasmid replication in the recipients. When used in facilitation experiments, pSP2 harboured either an *S. pneumoniae* insert of about 1.5 kb or a 1.4 kb *Escherichia coli* insert (Prats *et al.*, 1985b).

For electrotransformation experiments, cells were grown at 37 °C in CAT complete medium (Morrison *et al.*, 1983) supplemented with 2% bovine serum albumin (final concentration) and harvested in the early exponential growth phase (OD₆₀₀ 0.1–0.2). They were washed twice and concentrated tenfold in electroporation medium (0.5 M sucrose, 7 mM potassium phosphate, pH 7.5, 1 mM MgCl₂). A volume of 0.8 ml cell suspension was poured into a Bio-Rad cuvette. Plasmid DNA was added at a concentration of 1 µg ml⁻¹ and chromosomal DNA at 20 µg ml⁻¹. The mixture was kept at 0 °C for 10 min. A single impulse of current was given with the Bio-Rad apparatus set at maximum (6.25 kV cm⁻¹, 25 µF).

RESULTS AND DISCUSSION

Test for electrotransformation of chromosomal markers

Electrotransformation of wild-type strain 800 was performed as described in Methods. When DNA from plasmid pSP2 was used, transformants selected for their resistance to erythromycin were obtained at the usual frequency (Table 1). With chromosomal DNA carrying the *str41* marker, transformants could not be selected even at the highest concentration of DNA (20 µg ml⁻¹). Spontaneous mutants resistant to streptomycin (200 µg ml⁻¹) were also undetectable. Even upon increasing the number of bacteria plated to the upper limit allowing selection, transformants were undetectable. We could estimate that the frequency of marker rescue was below 10⁻⁹ per viable cell. Therefore, penetration of chromosomal DNA in *S. pneumoniae* by high-intensity electric fields was not sufficient to obtain transformants. We have previously reported that under these conditions dsDNA penetrates into these bacteria without a single-strand step in contrast to natural transformation (Lefrançois & Sicard, 1997). A possibility is that ssDNA is required to recombine with the chromosome. As denatured plasmid DNA yields electrotransformants, although at a reduced frequency (Lefrançois & Sicard, 1997), we tested boiled chromosomal DNA. Electro-

Table 1. Electrotransformation experiments using chromosomal or plasmid DNA

Strain R800 (Lefevre *et al.*, 1979) was used in all experiments. Induction of natural competence was obtained by addition of Csp. Natural competence was measured using chromosomal *str41* DNA at saturating concentrations ($1 \mu\text{g ml}^{-1}$) before and after shock to ensure that it was conserved during the operation. To prevent natural transformation, pancreatic DNase ($50 \mu\text{g ml}^{-1}$) was added immediately (< 2 s) after the electric pulse. A control experiment without electric shock was also performed under the same conditions and did not yield any transformants. Plasmid pSP2 is a replicative plasmid in *S. pneumoniae*. Plasmid pAmC is an *E. coli* plasmid that does not replicate in this bacterium; it contains the chloramphenicol resistance gene inserted in the *amiA* locus. The synthesized oligonucleotide 60 *str* carries the *str41* mutation, as does 480 *str* obtained by PCR amplification. DNA was used at a concentration of $1 \mu\text{g ml}^{-1}$ except in expts 3 ($4 \mu\text{g ml}^{-1}$) and 4 ($12 \mu\text{g ml}^{-1}$). When survival was in the 10% range, electroporation was performed in 0.5 M sucrose instead of the buffer described in Methods.

Expt	Processing of the recipient strain		Natural competence (%)	Survival after pulse (%)	Transformation frequency						
	Natural competence	Electric pulse			pSP2 (circular)	Native chromosomal <i>str</i>	Denatured chromosomal <i>str</i>	pAmC (ds circular)	pAmC (ss circular)	60 <i>str</i> (ss linear)	480 <i>str</i> (ss linear)
1a	—	+		10.9	2.3 × 10 ⁻⁵						
1b	—	+		10.6		< 10 ⁻⁸					
2	—	+		53.8			< 10 ⁻⁹				
3	—	+		73.0				< 10 ⁻⁸			
4	—	+		43.0					< 10 ⁻⁸		
5a	+	+	1.2	7.0	3.1 × 10 ⁻⁴						
5b	+	+	1.2	9.4		< 10 ⁻⁸					
6	+	+	3.6	67.2			< 10 ⁻⁹				
7	+	+	2.9	90.0				< 10 ⁻⁹			
8	+	+	3.5	49.7					< 10 ⁻⁸		
9	+	+	3.55	71.7						1.4 × 10 ⁻⁷	
10	+	+	3.5	56.5							9.6 × 10 ⁻⁷
11	—	+		53.9						< 10 ⁻⁸	
12	—	+		55.6							< 10 ⁻⁹

Table 2. Penetration of ds- and ssDNA during electroporation

Labelled DNA was produced as indicated in Methods. Each assay was performed with equivalent numbers of cells (0.4×10^8). The strain used was R800.

DNA (ng ml^{-1})	Electroporability	Radioactivity retained after washing (c.p.m.)	Total radioactivity in sample labelled DNA used in each assay (c.p.m.)
Chromosomal (210)	+	13 160	8.16×10^5
Chromosomal (210)	—	3 265	8.16×10^5
pLS1 (21)	+	12 944	15.5×10^5
pLS1 (21)	—	6 543	15.5×10^5
800 bp ds fragment 1 (560)	+	18 784	4.09×10^5
800 bp ds fragment 1 (560)	—	3 117	4.09×10^5
800 bp ds fragment 2 (5600)	+	52 142	4.06×10^6
800 bp ds fragment 2 (5600)	—	37 341	4.06×10^6
800 bases ss fragment (230)	+	47 911	3.65×10^6
800 bases ss fragment (230)	—	30 414	3.65×10^6

transformants were not observed (Table 1, expt 2). To increase the sensitivity of detection of rare transformants we cloned a fragment of DNA carrying a marker conferring resistance to chloramphenicol flanked by *S. pneumoniae* *amiA* sequences in the plasmid pAmC that replicates in *E. coli* but not in *S. pneumoniae*. This plasmid can be obtained either as ds- (replicative form) or ssDNA (as described in the Methods) and in naturally competent *S. pneumoniae* cultures it yields chloramphenicol transformants by insertion in the chromosome

at the *amiA* locus. Electrotransformants could not be observed using either ds or ss circular forms of this DNA (expts 3 and 4). Similar assays for chromosomal markers conferring either resistance to cefotaxime [carried on a 2 kb PCR fragment amplified from the gene encoding penicillin-binding protein 2x (Laible *et al.*, 1989)] or sensitivity to amethopterin [carried on phage M13 and screened for the ability to grow on a synthetic medium containing an excess of isoleucine (Sicard, 1964)] were also unsuccessful.

Therefore, penetration of ds- or ssDNA by electropermeabilization is not sufficient to obtain chromosomal recombinants. Other steps that are induced during natural competence might be required to insert the incoming DNA into the chromosome. The many proteins that are overexpressed or newly synthesized during natural competence (Morrison & Baker, 1979) might catalyse such steps.

Electrotransformation of naturally competent cultures

Exponential cultures were grown and treated with Csp as described in Methods. A sample was mixed with DNA carrying the *str41* marker to measure natural competence. The frequency of natural transformation was greater than 3% (Table 1, expts 5–10). High-intensity electric field treatment was applied in the following experiments. Pancreatic DNase was added at a concentration of 5 $\mu\text{g ml}^{-1}$ to the cuvette immediately after the electric shock (<2 s). DNase was also added to agar medium to avoid the effects of natural competence on plates, an event that we have observed when Csp is used. As a control, an aliquot of the same culture was mixed with the same DNA in the cuvette without electric shock and DNase was added within <2 s. Transformants were not detectable. This shows that under these conditions the cells were competent but during this very short period of time the marker carried by the DNA molecule did not enter the bacteria. This was expected since natural penetration of DNA is a relatively slow process (Kent & Hotchkiss, 1964). The first transformants can only be detected when DNase is added 20 s after the DNA is mixed with the cells and penetration of 50% of the molecules requires 7 min (A. M. Sicard, unpublished results). When aliquots of the same culture were electropermeabilized in the presence of the chromosomal *str41* marker and DNase added immediately, transformants were not observed (Table 1, expt 5b). ssDNA was also ineffective (expt 6). Both forms of the plasmids were also inefficient when natural competence was induced (expts 7 and 8).

We tried to increase the probability of obtaining electrotransformants by using medium-length oligonucleotides carrying a resistance marker. If such a short region of homology could produce transformants compared to a marker carried by a several kilobase plasmid, the sensitivity of the test could be significantly improved because of the increased molarity with respect to the mass. We synthesized a 60-base oligonucleotide carrying the *str41* marker (60 *str*). A PCR-amplified 480 nt ssDNA (99%) carrying the same mutation in a central position was also synthesized (480 *str*). It can be seen from Table 1 that few transformants were obtained (expts 9 and 10). Their identity was verified by further cultivation and resistance measurements. In the absence of competence, electrotransformants were not observed (expts 11 and 12). Therefore, it is very difficult or impossible to obtain electrotransformants with several kinds of DNA when chromosomal recombination is

required, even when natural competence is induced. Rare electrotransformants were obtained when an oligonucleotide carrying the streptomycin resistance marker was used after induction of natural competence.

One might wonder if chromosomal DNA penetrates during electroporation. To answer this question, we labelled several DNA preparations and measured the radioactivity of washed cells after electric pulse. The data in Table 2 show that chromosomal DNA does penetrate, as well as plasmid DNA. Therefore, the absence of transformants was not due to a defect of penetration. Short (800 bp) ds and ss fragments do penetrate, although less efficiently than chromosomal or plasmid DNA.

A hypothesis to account for the absence of transformants could be that the electric pulse inactivated the recombination machinery of the bacteria. It is possible to test for this activity by investigating plasmid facilitation that requires recombination (Lopez *et al.*, 1982).

Facilitation of plasmid transfer by electrotransformation

In natural transformation, plasmid donor DNA is converted on entry to a linear single strand of one genome length or less due to DNA degradation. Establishment of the plasmid requires the interaction of two overlapping fragments to reconstitute the replicon. When the plasmid carries a segment of DNA homologous to the host chromosome, the frequency of plasmid transfer is drastically increased (Table 3). This observation, named facilitation, depends on synapsis, homology and recombination of the entering ss plasmid molecule with the host chromosome (Lopez *et al.*, 1982). In the absence of homology with the chromosome, establishment of plasmids also requires pairing between two partial plasmid genomes. As there is no evidence for a single-strand step or DNA degradation during electropermeabilization, facilitation was not anticipated. Moreover, the interaction of two plasmid molecules to obtain complete plasmid replicons was supported by the observation that in naturally competent cells the frequency of plasmid transformation of monomeric plasmid DNA without homology with the chromosome depends quadratically on the DNA concentration (Saunders & Guild, 1981). In contrast, dependence of plasmid electrotransformation on DNA concentration is linear, arguing against interaction between plasmid genomes (Bonnassie *et al.*, 1989). Nevertheless, we looked for facilitation during electrotransformation.

We submitted to electric shock naturally competent or non-competent cultures mixed with pSP2 plasmid DNA carrying an erythromycin resistance gene and transformants were selected. When pSP2 containing a 1.5 kb fragment of chromosomal DNA was used in a first experiment, induction of natural competence increased the transformation frequency 56-fold compared to electrotransformants. With pSP2 carrying a heterologous fragment from *E. coli* of the same length there

Table 3. Plasmid facilitation during electrotransformation

Cultures of both strains were divided, one part was induced to natural competence by addition of Csp and then both were submitted to the electric pulse. To prevent natural transformation, pancreatic DNase (50 µg ml⁻¹) was added immediately (<2 s) after the electric pulse. A control experiment without electric shock was also performed under the same conditions and did not yield any transformants. In all cases when naturally competent cells were electroporated, natural competence was measured for the *str41* marker before and after shock to ensure that it was conserved during the experiment. Plasmid pSP2 homol contains approximately 1.5 kb chromosomal DNA; pSP2 hetero contains 1.4 kb *E. coli* DNA. Both plasmids were used at a concentration of 1 µg ml⁻¹. The mean survival after electrotransformation was 73%. In four independent experiments, the mean efficiency observed for pSP2 homol in a *recA*⁺ naturally competent background was 47.3 times the efficiency observed in the same non-competent strain (SD = 7.3). The efficiency observed for pSP2 hetero with competent cultures was 7 times that observed for non-competent cultures (SD = 2.9). The mean competence rate was 3.4% (SD = 1.8).

Strain	Natural competence	DNA	DNase	Electric pulse	Transformation frequency
<i>recA</i> ⁺	+	<i>str41</i>	+	—	<2 × 10 ⁻⁷
	+	<i>str41</i>	—	—	3.8%
	—	pSP2 homol	+	+	0.96 × 10 ⁻⁶
	—	pSP2 hetero	+	+	0.2 × 10 ⁻⁵
	+	pSP2 homol	+	+	5.43 × 10 ⁻⁵
	+	pSP2 hetero	+	+	2.32 × 10 ⁻⁵
<i>recA</i> ⁻	—	pSP2 homol	+	+	0.66 × 10 ⁻⁵
	—	pSP2 hetero	+	+	0.40 × 10 ⁻⁵
	+	pSP2 homol	+	+	0.36 × 10 ⁻⁵
	+	pSP2 hetero	+	+	0.44 × 10 ⁻⁵

was a 12-fold increase (Table 3). In three other experiments under the same conditions these factors were 53.2-, 42- and 38-fold for competence induction using plasmids with a homologous insert and 5.6- 4.4- and 6-fold when the insert was heterologous, respectively. Therefore, the induction of natural competence strongly increases establishment of replicative plasmids.

Among the many proteins overexpressed during competence (Martin *et al.*, 1995), *recA* is a good candidate to play a role in this phenomenon. We have used a *recA*⁻ strain that is completely blocked in recombination due to an insertion in the gene and consequently this strain is not naturally transformable. In Table 3 it is clear that in this *recA*⁻ strain, the efficiency of electrotransformation was constant whatever plasmid was used. As there was no natural transformation when the *recA*⁻ strain was used as receptor, even with the addition of Csp, it was necessary to ensure that DNA was taken up under these conditions. Labelled DNA was mixed with Csp-treated or untreated *recA*⁺ or *recA*⁻ strains under identical conditions and radioactivity was measured after washing the bacterial suspension. All tests were made with equivalent amounts of both DNA and cells and the total radioactivity used in each assay was 8.26 × 10⁴ c.p.m. The background radioactivity retained by non-competent cell surfaces was 304 c.p.m. for the *recA*⁺ strain and 407 c.p.m. for the *recA*⁻ strain. The radioactivity measured in each induced strain after transformation was 5716 c.p.m. for the *recA*⁺ strain and 4662 c.p.m. for the *recA*⁻ strain: they had incorporated

similar amounts of DNA. This shows that the *recA*⁻ strain was induced to natural uptake at the same level as the *recA*⁺ strain. The natural competence rate of the *recA*⁺ culture, as measured by natural transformation with chromosomal DNA containing a marker conferring streptomycin resistance, was 6.2%.

Surprisingly, the efficiency of *recA*⁺ electrotransformation was also increased by Csp treatment when the plasmid carrying a heterologous fragment of DNA was used. This increase was abolished in the *recA*⁻ background as shown in Table 3. The requirement of *recA* suggests that even for this plasmid, a recombination process facilitates its establishment.

A hypothesis could be that, similar to natural transformation, electrotransformation in a *recA*⁺ background may involve pairing between two plasmid genomes that have been more or less degraded. This could explain the increased number of transformed cells with regard to experiments driven with *recA*⁻ strains. If this was true, the transformation frequency would depend quadratically on DNA concentration because the probability of giving rise to a new replicon depends on the presence of at least two independently penetrated molecules inside the cell. Thus, facilitation of plasmid electroporation was measured at different DNA concentrations. Naturally competent cultures were mixed with DNA, electroporated and DNase was added immediately to prevent natural transformation as already described. In Fig. 1 we can see that the frequency of

Fig. 1. Linear dependence of facilitation on DNA concentration in electrotransformation of competent cells with pLS1. Solid line, experimental curve showing linear dependence; dotted line, theoretical curve, assuming that the interaction between two plasmids determines the enhancement of pLS1 transfer in competent cells. The equation of this curve is $F_n = [n \cdot (0.83 \times F_{0.5})]^2 + n(0.17 \times F_{0.5})$, where n is the ratio of the tested DNA concentration to the concentration tested initially ($0.5 \mu\text{g ml}^{-1}$), F_n is the theoretical frequency corresponding to a concentration n -fold greater than $0.5 \mu\text{g ml}^{-1}$ and $F_{0.5}$ is the frequency observed at $0.5 \mu\text{g DNA ml}^{-1}$. The $F_{0.5}$ multiplicative factor in the equation is calculated assuming that, as observed, the competence-specific enhancement produces a sixfold greater transformation frequency for $0.5 \mu\text{g pLS1 DNA ml}^{-1}$, the frequency ratio of competence-specific enhancement is 0.83 and the frequency ratio characteristic of non-competent cells is 0.17. The strain used was R800. Natural competence (1.5%) was obtained by addition of Csp. The apparatus settings were 25 μF and 2.5 kV using 0.4 cm wide cuvettes, the ionic buffer described in Methods and a 200 Ω shunter.

electrotransformation is proportional to DNA concentration. The theoretical curve for a quadratic function, taking into account that in this experiment 83% of the transformants result from an enhancement of the transfer in a competent background, clearly deviates from the experimental results. This suggests that inside the cell there is no important interaction between plasmid molecules. Therefore, it is likely that this *recA*-dependent facilitation proceeds through a partial pairing between plasmid DNA and the *S. pneumoniae* chromosome.

Search for homology between plasmids and the *S. pneumoniae* chromosome

To test the hypothesis of partial homology between the vector and the chromosome, pLS1 and pSP2 harbouring either the homologous or the heterologous insert were labelled as described in Methods and used as probes for autoradiography of chromosomal fragments separated by conventional electrophoresis or PFGE. Several hybridization bands were obtained for *EcoRI*-digested *S. pneumoniae* chromosomal fragments with all three

Fig. 2. Hybridization assay between fragments of the *S. pneumoniae* chromosome and several probes. Southern blot hybridizations were performed at 55 °C; pLS1 carries *tetL*; pSP2 homol and hetero are derived from pLS1 and contain 1.5 kb *S. pneumoniae* or 1.4 kb *E. coli* DNA, respectively, inserted between two erythromycin resistance genes. M13 was used as a control; *tetL* is a PCR product. All the probes were tested on *EcoRI* digests of the *S. pneumoniae* chromosome, pLS1 was also hybridized to *ApaI* fragments separated by FIGE.

probes as shown in Fig. 2. Under the same conditions an M13 probe did not show any hybridization bands. Three major bands of medium size (25–100 kb) separated by FIGE after *ApaI* digestion also hybridized with the pLS1 probe. Therefore, the plasmid genome contains sequences homologous to several regions of the chromosome. When plasmid pSP2 carrying a 1.5 kb *S. pneumoniae* insert was tested for hybridization, a very strong band corresponding to this insert was observed as expected. It is noteworthy that the pattern of hybridization is the same for all three plasmid probes. Plasmid

Psp2 is derived from pLS1 with the addition of two erythromycin resistance genes and either a 1.4 kb *EcoRI* insert or a 1.5 kb *S. pneumoniae* insert. Therefore, these DNA sequences, which are not shared by all the probes, cannot be responsible for the identical profile. Hence, they are not involved in the homologous regions. Moreover, a probe from the tetracycline resistance gene (*tetL*) harboured by all three vectors did not hybridize with the chromosome under the same conditions, suggesting that the remainder of the pLS1 vector, which is also a common part of all the probes, could be responsible for the profile identity and contains sequences homologous or partially homologous to the chromosome.

Conclusions

Attempts to obtain electrotransformants for chromosomal markers was unsuccessful even when the natural competence machinery was induced and/or when denatured DNA was used to mimic natural uptake. This suggests that the process of entry during natural competence is required to obtain chromosomal recombinants. In natural transformation all ssDNA is associated with proteins in the eclipse complex which protects the entering DNA from nuclease degradation (Morrison, 1978). In electroporation, this step might be impossible due to the very rapid penetration. Indeed, this process might be even more complex than the steps already described. It appears that one, several or may be all these events are necessary to yield chromosomal recombinants. During natural competence, not only does the DNA penetrate into the cell, but it is also processed to prepare for *recA*-dependent homologous recombination in the chromosome. This event is not required for plasmid replication. The other alternative of inactivation of the recombination apparatus by the electric pulse was excluded by the evidence of recombination-dependent plasmid facilitation during electrotransformation.

Failure to obtain chromosomal electrotransformants is generally observed. In *Haemophilus influenzae*, another naturally competent bacterium, this failure has also been reported (Mitchell *et al.*, 1991) and, as we have found, natural competence induction was no more successful.

It is noteworthy that in *recA*⁻ strains plasmid establishment is efficiently obtained by electrotransformation, in contrast with natural competence where *recA*-dependent recombination occurs between two ssDNA molecules containing part of the plasmid genome (Martin *et al.*, 1995). This is in agreement with the demonstration that dsDNA penetrates *S. pneumoniae* during electroporation (Lefrançois & Sicard, 1997) and suggests that this DNA contains an intact genome ready for replication.

We have observed that induction of natural competence markedly increases the establishment of a plasmid containing a fragment homologous to the chromosome. This facilitation, first described by Lacks and co-workers

in a slightly different system (Lopez *et al.*, 1982), is *recA*-dependent, confirming the requirement of recombination to explain the original facilitation experiments. When the vector used in our electrotransformation studies carries a heterologous fragment of the same size as the *S. pneumoniae* fragment, there is still an increased electrotransformation, although at a reduced level. This phenomenon is also *recA*-dependent. It cannot be explained by an interaction between two degraded plasmids, since the frequency of electrotransformation is proportional to DNA concentration. The more likely interpretation is that facilitation results from partial pairing of the plasmid DNA with the chromosome through some homologous sequences. This could account for the origin of plasmid resistance genes transferred from the bacterial chromosome either as wild-type or resistant alleles. Further investigations are in progress to test this hypothesis.

Facilitation of plasmid establishment when the natural competence machinery is turned on in a *recA*⁺ background might suggest that plasmids used in electrotransformation contain substantial lesions that are repaired by *recA*-dependent recombination to allow successful replication. These lesions could be single-strand interruptions, gaps or partial denaturation occurring during plasmid preparation or during the electric-pulse-dependent penetration of the DNA. However, the electric pulse by itself has no effect on DNA in solution (data not shown). It is possible that inside the cell, plasmid DNA not coated by the proteins induced by natural competence could be the target of DNases. Pairing in a *recA*-dependent way would protect these molecules.

Electrotransformation has been generally used to introduce plasmid DNA in hundreds of bacterial species for developing molecular genetic and biotechnological techniques. In this study we have used this method to obtain plasmid installation in recombination-deficient (*recA*⁻) strains and to investigate the mechanism of natural competence. Therefore, electrotransformation is not only a tool for biotechnological studies but is also a tool for fundamental research.

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