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Permanent Mycoplasma Removal from Tissue Culture Cells: A Genetic Approach

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Abstract Mycoplasma contamination of tissue culture cells easily evades detection and, thus, represents a continous threat to cell biologists. In cases where infected cells can not simply be replaced, attempts have to be made to eradicate mycoplasma from the tissue culture cells. A variety of anti-microbial agents have been shown to be toxic to mycoplasma strains; however, cell associated mycoplasmas are often protected from antibiotics at concentrations shown to be effective *in vitro*. Antibiotic concentrations high enough to be lethal to cell associated mycoplasmas frequently are also detrimental to the host cells, while moderately increased antibiotic levels tolerated by the host cells often lead to only temporary growth suppression and/or to the emergence of mycoplasma strains resistant even to high concentrations of the antibiotic applied. Here, a genetic approach for the elimination of mycoplasma from tissue culture cells that overcomes these limitations is described. By expression of a selection marker conferring resistance to an otherwise toxic agent, *Acholeplasma laidlawii* infected BHK-21 cells used as the model system were enabled to temporarily tolerate antibiotic concentrations high enough to be lethal to cell associated mycoplasma while leaving the host cells unharmed. Upon successful mycoplasma eradication, cultivation of the cured host cells in the absence of the selective agent yielded revertant cell clones that had regained susceptibility to the toxic agent Cessation of the selection marker expression was shown to result from the loss of the selection marker DNA, which is a consequence of the fact that the stable and permanent integration of foreign DNA in eucaryotic cell chromosomes is highly inefficient Thus, the cells were cured from mycoplasma yet remained biochemically unaltered

Keywords. mycoplasma, selection, cell culture techniques

INTRODUCTION

Mycoplasma infection of tissue culture cells/permanent cell lines represents one of the major threats to the cell biologist (for excellent reviews see 1-4). Without causing obvious phenotypic changes in the affected cell, mycoplasma contamination often evades detection Yet, mycoplasma can affect virtually any measurable cell parameter and exert significant effects on the metabolism of the host cell [4]; experimental data from affected cells might yield unpredictable results caused by the contaminating mycoplasma rather than true effects by the cells and, hence, lead to misinterpretation. Pronounced effects caused by mycoplasma infection might be as diverse as chromosome breakage and other karyologic changes [5], DNA degradation [6], changes in virus propagation, cell growth interference, morphological changes, membrane modifications, as well as amino and nucleic acid metabolism alterations [2]. The major causes for the contamination of tissue culture cells with mycoplasma have been shown to be the result of non-sterile solution, equipment, and inappropriate handling [7]. Depending on the individual survey, between 4 and 92% of the cell lines investigated in different cell culture laboratories have been reported to be contaminated [2,5,8]. Since mycoplasma infection can lead to serious diseases, mycoplasma contamination of cell lines used for the production of therapeutic agents obviously represents a serious health hazard to the patient treated with pharmaceuticals derived from biotechnological production [9,10].

Frequent and routine mycoplasma testing is a prerequisite for appropriate cell culture practices. A variety of tests are available [2,11], the most common of which are based on DNA fluorochrome staining (Biochrom/Seromed, Boehringer Mannheim), specific mycoplasma DNA hybridization (Biological Industries), ELISA (Boehringer Mannheim), and adenosine phosphorylase activity (Gibco); other and technically more sophisticated tests include PCR (Stratagene, Boehringer Mannheim; 12), arginine deaminase activity [13] and *in vitro* culturing [14]. Since no test is likely to detect all mycoplasma species, a combination of them is highly advisable for routine testing.

Upon detection of mycoplasma contamination, the best choice is to immediately discard the infected cells

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and thaw a sterile back-up stock vial. In instances, however, where no stock is available and mycoplasma free cells can not be replaced by other sources, attempts need to be made to rid the cells of the mycoplasma.

Different methods have been employed in order to remove mycoplasma from tissue culture cells [15]. These include hypotonic treatment, exposure to elevated heat, the use of mycoplasma-specific anti-serum [16], and, most commonly, the application of antibiotics [6].

A wide variety of antibiotics have been demonstrated to be lethal, even at very low concentrations, to individual mycoplasma grown in broth media [17]. When associated with tissue culture cells, however, mycoplasma often are protected [26], requiring much higher antibiotic concentrations for successful elimination. In this instance, at the concentration necessary to eliminate mycoplasma, the antibiotic often becomes toxic to the host cells also [15,16,18]. Moderate antibiotic concentrations tolerable to the host cell often suppress mycoplasma growth temporarily but do not lead to successful permanent elimination [18,19,26] Upon removal of the antibiotic, mycoplasma become detectable again. In addition, moderate antibiotic concentrations were reported to lead to the selection and outgrowth of mycoplasma substrains highly resistant to the antibiotic applied [15,20,21].

A variety of toxic agents specifically designed for mycoplasma removal are commercially available [3], including the Mycoplasma Removal Agent (ICN/Flow Laboratories), BM Cyclin (Boehringer Mannheim), and Anti-PPLO Agent (Gibco) These cures meet with only limited success [3]

Here, a genetic approach for the elimination of mycoplasma from tissue culture cells is described Ninety-five percent of mycoplasma contaminants in tissue culture reportedly are caused by only four mycoplasma species, one of which is *Acholeplasma laidlawii* [2]. *Acholeplasma* commonly are introduced into tissue culture cells by contaminated bovine serum [1]. In this study, baby hamster kidney BHK-21 cells infected by *Acholeplasma laidlawii* were thus chosen as the model system. The infected cells were genetically manipulated so as to temporarily tolerate antibiotic concentrations high enough to be lethal to associated mycoplasma while leaving the host cells unharmed Withdrawal of the antimicrobial agent and subcultivation of the cured host cells led to the identification and subsequent isolation of cell clones that had lost the foreign DNA previously conferring increased antibiotic tolerance to the cell. Thus, the cells were cured yet remained biochemically unaltered.

MATERIALS AND METHODS

Mycoplasma Detection

Routinely, three different tests were employed.

DNA detectable in the cytoplasm of cells is a strong indication of mycoplasma contamination and was assayed employing a DNA fluorochrome staining kit (Seromed/Biochrom, Germany, cat. no. D 7001). The tissue culture cells to be tested were grown to 30% confluency on slideflasks (Nunc, Denmark, cat. no. 170920). Subsequently, the cells were treated according to the manufacturer's instructions.

For the Hybricomb test (Biological Industries, Israel; cat. no. MTK-12), the cells to be tested were seeded on a tissue culture petri dish at low density so as to reach 30% confluency within four days of incubation without requiring any further medium change. Thereafter, an aliquot of the supernatant was processed according to the manufacturer's description The Hybricomb test is based on specific mycoplasma DNA hybridization.

Isolation of mycoplasmas from infected tissue culture cells and subsequent *in vitro* cultivation was performed as follows:

10 mL of 96 h conditioned medium from confluent cells were inoculated into 100 mL of Mycoplasma Liquid Media BT1/1 and BT2/1 (Amimed/Biotrade, Austria), respectively. Subsequently, the samples were incubated for 14 days at 37°C, 5% CO_2 in a regular cell culture incubator, in order to allow for growth/division of mycoplasma potentially present. After 3, 7, and 14 days of incubation, 800 µL were taken from the incubation reactions and spread onto two BT3 and two BT4 Mycoplasma agar plates. These media were prepared by autoclaving 35 g of PPLO agar (Difco, USA; cat. no. B412) in 1,000 mL H_2O, and, thereafter, adding 400 mL of either Mycoplasma Nutrient Component BT3 or BT4 (Amimed/Biotrade, Austria) prior to solidification.

One plate was incubated aerobically and the other anaerobically for three weeks. They were regularly inspected microscopically for growth and the presence of mycoplasma, which exhibit a typical 'fried egg' appearance.

Tissue Culture

Mycoplasma negative BHK-21 cells (ATCC CCL 10, BHK/Myco–cells) were routinely grown in DMEM: Ham's F12 medium (BioWhittaker, Belgium; cat. no 12-719F) supplemented with 10% fetal calf serum, 2 mM glutamine, and 0 075% bicarbonate Acholeplasma laidlawii [21] infected BHK-21 cells (BHK/Myco+) were grown under identical conditions

Plasmids, Transfection and Selection

1 µg pUCSV-Neo [22], in combination with 4 µg pUCSV-dhfr where applicable, was introduced into 30% confluent cells on a 5 cm tissue culture petri dish by the $CaPO_4$ coprecipitation technique essentially as described [23]. pUCSV-dhfr was constructed by cloning the *KpnI/SalI* fragment from pNUT [24] into analogously digested pUC19. The volume of the DNA containing solution was adjusted to 250 µL with 1 mM Tris/Cl pH 8.0, 0.1 mM EDTA, and 25 µL 2.5 mM $CaCl_2$ were added. This solution was added to 250 µL 2xHBS (280 mM NaCl, 2.5 mM Na_2HPO_4, 50 mM Hepes/NaOH pH 7.12), mixed, and spread onto the cells. After three hours, the medium was removed, and 1 mL of PBS containing 15% glycerol added. After one minute, the PBS/ glycerol was removed by aspiration, the cells were thoroughly rinsed twice with PBS, and fresh medium was added.

Two days after transfection, the cells were split into large tissue culture petri dishes at different densities,

and either 1 mg G418 (Gibco, USA; cat. no. 11811-049, 10 mg G418/mL PBS sterile filtered stock solution) or, in the case of double selection, 0 5 mg G418/mL medium and 400 nM methotrexate (Sigma, cat. no. M 8407) were added. Approximately two weeks post transfection, cell clones became clearly visible whereas unmanipulated cells did not survive under these conditions. The cell clones were trypsinized, and seeded onto new petri dishes. For continued passage, these cells were grown either in the presence or absence of G418

For isolation of individual cell clones, cells were split into tissue culture dishes at very low densities. Individual colonies that arose were transferred, by means of trypsin/EDTA saturated cotton swabs, to 24 well dishes, grown to confluency, and split equally into 6 well dishes in the absence and presence of G418 as described in the text.

PCR Testing for Foreign DNA

In order to test for the presence of the selection marker plasmid in the cells by PCR, cellular DNA was prepared essentially as described [25]· 5×10^5 cells were pelleted, 50 μL of 2× lysis buffer (with Proteinase K) added and incubated for 4 h at 56°C, yielding 100 μL lysate. Thereafter, Proteinase K was inactivated by incubating the sample for 10 min at 95°C, followed by vigorous shaking of the sample for 5 mins at 95°C. Samples were stored at -20°C. PCR was performed with 1 μL lysate in a 100 μL reaction consisting of 1 × PCR buffer (Boehringer Mannheim), 200 μM of dATP, dGTP, dTTP, and dCTP each, 1 μM of each primer, and in the presence of 1 μL Taq polymerase (Boehringer Mannheim) The PCR primers were 5'-GACGTTGTCACTGAAGCGGGAA-3' and 5'-TCACGGGTAGCCAACGCTATGTC-3', and the PCR cycle consisted of 1 min at 55°C for annealing, 1 min at 72°C for extension, and 1 min at 95°C for denaturation. Thirty cycles were run.

RESULTS

The goal of this study was to investigate whether simple genetic manipulation of the infected cell could confer sufficient antibiotic resistance to the host cell, such that these cells would tolerate high concentrations of the antibiotic without suffering any adverse effects, while simultaneously ensuring the permanent elimination of mycoplasma. Since, by conventional antibiotic susceptibility determination of mycoplasma *in vitro*, the deoxystreptamine group of antibiotics (*e.g.* hygromycin B, kanamycin, and neomycin) had previously been shown to be lethal to a wide variety of mycoplasma strains [6,17,21,27], neomycin phoshotransferase was chosen as a model selection marker. Neomycin phosphotransferase mediates resistance to neomycin and kanamycin in bacteria as well as to the neomycin analog G418/geneticin in tissue culture cells. The latter selection system is popularly used in many laboratories for the establishment of stably transfected cell lines, hence widely available, and many experimentors are familiar with its use.

Mycoplasma Eradication from Tissue Culture Cells

BHK/Myco+ cells, harboring *Acholeplasma laidlawii*, were shown to test positive for mycoplasma by DNA staining, hybridization, and *in vitro* culturing. Mycoplasma free BHK (BHK/Myco–) cells tested negative in all three tests. These cells were passaged and served as the positive and negative controls for subsequent tests

Plasmid pUCSV-Neo, mediating expression of the bacterial neomycin phosphotransferase, was introduced into BHK/Myco+ cells (Fig. 1(A)) Stably transfected colonies resistant to 1 mg G418/mL were selected; cells that had not taken up the plasmid died. Approximately 200 colonies were pooled and grown to confluency. After 18 cell divisions post transfection under permanent exposure to G418 selection, the cells were subjected to mycoplasma detection. In the three tests employed, the cells were found to be negative (Fig 1(G)). The generation time of untransfected cells (in the absence of G418) was found to be equal to that exhibited by the transfected cells resistant to G418 when cultivated in the presence of G418 This finding shows that the transfected cells grew well in the presence of the high G418 concentration.

In order to determine whether the inability to detect mycoplasma was only due to the suppression of mycoplasma growth below the limits of detection or, rather, to a permanent eradication, the cells were subsequently cultivated for 19 cell divisions in the absence of selection pressure. Thereafter, the cells still tested negative for mycoplasma (Fig 1(I)). However, after passaging the cells for an additional 11 cell divisions without G418, they were found to be mycoplasma positive again (Fig. 1(B)) Thus, mycoplasma recurrence had occurred 30 cell divisions after omission of the selective agent G418. This finding indicated that exposure to G418 for 18 generations was not sufficient to permanently remove mycoplasma Rather, this temporary exposure of mycoplasma to G418 had only resulted in growth suppression to the extent that allowed them to evade detection.

Historically, it has been a matter of dispute when a cell was considered cured. Our observation of mycoplasma recurrence after 30 cell divisions in the absence of the antimicrobial agent confirms the necessity for a 30 cell division post-treatment follow-up testing, as has been suggested by some reports [2,15].

Transfected cells permanently cultivated in selective medium for 110 cell divisions after transfection remained mycoplasma negative (Fig. 1(F)) Cells exposed to G418 for a prolonged period of time after transfection, *i.e* 53 generations (Fig. 1(H)), tested mycoplasma negative when cultivated for additional 64 cell divisions without G418 (Fig. 1(C)). Since growth-suppressed mycoplasma in tissue culture had been shown to regain detectability at approximately 30 cell divisions after withdrawal of the selective agent (see above), this finding suggests that the mycoplasma were, in fact, permanently eliminated from the transfected infected cells when exposed to the toxic agent for 53 cell divisions prior to its withdrawal.

Thus, expression of an antibiotic resistance marker in infected cells allows exposure to antibiotic concentra-

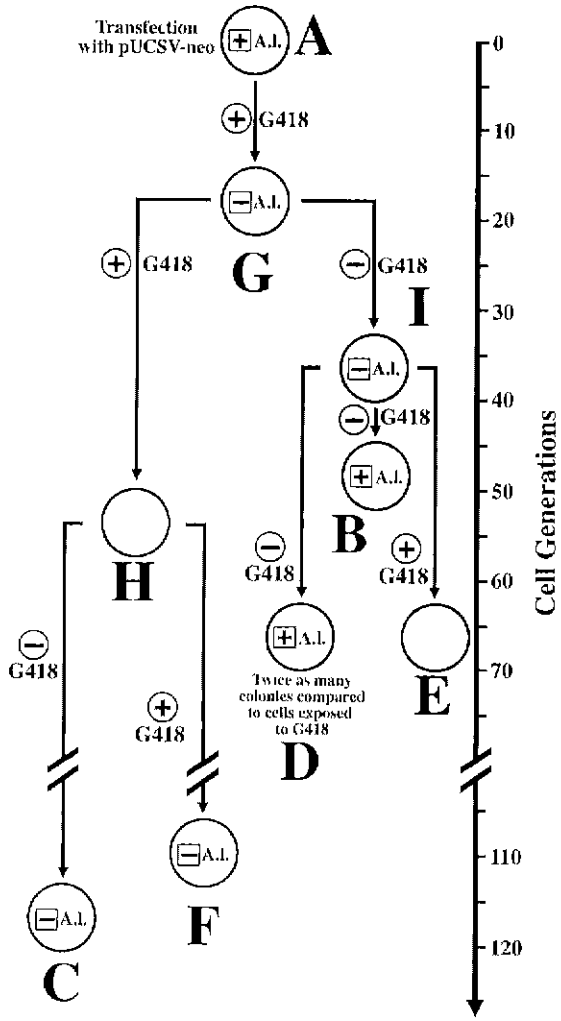

Fig. 1 Eradication of mycoplasma from infected cells by use of a selection marker plasmid and subsequent selection. *Acholeplasma laidlawii* contaminated BHK-21 cells were transfected with the plasmid pUCSV-Neo, which mediates resistance to the selective agent G418 (A). Stably transfected cells were selected (G) and subsequently cultivated either in the presence (H) or absence (I) of G418. Although mycoplasma could not be detected after the host cells had been exposed to G418 for 18 cell divisions (G), the mycoplasma had not been eliminated, but could be readily detected after an additional 30 host cell divisions in the absence of G418 (B). Cells seeded, after initial selection and subsequent omission of G418 (I), into medium lacking the selective agent yielded twice as many clones (D) compared to the number of clones detected in medium containing G418 (E). Similarly to cells cultivated for 110 divisions in medium harboring G418 (F), cells initially exposed to G418 for 53 generations post transfection (H) and passaged for 64 cell divisions in the absence of the toxic agent thereafter remained mycoplasma negative (C). Large circles represent cells at individual time points during anti-mycoplasma treatment. The distance between the cells is proportional to the number of cell divisions '+' and '−' in squares represent positive and negative *Acholeplasma laidlawii* (A.I.)/mycoplasma test results, and cultivation of cells in the presence and absence of G418 when in small circles.

tions high enough to lead to the permanent eradication of mycoplasma while ensuring host cell survival.

Genetic Instability of the Transfected Resistance Marker Gene

Exposure to the toxic agent selects for those cells retaining the resistance marker expression independent of whether the corresponding genetic information is stably or instably maintained in the cell population. Upon removal of selection pressure, expression of the foreign protein often ceases quickly unless the heterologous genetic information has integrated stably into a host cell chromosome. Foreign genetic information is thought to be genetically labile when present episomally in the nucleus, or when integrated within a structurally instable chromosomal locus [28-30].

When equal numbers of cells, having undergone 18 cell divisions in the presence and 30 cell divisions in the absence of the toxic agent (Fig. 1(B)), were cultivated for an additional 30 cell divisions either in the presence or absence of G418 (Fig. 1(E) and (D)), twice as many cell clones were present in the medium lacking G418. This finding suggested potential genetic instability of the selection marker in the transfected BHK/Myco+ cells: when grown in the presence of the toxic agent, all cells having lost the vector harboring the selection marker gene died, resulting in a reduced number of clones (Fig. 1(E)). In contrast, in the absence of the toxic agent, cells survive even when they have lost the selection marker plasmid.

Rescue of Cured Albeit Antibiotic Susceptible Revertant Cells

The permanent expression and presence of the selection marker protein in the cured cells may influence intracellular processes directly or indirectly and, thus, might be undesirable in certain instances. If, after successful mycoplasma eradication, the absence of the selection marker protein is required, the fact that stable integration of foreign DNA into the host cell chromosomes occurs very inefficiently can be exploited. Given the potential genetic instability of the resistance marker gene, as described above, it should be possible to identify individual cell clones having lost neomycin phosphotransferase expression after successful anti-mycoplasma treatment.

BHK/Myco+ cells cotransfected with vectors pUCSV-Neo and pUCSV-dhfr, mediating resistance towards G418 and methotrexate (MTX), were used in order to investigate the postulated loss of the selection marker expression vector (Fig. 2(A)). Based on the previous experiment, exposure of the transfected cells to the toxic agent for 53 cell divisions is considered sufficient for complete mycoplasma eradication (Fig. 1(C)).

Stable anchorage of foreign DNA in the host cell is generally known to increase with extended cultivation in the presence of the selective agent. In order to investigate whether, even under stringent conditions (i.e. even upon cultivation in the presence of G418 in significant excess to 53 generations) cell clones having lost the resistance marker DNA upon permanent myco-

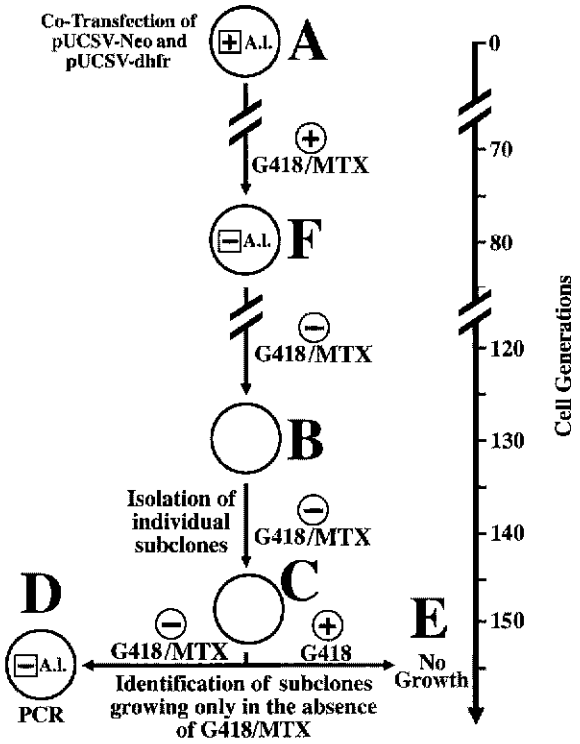

Fig. 2 Isolation of cell clones susceptible to the selective agent upon withdrawal of the latter after anti-mycoplasma treatment *Acholeplasma laidlawii* infected BHK-21 cells were co-transfected with plasmids pUCSV-Neo and pUCSV-dhfr, mediating resistance against G418 and increased concentrations of methotrexate (MTX), respectively. Transfected cells were selected (F) and subsequently cultivated in the absence of the selective agents (B). Individual clones were isolated (C) and tested for their ability to grow either in the presence (E) and absence (D) of the toxic agents. Large circles represent cells at individual time points during anti-mycoplasma treatment. The distance between the cells is proportional to the number of cell divisions '+' and '−', in the squares represent positive and negative *Acholeplasma laidlawii* (A.I.)/mycoplasma test results, and cultivation of cells in the presence and absence of G418 when in small circles

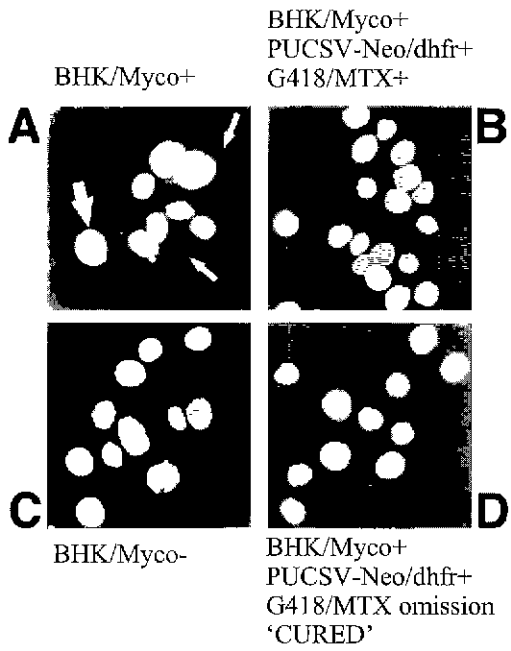

Fig. 3. Mycoplasma detection by DNA staining. mycoplasma free, contaminated, and permanently cured BHK cells. (A) *Acholeplasma laidlawii* infected BHK-21 cells; (B) transfected cells constantly cultivated in the presence of the selective agents; (C) sterile BHK cells; (D) a cured BHK cell clone, after treatment for permanent mycoplasma eradication and subsequent prolonged cultivation in the absence of the selective agent, resulting in loss of resistance (corresponding to the clones in Fig. 2(D)) BHK/Myco +, −, mycoplasma infected and sterile BHK-21 cells, pUCSV-Neo/dhfr +, transfected with selection marker plasmids pUCSV-Neo and pUCSV-dhfr and selected thereafter, G418/MTX, cultivated either in the permanent presence (+) or, after initial presence, upon omission of G418/methotrexate. The large arrow depicts a nucleus; the small arrows point at intracellular fluorescence typical to mycoplasma located in the cytoplasm

plasma eradication could be identified, the transfected BHK/Myco+ cells were exposed to G418/MTX for 80 cell divisions. As expected, mycoplasma could not be detected in the pool of resistant cells (Fig. 2(F)). Thereafter, the selective agents were omitted. After additional 50 cell divisions, the cells were split in order to allow for clone isolation (Fig. 2(B)). Individual subclones were isolated (Fig. 2(C)) and cultivated in medium either lacking any toxic agents (Fig. 2(D)) or containing G418 (Fig. 2(E)). Several clones were shown to be able to grow only in the absence of G418 (Fig. 2(D)). When assayed for the presence of mycoplasma, these clones tested negative, even after cultivation for 73 cell divisions in the absence of selective agents, and thus were demonstrated to be cured from mycoplasma. Employing the DNA-staining detection test, a representative clone, cured from mycoplasma and incapable of growing in the presence of G418, is shown in Fig. 3(D). The control cells used in this assay were mycoplasma infected (Fig. 3(A)) and mycoplasma free (Fig. 3(C)) cells, as well as treated cells permanently exposed to the selective agents (Fig. 3(B)).

This finding demonstrated that, upon successful mycoplasma eradication, cell clones could be identified whose selection marker expression had ceased.

Antibiotic Sensitivity Revertance as the Result of Loss of the Antibiotic Resistance Mediating Expression Vector

In order to test whether clones exhibiting G418 sensitivity after mycoplasma eradication had regained sensitivity to G418 as a consequence of the loss of the resistance marker gene, rather than due to simple cessation of expression by other cellular mechanisms (*e.g.* transcriptional silencing subsequent to methylation), polymerase chain reaction (PCR) analysis was performed with four clones. Total cell lysates were prepared, and primers specific for the neomycin phosphotransferase

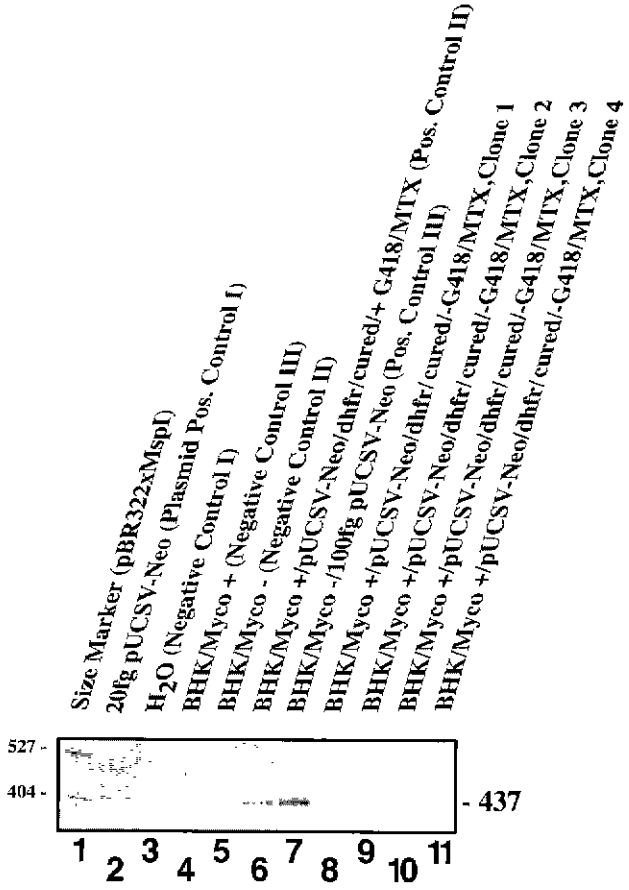

Fig. 4 Detection of selection marker vector DNA in mycoplasma contaminated, free, and treated BHK-21 cells PCR reactions for the amplification of a DNA fragment, 437bp in length and specific for the expression vector conferring G418 resistance to the cells, were performed in the lysates of mycoplasma free, mycoplasma contaminated, and 'cured' cells as well as in appropriate control reactions (lanes 2-11) 1, DNA molecular weight marker; 2, 20 fg selection marker vector DNA (pUCSV-Neo); 3, No DNA added; 4, mycoplasma infected BHK cell lysate; 5, sterile BHK cell lysate, 6, lysate from mycoplasma positive cells after treatment and grown in the presence of the selective agent; 7, mycoplasma free cell lysate supplemented with 100 fg selection marker plasmid DNA; 8 - 11, lysates from clones of infected cells, exhibiting regained selective agent susceptibility upon successful treatment and subsequent cultivation in the absence of the selective agent

gene in pUCSV-Neo were used for amplification The size of the fragment to be amplified was expected to be 437 base pairs.

PCR of DNA derived from transfected BHK cells, permanently exposed to G418/MTX for 153 generations and used as a control (Fig. 3(B)), exhibited the expected PCR product (Fig 4, lane 6 'Positive Control II') Similarly, this fragment could be detected by PCR reactions employing either the neomycin resistance mediating plasmid alone (Fig. 4, lane 2 'Plasmid Positive Control I') or a mycoplasma negative BHK lysate to which the plasmid DNA was added (Fig. 4, lane 7 'Positive Control III') In contrast, a PCR fragment was not obtained from mycoplasma negative (Fig. 4, lane 5 'Negative Control II'; Fig. 3(C)) or from mycoplasma positive but untreated BHK cells (Fig. 4, lane 4 'Negative Control III'; Fig. 3(A)). Likewise, no fragment was detected by PCR in the four cell clones that originally had been mycoplasma positive but were considered cured upon treatment and shown to have regained G418 susceptibility upon prolonged omission of the selective agents (Fig. 4, lanes 8-11). These findings strongly suggest that loss of G418 resistance resulted from the loss of the expression marker DNA previously mediating the resistance to G418.

Assuming the presence of one neomycin phosphotransferase plasmid per cured cell, cultivated in the permanent presence of the selective agents, the resulting PCR band was expected to exhibit an intensity corresponding to that shown in the PCR reaction employing 20 fg pUCSV-Neo plasmid (Fig. 4, lane 2). The PCR reaction in these cells, however, revealed quantities of the PCR fragment (Fig. 4, lane 6) slightly less than that shown by 100 fg plasmid (Fig. 4, lane 7). 100 fg plasmid corresponds to 2.5×10^4 copies of the neomycin phosphotransferase gene, whereas 1 μL of lysate is derived from from 5×10^3 cells. Thus, the experimental data suggest that each G418 resistant cell contains approximately four (*i.e.* slightly less than five) copies of the resistance marker plasmid.

DISCUSSION

When cultured in broth media, mycoplasma are very sensitive to a wide range of antimicrobial agents Upon infection of tissue culture cells, however, cell associated mycoplasma often are protected from these agents. The concentration of drugs, *e.g.* kanamycin and neomycin, necessary to permanently eliminate mycoplasma from tissue culture cells frequently is toxic for the cells [31]. Antibiotic concentrations low enough to be tolerated by the cell often are reported to suppress mycoplasma growth only temporarily (rather than causing their eradication); in many cases, *e.g* when *Acholeplasma laidlawii* is exposed to neomycin, mutant strains occur which have developed resistance to high concentrations of the antibiotic in use [21].

In this report, a genetic approach of mycoplasma eradication from tissue culture cells is described. Transfection of the neomycin phosphotransferase gene confers resistance against high concentrations of the antimicrobial agent G418, a neomycin analog, to the host cells. Thus, the cells can be exposed to antibiotic concentrations high enough to permanently eliminate mycoplasma while simultaneously tolerating high concentrations of the antibiotic chosen without suffering from any adverse effects.

Exposure of mycoplasma infected baby hamster kidney cell line BHK-21, chosen as the model system cells, to G418 for 18 cell divisions after transfection suppressed growth of cell associated mycoplasma below the limit of detection. However, this period of time was not sufficient for permanent mycoplasma removal, since mycoplasma reccurrence was detected after growth of the cells in the absence of G418 for an additional 30 cell

generations. Successful eradication of mycoplasma from infected cells was, however, achieved by exposure of the cells to G418 for 56 cell divisions prior to omission. These findings imply that short exposure to G418, although shown to severely suppress mycoplasma growth, was not sufficient to permanently eliminate the mycoplasma contamination. Rather, prolonged exposure to the antibiotic was compulsory. This finding emphasizes that long-term monitoring, a criterion which is often not obeyed strictly enough [20], is absolutely mandatory to ensure the permanent eradication of mycoplasma.

Presumably, no given antibiotic is likely to inhibit all mycoplasma strains [32]. However, quite a few cell culture expression systems are available that mediate resistance against cytotoxic agents other than G418, such as hygromycin B, puromycin, or bleomycin, which are toxic to mycoplasma and which provide potentially useful alternatives to the use of neomycin resistance for the described approach [33].

For mycoplasma strains that are known to easily develop resistance to antibiotics, a double selection protocol analagous to a conventional mycoplasma removal approach, combining or alternating different antibiotics, could be employed [15]. Plasmids mediating resistance to different antibiotics, e.g. hygromycin and neomycin, could be introduced into the infected cells simultaneously [21], and the transfected cells could be doubly selected [33,34]. The double selection approach might even further minimize the risk of selecting for mycoplasma substrains developing increased resistance to any given single antibiotic.

In cases in which, after curing, the presence of the selection marker used for mycoplasma eradication is undesirable, the mammalian cells' low rate of stable integration of foreign DNA into their chromosomes can be exploited. In this study, we demonstrate that the transfected cells expressed the resistance marker after transfection for as long as the selection pressure persisted. Upon cultivation of the cured cells in media lacking the selective agent, a significant fraction of the cells had regained susceptibility to the antimicrobial agent. Employing PCR, it was shown that the abolished selection marker expression was due to the loss of the selection marker DNA. It might be argued that point mutations in a PCR primer binding site may cause the selection marker to become non-functional and, in addition, reduce the PCR efficiency below the limit of detection. Given the presence of four selection marker plasmids in one cell prior to omission of the selection pressure, as shown, it is, however, highly unlikely that such a rare mutational event would affect all four plasmids in this cell alike, and that this would occur, to an extent entirely abolishing detection by PCR, in all clones investigated. Rather, the findings most plausibly demonstrate the transient presence of the heterologous DNA in the cell undergoing treatment. The approach for mycoplasma eradication described here thus yielded mycoplasma free cells unaltered by the treatment.

The nature of the physical association between mycoplasma and cells is a matter of debate, postulating either an intimate extracellular interaction of mycoplasma with their host cell by means of specialized tip organelles or, alternately, an intracellular manifestation [37] In this investigation, the fluorescence images of the mycoplasma infected cells may suggest an intracellular location (Fig. 3)

The reason for the extraordinary resistance of mycoplasma to antibiotic agents when associated with eucaryotic cells is unclear. G418, blocking translation by interacting with 80S ribosomes, is inactivated by neomycin phosphotransferase mediated phosphorylation. Why G418 taken up from the medium may be toxic to mycoplasma, if intracellular, while leaving the surrounding host cell unharmed remains to be determined. Given the significant antibiotic concentrations used, most plausibly a substantial portion of the G418 molecules entering the host cell may proceed into the mycoplasma, before being exposed to the neomycin phosphotransferase present in the cytoplasm, and therefore evade inactivation. Once within the mycoplasma, G418 is protected from neomycin phosphotransferase action, and thus can exert its lethal effects on the mycoplasma. In contrast, G418 remaining in the cytoplasm of the host cell is exposed to the neomycin phosphotransferase and inactivated.

The G418/neomycin phosphotransferase system is popularly used in many laboratories for the establishment of stably transfected cell lines; hence, this system is widely available and many experimentors are familiar with its use. Given the varying success met by the conventional antibiotic mediated mycoplasma elimination from infected cells, the described approach represents a promising means for the eradication of particularly refractory mycoplasma from cells that are highly valuable yet not replaceable by other sources, all awhile minimizing the risk of developing resistant mycoplasma substrains.

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