Fusion-Mediated Transfer of Plasmids into Spiroplasma floricola Cells

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We have developed and characterized a system for the transfer of plasmids encapsulated in large unilamellar vesicles (LUV) into Spiroplasma floricola BNR1 cells. The approach is based on the ability of S. floricola-derived LUV to fuse with S. floricola cells. The fusion was continuously monitored by an assay for lipid mixing based on the quenching of the fluorescent probe octadecylrhodamine B (R18) that was incorporated into LUV at self-quenching concentrations. The fusion was also evaluated by fluorescence-activated cell sorter measurements and by sucrose density gradient analysis. LUV-cell fusion occurred only in the presence of low concentrations (5%) of polyethylene glycol (polyethylene glycol 8000) and depended on temperature, the LUV/cell ratio, and divalent cations in the incubation medium. Throughout the fusion process, spiroplasma cells remained intact and viable. Under optimal fusion conditions, the plasmid pACYC, encapsulated in LUV by reversed-phase evaporation, was transferred into live S. floricola cells and expressed chloroplastic acetyltransferase activity. The expression was transient with maximal chloroplastic acetyltransferase activity observed after 6 h of incubation of the transfected cells.

In recent years, there has been a great deal of interest in the use of liposomes as carriers for delivery and introduction of biological macromolecules into cells (13). This approach is based on the ability of liposomes to adsorb on the cell surface with subsequent efficient fusion that facilitates the entry of liposome-encapsulated genetic material into the cells (12). Although the lack of a cell wall in Mollicutes species would seem to favor genetic exchange by transfection following direct contact and fusion of cell membranes (14), most experiments performed so far to transform Mollicutes species have given inconsistent results (4, 7, 21). It was suggested that the potent endonuclease activity of mycoplasmas, as well as their tendency to lose viability in buffer solutions, may hinder transformation experiments (19, 20). Reports on the polyethylene glycol (PEG)-mediated transfer of chromosomal genes between Spiroplasma citri strains or between the membrane-bound Acholeplasma virus and Acholeplasma laidlawii cells have been recently published, and membrane fusion was suggested as a prerequisite step (1, 2, 9, 27, 29). Recently, we showed that Mycoplasma capricolum cells were capable of fusing with small unilamellar vesicles, provided that the vesicles contained cholesterol and that low concentrations of PEG were present in the incubation medium (26). This observation opened the way for the development of a fusion-mediated delivery system of genetic material into mycoplasma cells. In the present study, we describe the optimal conditions for the fusion of Spiroplasma floricola cells and show a transient expression of the chloroplastic acetyltransferase (CAT) gene transferred to S. floricola cells by the fusion-mediated process.

MATERIALS AND METHODS

Growth conditions. S. floricola BNR1 cells were grown in Saglio medium (25) supplemented with 10% horse serum and adjusted to pH 8.0. To label membrane lipids, the cells were grown with 0.02 μCi of [9,10(α)-3H]palmitic acid (40 to 60 Ci/mol; Radiochemical Centre, Amersham, United Kingdom) per ml. To test for cell leakage during the fusion process, 0.25 μCi of [6-methyl-3H]thymidine (35.2 Ci/mol; Nuclear Research Center, Negev, Israel) was added (10). The cells were harvested at the late exponential phase of growth (A640 = 0.25) by centrifugation at 12,000 × g for 15 min, washed once, and suspended in a solution containing 250 mM NaCl, 10 mM MgCl2, and 10 mM Tris-HCl, pH 7.4 (A buffer). The viability of the cell suspension was measured by the colony-counting technique and expressed as colony-forming units.

Preparation of LUV. Large unilamellar vesicles (LUV) were prepared by the reversed-phase evaporation method, according to Nagata (17), with slight modifications. About 4 mg of S. floricola lipids, obtained by extracting intact S. floricola cells according to Bligh and Dyer (3), was dissolved in 5 ml of chloroform, evaporated under nitrogen, and redissolved in 0.5 ml of double-distilled diethyl ether. An aliquot of 0.17 ml of TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (2 mM TES, 2 mM L-histidine, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]) was then added to the lipid suspension. The resulting two-phase system was shaken vigorously for 1 min on a Vortex mixer and then sonicated for 5 s by a W-350 probe sonicator (Heat Systems, Plainview, N.Y.) under nitrogen to make the water phase opalescent. The mixture was placed in a conical centrifuge tube and evaporated in a rotary evaporator (Buchi, Flawil, Switzerland) at 400 mm Hg0 and 120 rpm until the suspension became a gel. After brief vortexing, evaporation was carried out at 730 mm Hg0 until a homogeneous suspension was obtained. The resulting LUV preparation was diluted 1:3 with TES buffer and passed through a 1-ml Sephadex G-50 column to remove impurities.

Plasmid encapsulation in LUV. The Escherichia coli plasmid pACYC 184 was purified by CsCl gradient centrifugation (16). Primer labeling of the plasmid with [α-32P]dCTP was performed with the random primer labeling kit (Boehringer GmbH, Mannheim, Germany) according to the manufacturer’s instructions. LUV were prepared as described

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above, except that 10 μg of DNA per mg of lipid was added to the lipid mixture before sonication. The added DNA was either 32P-labeled or unlabeled native pACYC 184 plasmid. To determine the efficiency of the encapsulation, the plasmid-encapsulated LUV preparations were incubated with DNase I (5 μg/ml; Sigma) in a buffer containing 20 mM MgCl₂ at 37°C. At various time intervals, samples (20 μl each) were withdrawn, and the DNase I activity was terminated by adding 80 μl of a buffer containing 20 mM EDTA. The LUV-encapsulated plasmid was separated from the untrapped, digested plasmid by passing 100-μl samples through a Sephadex G-50 column in a 1-ml tuberculin syringe, as previously described (24). The amount of residual DNA in the LUV preparations was estimated by counting the radioactive or determining total DNA and comparing with controls containing nonecapsulated plasmids. The total DNA was assayed, after binding of Hoechst 33258, by measuring the relative fluorescence at 460 nm with the TKO minfluorimeter (Hoefer Scientific Instruments, San Francisco, Calif.).

Fusion of LUV with S. florica cells. LUV were labeled by octadecylrhodamine B chloride (R18; Molecular Probes, Eugene, Ore.) as previously described (5). R18-labeled LUV (30 to 50 μg) were added to 300 to 400 μg of nonlabeled S. florica cells in a buffer (final volume of 200 μl) containing 5% PEG 8000 (Merck, Darmstadt, Germany). The reaction was stopped with 2 ml of cold A buffer, and the intensity of R18 fluorescence dequenching was measured with excitation and emission wavelengths at 560 and 590 nm, respectively, and with correction on light scattering (5). To determine the transfer of a LUV-encapsulated plasmid into the cells, LUV with encapsulated 32P-labeled pBR 202 plasmid were fused with S. florica cells as described above. Samples, in duplicate, were layered onto the surface of 0.3 ml of silicone oil (550:556 grade; Dow Corning Corp.) in 1.5-ml plastic microcentrifuge tubes and centrifuged at 13,000 × g for 2 min. Under these conditions, the cells passed through the oil, forming a pellet at the bottom of the tube (24). The cell pellet was resuspended, and the amounts of residual radioactivity and fluorescence in the cells were determined.

Transfection of S. florica. pACYC 184 (S4) plasmid harboring a CAT gene with resistance to chloramphenicol was propagated in E. coli grown in a Luria broth medium containing 10 μg of chloramphenicol per ml and purified by the alkaline lysis method (28). The pACYC 184 plasmid was encapsulated in LUV (0.5 to 25 μg of DNA encapsulated per mg of lipid), and 100 μg of LUV was fused with S. florica cells (800 μg of cell protein; 4 × 10⁹ CFU). The fused cells were incubated at 32°C in 5 ml of a growth medium supplemented with 8% horse serum. After various time intervals (3, 6, 9, and 12 h), the cells were harvested, washed twice, and disrupted by three cycles of quick-freezing and thawing. The cell extracts were then assayed for CAT activity according to Gorman et al. (8), with CAT (Pharmacap, Uppsala, Sweden), acetyl coenzyme A, lithium salt (Sigma), and d-threo-[dichloroacetyl-1-14C]chloramphenicol (55 mCi/nmol; Radiochemical Centre). Attempts to select chloramphenicol-resistant strains were made with Saglio solid medium (25) containing various concentrations of chloramphenicol (2, 10, and 25 μg/ml). The presence of the pACYC 184 plasmid within spiriplasma cells was determined by Southern blot hybridization techniques (16).

Miscellaneous procedures. Right-angle light scattering and endogenous fluorescence were performed according to Nahas et al. (18) by a fluorescence-activated cell sorter (FACS-440; Beckton Dickinson, Sunnyvale, Calif.). Labeling of S. florica cells with 12-(1-pyrene)dodecanoic acid (P12) was carried out by adding 10 μl of 2 mM P12 to 0.25 mg of cells in 0.5 ml of A buffer. After 20 min of incubation at 32°C, the cells were washed twice with A buffer containing 0.4% bovine serum albumin (fraction V; Sigma) and resuspended in a 1-ml volume of A buffer. The fluorescence intensity of cells was measured with excitation and emission wavelengths at 345 and 378 nm, respectively, with the correction on light scattering. To determine whether the cells remained intact in the fusion reaction mixture, the retention of [3H]thymidine-labeled components within the cells was determined as described previously (10).

RESULTS

Fusion between intact S. florica cells and LUV was investigated. The LUV were prepared from the total lipid fraction of S. florica cells. This lipid fraction was composed mainly of polar lipid and cholesterol. The polar lipid fraction consisted mainly of phosphatidyglycerol (55%), sphingomyelin (30%), and phosphatidylcholine (10%), with a molar ratio of cholesterol to phospholipid of 0.82. The diameter of the LUV, as determined by transmission electron microscopy after negative staining with 2% uranyl acetate, ranged from 80 to 300 nm. The size distribution of the LUV was also determined with a Coulter Counter (Model N4SD; Coulter Electronics, Inc., Hialeah, Fl.). About 40% of the LUV was found to have a diameter of 275 ± 43 nm, whereas about 50% showed a diameter of 1,480 ± 250 nm, apparently representing aggregates of smaller vesicles (17).

Kinetics of PEG-stimulated fusion. Fusion was evaluated by measuring the increase of fluorescence of R18 following incubation of fluorescently labeled LUV and intact S. florica cells. This probe is incorporated into membrane at self-quenching concentrations and, upon fusion or lipid transfer, is diluted in the enlarged membrane space and dequenched (13). When S. florica cells were incubated with R18-labeled LUV prepared from the total S. florica lipid fraction, a marked increase in the degree of fluorescence was observed. The fluorescence dequenching depended on the PEG concentration in the fusion mixture reaching its highest levels after 60 to 100 min of incubation at 32°C with 5% PEG (Fig. 1). At higher PEG concentrations, the R18 dequenching dropped, reaching a low basic level at 20% PEG. No dequenching was observed in a reaction mixture not containing cells (data not shown). Glutaraldehyde, at concentrations of 0.01 to 1.0%, or chlorpromazine (20 to 100 μM) completely suppressed R18 dequenching and, therefore, was used as a control throughout all further experiments.

Further evidence of LUV-S. florica fusion was obtained by entrapping the water-soluble fluorescence probe 5(6)-carboxyfluoresceine at self-quenching concentrations in the LUV. Upon incubation of the LUV with intact S. florica cells, a 32% increase in fluorescence was observed, supporting the notion that intermixing of LUV content with the content of S. florica cells had occurred. When LUV were incubated with cells in the absence of PEG or when LUV were incubated without cells in the presence of PEG, only a moderate (7 to 10%) increase in fluorescence was obtained. This may represent a slow leakage of the probe from the vesicles.

Optimal fusion conditions. The constant ratio of 1:9 between LUV lipids and total cell lipids was utilized throughout our study, producing a constant degree of R18 de-
quenching. The rate of LUV-spiroplasma cell fusion was temperature dependent, reaching maximal values at 36°C (data not shown). Under nonfusing conditions (without PEG or with 0.1% glutaraldehyde), a very low level of R18 dequenching was observed. As with *M. capricolum* (26), divalent cations were required for the fusion of *S. floricola* cells with LUV. High levels of fluorescence were obtained with 10 mM MgCl₂ or CaCl₂ (29.1% ± 1.3% and 23.5% ± 2.7% dequenching, respectively). The highest fusion levels were obtained in a medium of low osmolarity (300 to 400 mosM), adjusted to a pH between 7.0 and 7.6, in the presence of 10 mM MgCl₂. During the 1- to 5-h incubation periods in the fusion medium, *S. floricola* cells remained intact and viable, as was determined by the retention of 96% of [³H]thymidine-labeled components within the cells and by colony counting (10).

Figure 2 shows the results of a right-angle-light-scattering analysis made by the FACS-440 of LUV-spiroplasma fused material. The FACS analyzes and sorts cells according to light-scattering properties, which reflect the natural diversity of cell size among the population. When fusion was completed, the light-scattering histogram shifted to the right end. This represents an increase in cell size and a more heterogeneous distribution of cells.

The local anesthetic chlorpromazine (100 μM) completely suppressed the LUV-*S. floricola* fusion. Fusion was partly inhibited (44% inhibition) by treating the cells with 5 μM carbonyl cyanide m-chlorophenylhydrazone, whereas dicyclohexylcarbodiimide (1 mM), known as a specific inhibitor of Mg²⁺-dependent ATPase, or the SH reagent N-ethylmaleimide (1 mM) had no effect on fusion. Proteolytic digestion of intact spiroplasma cells with either trypsin or pronase (both at 30 μg/mg of cell protein) partially inhibited the fusion. This suggests that *S. floricola* cells possess protease-sensitive receptors that are responsible for a tighter contact with the lipid vesicles.

Table 1 shows the marked dependence of fusion activity on the growth phase of *S. floricola* cells. Mid-exponential-phase cells (*A₆₅₀ = 0.155*) demonstrated a strong ability to fuse with LUV, whereas the fusion activity of stationary-phase cells (*A₆₅₀ = 0.260*) was very low, although these cells were viable.

**Transfection of *S. floricola* cells.** To test the efficiency of plasmid encapsulation in LUV, ³²P-labeled plasmids were encapsulated during LUV preparation (5 μg of DNA encapsulated per mg of lipid). The plasmid-encapsulated LUV were then exposed to DNase I for various periods of time and chromatographed on Sephadex G-50 to separate plasmid containing LUV from free extravesicular oligonucleotides. Approximately 50% of the radioactivity was recovered in the LUV fraction, even after prolonged digestion periods (up to 6 h). In control experiments, in which radioactive plasmids were added to preformed LUV, the level of residual radioactivity observed in the LUV fraction after short digestion periods (30 min) was very low (1 to 5%). A similar encapsulation efficiency (~46%) was obtained by utilizing a nonra-

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**FIG. 2.** Right-angle light scattering of LUV-*S. floricola* cell fusion. PEG-stimulated fusion was performed as described in Materials and Methods, except that double amounts of LUV (20 μl) and cells (700 μg) were used in a final volume of 200 μl. After incubation with 5% PEG at 32°C for 90 min, the mixture was diluted to 600 μl by A buffer, and the resulting dilution was FACS analyzed. 1, cells plus LUV at zero time; 2, cells plus LUV after 90 min of incubation.

**TABLE 1.** The effect of the age of the *S. floricola* culture on the LUV-cell fusion

<table>
<thead>
<tr>
<th>Age of culture (h)</th>
<th><em>A</em>₆₅₀</th>
<th>pH of medium</th>
<th>CFU/ml (10⁶)</th>
<th>Fusion activity (% dequenching ± SD)</th>
</tr>
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<tbody>
<tr>
<td>15</td>
<td>0.155</td>
<td>6.2</td>
<td>1.60</td>
<td>24.4 ± 2.1</td>
</tr>
<tr>
<td>20</td>
<td>0.198</td>
<td>5.8</td>
<td>1.64</td>
<td>20.2 ± 1.8</td>
</tr>
<tr>
<td>25</td>
<td>0.260</td>
<td>5.5</td>
<td>0.98</td>
<td>3.0 ± 0.1</td>
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* Cells were grown in Saglio medium (25) containing 4% horse serum. Growth was monitored by measuring the *A*₆₅₀, pH, and CFU. At the various growth phases, the cells were fused with LUV as described in Materials and Methods.
dioactive pACYC 184 plasmid and measuring the intravesicular DNA content by the fluorimetric assay.

When LUV containing radioactive pACYC 184 plasmid were fused with S. floricola cells, about 20 to 25% of the radioactivity was found to be associated with the cells, suggesting a fusion-mediated microinjection of the plasmid into the cells. Figure 3 shows the pACYC 184 hybridization pattern of total DNA from S. floricola cells cleaved by HindIII. The linear (4.2-kb) and supercoiled forms of the pACYC 184 plasmid were clearly detected in the DNA from cells that were transfected with LUV containing nonradioactive pACYC 184 plasmid. The plasmid DNA was detected in transfected S. floricola cells incubated for up to 9 h at 32°C in the Saglio growth medium (25) but was not detected after prolonged incubation periods (≥12 h) or in control cells that were fused with empty vesicles (Fig. 3). Our attempts to select chloramphenicol-resistant S. floricola strains have failed. Nonetheless, CAT activity was expressed by the transfected S. floricola cells (Fig. 3). Figure 4 shows an autoradiogram of a thin-layer chromatography analysis of [14C]chloramphenicol and its acetylated derivatives obtained by incubation of S. floricola cells transfected with the LUV-encapsulated plasmid. The activity was best expressed when the cells (800 µg of cell protein) were transfected with 0.5 µg of encapsulated DNA (10 µg of DNA per mg of LUV lipids). Lower CAT activities were observed when transfection was performed with 0.1 µg (1 µg/mg of LUV lipids) or 2.5 µg (50 µg/mg of LUV lipids) of encapsulated DNA (Fig. 4). The highest level of CAT activity was detected after 6 h of incubation on Saglio growth medium (25). After prolonged incubation periods (≥12 h), CAT activity could not be detected anymore, although the cells remained viable (data not shown).

CAT expression depended on the growth phase of S. floricola cells utilized for the transfection experiments. Cells harvested in the early exponential phase of growth (10 h [A_{650} = 0.090]) were successfully transfected by LUV-encapsulated pACYC 184 plasmid and expressed high CAT activity. Lower CAT activities were detected when mid- or late-exponential-phase cells (15 h [A_{650} = 0.155] or 20 h [A_{650} = 0.198] of growth, respectively) were transfected. No CAT activity was detected in transfected stationary-phase cells (25 h [A_{650} = 0.260]). CAT activity was not detected in control S. floricola cells incubated either with free pACYC 184 plasmid or with empty LUV (data not shown).

FIG. 3. Hybridization of a {³²}P-labeled pACYC 184 probe with total DNA from transfected S. floricola cells. Two micrograms of total DNA was digested with HindIII, separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with a {³²}P-labeled pACYC 184 probe. Autoradiography was carried out at −70°C for 12 h. Lanes: A, control of cells fused with empty LUV; B to F, cells fused with LUV containing the pACYC 184 plasmid after incubation at 32°C for 0, 3, 6, 9, and 12 h, respectively.

FIG. 4. CAT expression in transfected S. floricola cells. CAT expression was determined following fusion-mediated transfection of early-exponential-phase (A_{650} = 0.09) S. floricola cells. The transfected cells were incubated for 6 h at 32°C in a complete Saglio medium (25), and CAT was assayed as described in Materials and Methods with [14C]chloramphenicol. The reaction products were analyzed by thin-layer chromatography and autoradiography. Lanes: A to D, expression of the CAT gene in cells fused with LUV containing various amounts of pACYC 184 plasmid (0, 1, 10, and 50 µg of plasmid DNA per mg of LUV lipid, respectively); E, a positive control with commercial CAT. CM, chloramphenicol; 1-ACM, 1-acetylchloramphenicol; 3-ACM, 3-acetylchloramphenicol; 1,3-ACM, 1,3-diacylchloramphenicol.

DISCUSSION

In the present study, we have for the first time shown fusion-mediated microinjection of plasmids into S. floricola cells. The fluorescence probe R18 was utilized to monitor the fusion. Once the probe is inserted into the lipid bilayer, it is not dissociated from it either by spontaneous diffusion through the aqueous phase or by a collision-mediated reaction (13). Since preincubation of LUV with glutaraldehyde and chlorpromazine resulted in a very low, if any, degree of R18 fluorescence quenching, it seems that membrane fusion but not lipid-exchange reaction occurred.

Cell fusion may be induced by various agents, such as viruses, alcohols, fatty acids, lysolceithins, certain proteins, and PEG (6). In our experiments, PEG was found to be a very efficient fusion promoter, and the effective PEG concentrations were much lower than the concentration used in other studies (5% versus 20 to 40%) (15). It was suggested that the dehydrating agent PEG is responsible for the close association of the two participating membranes (6). It therefore seems that only partial dehydration suffices to destabilize the membrane lipid phase of S. floricola and to trigger the fusion process.

The divalent cation requirement for LUV-spiroplasma cell fusion is expected in view of the negatively charged cell surfaces of both S. floricola cells and the LUV utilized (22). The divalent cations are capable of bringing membrane bilayers into close proximity by neutralizing the repulsive forces between phospholipids and destabilizing the attached bilayers (15). The control results obtained by the LUV and Spiroplasma cell mixing assay, which monitored dequenching of carboxyfluoresceine incorporated into LUV intravesicular space, are in agreement with those obtained by the R18-dequenching
assay as well as by FACS and sucrose density gradient analysis.

Like other types of intracellular processes, fusion of LUV and S. floridica cells may require a membrane potential or a pH gradient across the membrane, as was suggested by showing that carbonyl cyanide m-chlorophenylhydrazone, which dissipates membrane electrochemical potential, partially inhibited the fusion process. The inhibition of fusion by proteolytic enzymes supports the idea that S. floridica cells possess protease-sensitive receptors responsible for a closer contact with LUV. However, one cannot exclude the possibility that following digestion, membrane lipids tend to aggregate in protein-free membrane areas that may prevent them from fusion (30).

The efficient plasmid encapsulation in LUV corresponds to data obtained by others (17). Our inability to obtain chloramphenicol-resistant transformants of S. floridica by microinjection of pACYC 184 plasmid into the cells was expected, since the origin of replication of E. coli plasmids cannot be recognized by mycoplasma cells (20). Nonetheless, we have succeeded in obtaining a transient expression of LUV-encapsulated CAT gene in chloramphenicol-sensitive and CAT-lacking S. floridica cells. Only after 6 h of incubation of the transfected cells was enough enzyme to make the reaction products detectable on thin-layer chromatography plates produced. After longer incubation periods, CAT activity could not be detected anymore, apparently because of the degradation of the pACYC 184 plasmid within S. floridica cells and the turnover rate of cell proteins. This was suggested by our inability to detect the plasmid by Southern blot hybridization after 12 h of incubation.

The low or nonexistent CAT activity of stationary-phase S. floridica cells fused with LUV-encapsulated pACYC 184 plasmid may reflect the low extent of fusion of LUV with aged S. floridica cells (Table 1). Changes in the chemical composition and biophysical characteristics of mycoplasmas upon aging have been reported before (11, 23). These changes include a decrease in the ratio of phospholipid to protein that was attributed to an increase in membrane protein content. In addition, aged M. capricolum cells show a higher ratio of unsaturated to saturated fatty acid and a higher ratio of diphosphatidylglycerol to phosphatidlyglycerol (11). The possibility that the low extent of fusion obtained with aged S. floridica cells is due to changes in membrane composition and/or biophysical characteristics is now under investigation.

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REFERENCES


