Fusion of mycoplasmas: the formation of cell hybrids

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1. SUMMARY

Poly(ethylene glycol) (PEG 8000) can induce cell-cell fusion of Mycoplasma capricolum cells, and it can promote the formation of intergeneric hybrids of various Mycoplasma, Acholeplasma and Spiroplasma species. The extent of fusion was quantitatively evaluated by following the dequenching of octadecylrhodamine fluorescent label incorporated into donor cell membranes after their incubation with recipient cells. The results of dequenching experiments were confirmed by electron microscopy, as well as by angle light-scattering measurements. Fusion appeared to require the presence of Mg$^{2+}$, but was completely inhibited by either 0.1% glutaraldehyde or 100 $\mu$M chlorpromazine, and was partially suppressed by proteolytic enzymes, carbonyl cyanide-m-chlorophenylhydrazone, or thiol reagents.

2. INTRODUCTION

The introduction of exogenous macromolecules, such as DNA and proteins, into mycoplasmas is of considerable potential importance for studying the biological function of the macromolecules. One possible method for achieving this might be by fusion of two cells, forming intergeneric hybrids [1]. Mycoplasmas lack a rigid cell wall and would seem to be amenable to cell-cell fusion [2] and this has been suggested in several works [3–5]. The major obstacles for fusion studies have been the semi-quantitative and indirect methods used. This disadvantage has been overcome recently by the introduction of fluorescent dequenching methods [6].

In this communication, we demonstrate that poly(ethylene glycol) (PEG) stimulates fusion between various mycoplasma species. We suggest that intergeneric mycoplasma fusion is an appro-
appropriate approach for the formation of polyploid heterozygotic hybrids that will contain the complete genomes of the two parental cells.

3. MATERIALS AND METHODS

3.1. Organisms and growth conditions.

*Mycoplasma capricolum* (California kid), *M. gallisepticum* and *Acholeplasma laidlawii* (BC-9) were grown at 37°C in a modified Edward medium supplemented with 4% horse serum [7]. *Spiroplasma floricola* (BNR-1) was cultivated at 32°C in the medium of Saglio [8] with 10% horse serum. The cultures were harvested as previously described [9], washed once, and resuspended in a solution containing 250 mM NaCl, 10 mM MgCl₂, and 10 mM Tris·HCl, pH 7.4 (‘A-buffer’). To test for cell leakiness, 0.25 μCi of 6-methyl-[³H]thymidine per ml (35 Ci/mol, Nuclear Research Center, Negev, Israel) were added to the growth medium.

3.2. Fusion measurements

Fusion was carried out as described [10]. For the labeling of cells, 3 μl of a solution of 600 μg octadecylrhodamine (R18)/ml (Molecular Probes, U.S.A.) were injected into 200 μl of A-buffer containing 0.4 mg of cell protein, incubated for 15 min at 37°C in the dark, cooled rapidly and washed once with cold A-buffer. The R18-labelled cells (about 40 μg of protein) were mixed with unlabelled cells (about 350 μg of protein) and PEG 8000 (Merck, F.R.G.) (5% final concentration) in 0.5 ml of A-buffer, and incubated at 37°C for various times. The reaction was stopped by adding 2 ml of cold A-buffer and the intensity of R18-fluorescence dequenching was measured with excitation and emission wavelengths 560 and 590 nm respectively [10], and with correction for light scattering. Angle light scattering (90°) and endogenous fluorescence were done according to Nahas et al. [11] using FACS-440 (Becton Dickinson FACS Systems, Sunnyvale, CA).

4. RESULTS AND DISCUSSION

An R18-fluorescence dequenching assay was used to follow membrane lipid mixing as a result of fusion. This probe, once inserted into a membrane at self-quenching concentrations, will not dissociate from the membrane, either by spontaneous diffusion of the free monomers through the aqueous phase, or by a collusion-mediated transfer process [12]. Incubation of R18-labeled *M. capricolum* cells with non-labeled cells for 40–60 min at 37°C in the presence of 5% PEG resulted in dilution of the probe in the recipient cell membrane, as indicated by over 30% dequenching of R18 fluorescence. In the absence of PEG, no dequenching was observed, whereas a very low degree of dequenching was induced by high concentrations (> 15%) of PEG. No dequenching was observed in fusion mixtures where PEG was replaced with other known fusogenic agents, such as oleic acid (10–50 μM), spermidine (5 mM), or bovine serum albumin (0.4–2.0%). It has been suggested that PEG, as a dehydrating agent, acts through dehydration and solubilization of intimately juxtaposed cell membranes inducing bilayer destabilization and consequent fusion of cells [13,14]. The low PEG concentrations required in our study suggest that only partial dehydration of *M. capricolum* membranes is sufficient for optimal fusion. The PEG-stimulated fusion was completely suppressed by 0.1% glutaraldehyde or 100 μM chlorpromazine, which were therefore used as controls. As was expected, the extent of R18-fluorescence dequenching directly correlated with the labeled/non-labeled cell ratio in the incubation medium, with a maximal fluorescence obtained at a ratio of 1:9–1:10. This ratio was therefore used throughout subsequent experiments. The fused *M. capricolum* cells retained 93–96% of intracellular [³H]-thymidine-labeled components, suggesting that the hybrids thus obtained were non-leaky.

Thin section electron microscopy (Fig. 1A,B) of *M. capricolum* cells incubated under fusogenic conditions further confirmed the fusion process by showing that upon the completion of fusion, the membrane structures of both cells in the
Fig. 1. Electron microscopy of mycoplasma cell fusion. Thin-sectioned preparations were stained with uranyl acetate. Fusion areas are shown by arrows. A, *M. capricolum* cell fusion; bar = 200 nm. B, *M. capricolum* cell fusion, bar = 100 nm. C, *M. capricolum* (1) - *S. floricola* (2) cell fusion; bar = 50 nm.

contact areas could not be identified. Nonetheless, both cells appeared to be connected by a single continuous plasma membrane. The notion that the R18-fluorescence dequenching observed reflects a fusion process was also supported by angle light-scattering analysis (FACS) (Fig. 2). This method allows measurements of cell distribution according to size in heterogeneous populations. After fusion was completed, light scattering histogram of non-fused cells shifted to the right edge, representing an increase of cell size, as well as more heterogenous cell distribution (new subpopulations), apparently as a result of fusion.

Maximum homologous fusion was observed at 37°C, at pH values between 6.5 and 8.0. Fusion was stimulated in a medium of low (250 mOsm) osmolarity, indicating that partial cell swelling promotes cell fusion. Without PEG, the osmotic swelling or shrinking had very little, if any, effect on fusion.

Homologous *M. capricolum* cell-cell fusion was strongly inhibited by EDTA (10 mM), an inhibition that could be reversed completely by 10 mM MgCl₂ or CaCl₂. The divalent cations may neutralize the negative surface charges of mycoplasma membranes and dehydrate the intercellular space of adjacent cells, thus inducing membrane lipid aggregation and then triggering the cell fusion [15].
Table 1
Intergeneric fusion of various mycoplasmas

<table>
<thead>
<tr>
<th>Donor Species</th>
<th>Fusion (%)</th>
<th>Recipient Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R18 labeled)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. capricolum</td>
<td>100</td>
<td>M. gallisepticum</td>
</tr>
<tr>
<td>M. gallisepticum</td>
<td>211</td>
<td>A. laidlawii</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>253</td>
<td>S. floricola</td>
</tr>
<tr>
<td>S. floricola</td>
<td>164</td>
<td></td>
</tr>
</tbody>
</table>

Fusion was performed with 5% PEG as described in MATERIALS AND METHODS. The ratio between donor (R18-labeled) and acceptor (non-labeled) strains was about 1:9. For each species, the homologous fusion was taken as 100%.

Fusion was suppressed by 57% (as judged by R18 dequenching) when cells were pretreated with trypsin (30 μg/mg cell protein) for 1 h at 37°C. Similar results (53% inhibition) were obtained following treatment of intact cells with pronase (25 μg/mg cell protein). These observations support the notion that partial removal of surface proteins may trigger membrane lipids to aggregate in protein-free areas, preventing membranes from fusing [15,16]. The 32% suppressive effect of ascorbate (50 μM) + FeCl₃ (50 μM) can perhaps be explained by the formation of hydroperoxides and lipid peroxides as the primary oxidation products of membrane unsaturated lipids which decrease the membrane fluidity and the ability of cells to fuse [17]. A partial inhibition of fusion (35%) was obtained with the thiol reagent N-ethylmaleimide (1 mM), or (56%) with the proton ionophore carbonyl cyanide-m-chlorophenylhydrazone (5 μM), but not with the Fₒ/Fᵢ⁻ ATPase inhibitor dicyclohexylcarbodiimide (100 μM), or with the cation specific ionophores nigericin and monencin (2 μM of each).

Fusion of various mycoplasmas was also obtained (Table 1). These data, obtained with the help of R18-dequenching fluorescence technique, were supported by electron microscopy, which showed a fusion of M. capricolum cells with S. floricola cells (Fig. 1C). The intergeneric fusion was PEG- and Mg²⁺-dependent, with maximal activities obtained with 5% PEG and 10 mM MgCl₂. Among the representative species tested, highest fusogenic activities were obtained when M. capricolum served as either the donor or recip-
ient cells, whereas lower fusogenic activities were obtained with *A. laidlawii*. Intergenic mycoplasma fusion may open the way to utilize the fusion process as a means of constructing heterozygotes with chimeric properties.

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REFERENCES