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Improved Method for Conjugative Transfer by Filter Mating of *Streptococcus pneumoniae*

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The frequency of conjugation during filter mating of pneumococcus was increased 10- to 100-fold when the filter was embedded in agar during incubation instead of being on the surface. The major effect was not due to protection from oxygen. The factor of increase was similar for transfer of plasmids and of chromosomal insertions of drug resistance elements.

We recently described two kinds of conjugation among strains of pneumococcus (*Streptococcus pneumoniae*). One was the transfer of *cat* and *tet* genes present in the chromosomes of some clinical isolates in an insertion, Ω(*cat-tet*), of DNA that is not homologous to the normal genome (4, 5); the other was transfer of plasmids introduced from other streptococcal species (6). Both results came from mating of donor and recipient strains on nitrocellulose filters incubated on the surface of blood agar that contained high levels of DNase I to prevent transformation. Transfer did not occur in liquid culture (5, 6).

Although plasmid transfers were reasonably high and reproducible, the chromosomal transfers were highly variable and often quite low, in the range of 5 to 70 transconjugants per ml of resuspended cells, whereas occasional experiments gave 500 to 1,800 per ml. We have now found that if the filters were embedded in agar rather than resting on it during the incubation period, the transfer frequencies were 10- to 100-fold higher both for chromosomal genes and for plasmids. This method allows a much clearer distinction between positive and negative results and further examination of some of the parameters affecting the transfers. Because it may be useful for other systems, we describe the procedure in some detail.

Cultures were grown without aeration to about 5×10^8 cells/ml in CAT medium (3), containing 1.5% mixed casein hydrolysates, 0.1% yeast extract, 0.5% NaCl, 0.2% glucose, and 15 mM K_2HPO_4 (initial pH near 7.5). The recipient culture was then adjusted to contain 10 mM $MgSO_4$, 2 mg of bovine serum albumin (BSA) per ml, and 10 μg of DNase I (Worthington Biochemicals Corp.) per ml. Donor culture (usually 1:10 vol) was added, and 2 to 3 ml of the mixture was filtered onto sterile 13-mm nitrocellulose filters (HA; Millipore Corp.) to about the cell density that caused flow to cease. Quarter-sectioned 10-cm plastic petri dishes (1009X; Falcon Plastics) were prepared with 7 ml per section of 2% agar in CAT containing 10 mM $MgSO_4$, BSA (2 mg/ml), DNase I (70 μg/ml), 2% blood, and 20 mM Tris (pH 7.5). Filters were placed cell-side-up (but see below), one per section, overlaid with 7 ml of the same agar as the base layer, and incubated at 37°C for 4 h unless otherwise indicated. The filters were covered with about 5 mm of agar. Later tests (not shown) showed that the blood was not needed in the overlay agar.

After incubation, the agar over the filter and the filter itself were removed with sterile flat-bladed forceps and mixed with 2 ml of CAT plus 10 mM $MgSO_4$, BSA (2 mg/ml), and DNase (10 μg/ml), for about 10 s on a Vortex mixer. The wash was decanted, diluted, and plated to score for donors, recipients, and recombinants as described previously (5, 6).

Table 1 shows results for matings done when the filter was: (i) placed on the surface of previously prepared plates, (ii) pressed gently onto freshly poured warm agar, (iii) overlaid with agar as described above, or (iv) made of a different material (see below). Because initial results had suggested the enhancement might be due to exclusion of atmospheric oxygen, to which pneumococcus is sensitive, parallel plates were incubated in an anaerobic chamber. The level of transfer was not increased for filters incubated on the surface, and there was no correlation with the anoxic atmosphere during incubation. Whether the agar effect is due to forcing closer cell contacts or to another mechanism is unknown. Although in this experiment only 520 Ω*cat-tet* transconjugants were seen "in air," numbers from 2,000 to 17,000 were not uncommon (see below and Fig. 1 and Table 2). Plasmid transfer was also enhanced (Table 1), and we have seen pIP501 transconjugants at frequencies up to 0.3 per donor. On hindsight, the occasional high levels of transfer seen in reference no. 5

were in experiments in which the filters were pressed down on freshly poured warm agar.

Polycarbonate membranes (Unipore; Bio-Rad Laboratories) gave results similar to nitrocellulose. However, because cells do not stick to them, they were placed cell-side-down on the agar and then overlaid as above. After incubation, the agar below the membrane contained essentially all the cells, and it was removed with the filter and washed as above. This method also works with nitrocellulose filters, avoids washing the cells off the filter when applying the overlay, and is now our standard procedure. The agar overlay is still required for enhancement (not shown).

These manipulations inherently introduce some variability of recovery, and unknown factors increase the variability further. In one test, four replicate filters were incubated on the same plate for 4 h. Recovery of donors and recipients varied nearly twofold, and transconjugants ranged from 870 to 2,600 per ml, giving frequencies of 2.3, 3.5, 4.6, and 5.0 per 10^5 donors recovered. The results of examining dependence on time of incubation (Fig. 1) also varied somewhat, but in each case transfer rose rapidly in the first hour and was near maximum at 4 to 6 h. Longer incubations, particularly overnight, usually resulted in lower recovery and more variable frequencies, for reasons which are unknown at this time.

TABLE 1. *Filter mating of pneumococcus*

Filter[a]	Incubation[b]	Transconjugants per donor[c]	
		Ω(*cat-tet*)	pIP501
On surface	In air	6×10^{-7}	4.6×10^{-3}
	Anaerobic	2×10^{-7}	
Pressed onto warm agar	In air	7×10^{-6}	
	Anaerobic	9×10^{-6}	
Overlaid with agar	In air	1.7×10^{-5}	3.6×10^{-2}
	Anaerobic	1.9×10^{-5}	
Polycarbonate, overlaid	In air	2.6×10^{-5}	2.1×10^{-2}

[a] See text. Filters were nitrocellulose except where indicated.

[b] Plates with filters were incubated 4 h at 37°C either in a standard incubator or in an anaerobic glove box with access through an airlock (Coy Products).

[c] The recipient was strain DP1004 (*str-1*). Transconjugants were scored as Str^r Cm^r or Str^r Tc^r for donor DP1302 [*ery-2* Ω(*cat-tet*)] (4) and tested by transfer to the other drug as described (5). Cotransfer was nearly 100%. Plasmid transconjugants for pIP501 and pMV158 (not shown) were selected as described (6) from matings with DP3201 donors which contain pIP501 and pMV158. Mobilization of the nonconjugative pMV158 (6) was also increased by the overlay method.

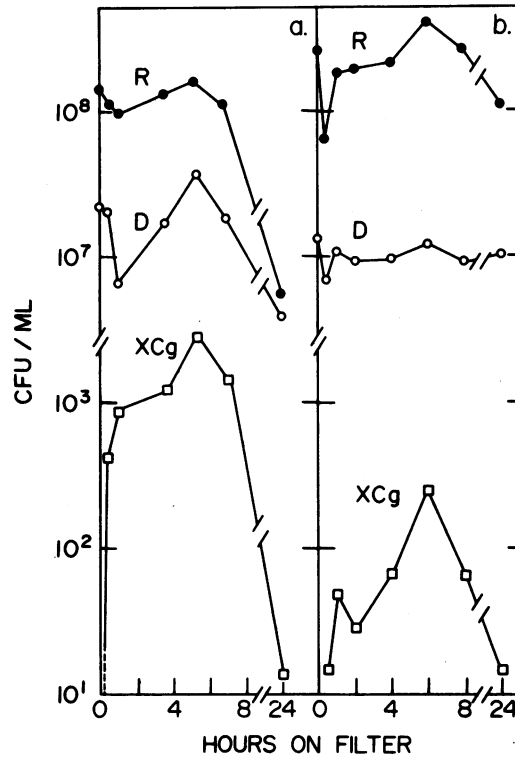

FIG. 1. *The time course of transfer of Ω(cat-tet) during filter mating when the filters were embedded in agar as described in the text. DP1302 donors (D), DP1004 recipients (R), and transconjugants (XCg) were scored as described in Table 1, footnote c. No Str^r Ery^r recombinants were found. The two experiments (a and b) illustrate that the system is still variable from day to day.*

TABLE 2. *Conjugative transfer of Ω(cat-tet) is independent of hex and end mutations in the recipient*

Recipient[a]	Phenotype	Transconjugants/ml[b]
DP1004	Str^r Hex$^-$ End$^+$	9,000
DP1002	Nov^r Hex$^-$ End$^+$	4,000
DP1609	Str^r Hex$^+$ End$^+$	17,000
DP1617[c]	Str^r Hex$^+$ End$^+$	8,000
DP1225[d]	Str^r Hex$^-$ End$^-$	16,000

[a] Donor was strain DP1302 [*ery-2* Ω(*cat-tet*)].

[b] Frequencies were 1×10^{-4} to 4×10^{-4} per donor recovered.

[c] DP1617 carries several other resistance genes. Str^r was used for selection in this experiment.

[d] DP1225 carries Lacks' *noz-19* allele of the *end* gene, which reduces entry of DNA and transformation more than 200-fold relative to End$^+$ strains (2).

Table 2 shows that any of several laboratory strains served as recipients with good yields, that the yield was unaffected by the status of the mismatch correction system (Hex) that acts at the heteroduplex stage of transformation (1), and that the transfer was independent of the membrane endonuclease that is needed for entry of DNA by the transformation pathway (2). Conjugative transfer of pIP501 was also independent of this endonuclease (6). These results reinforce the evidence that the transfer process differs entirely from transformation (5).

The use of the overlay procedure for filter mating should be of particular benefit when the frequencies of transfer are so low that they are difficult to distinguish from background mutations of the donor to the recipient phenotype.

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