Plasmid transformation and replica filter plating of *Acholeplasma laidlawii*

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1. SUMMARY

The restriction deficient mutant 8195 of *Acholeplasma laidlawii* strain JA1 was transformed by the promiscuous streptococcal plasmid vector pNZ18 at a frequency of $4 \times 10^{-4}$/cfu. The plasmid was maintained without structural rearrangements but was lost in the absence of a selection pressure, i.e. kanamycin or neomycin. Transformed primary colonies were easily recognized due to a different colony morphology. Replica filter plating, previously not obtained with mycoplasmas, was achieved using pNZ18 as a marker by incubating the replica filters with the cell side *down* on the new agar plates. These findings should greatly facilitate the genetic and functional analysis of *A. laidlawii*.

2. INTRODUCTION

The mycoplasmas are the smallest free-living bacteria known, they lack a cell wall and have evolved from the Gram-positive bacteria [1]. *Acholeplasma laidlawii* has in biophysical terms, the most well characterized membrane of all cells and organelles [2]. A more detailed analysis of the structural and physiological interplay between membrane proteins and lipids can probably be achieved through the use of genetic techniques. However, at present no natural plasmids have been found in *A. laidlawii*. There is no suitable plasmid vector for cloning in mycoplasmas but promising results have been obtained utilizing two separate integrating transposons (Tn916 and Tn4001) albeit with low frequencies [3,4]. Recently, two streptococcal plasmids were also shown to replicate in *A. laidlawii* [5]. Unfortunately, these plasmids formed unstable deletion derivatives. We have sought a shuttle vector system that is maintained in a stable state without spontaneous genetic rearrangements, and this report demonstrates the feasibility of the promiscuous lactic streptococcal vector pNZ18 for these purposes.

In this report we also present a modified method for replica filter plating using pNZ18 as a marker. Replica filter plating has previously not worked for the mycoplasmas. Utilizing our new technique *A. laidlawii* colonies could easily be replica plated by incubating the replica filters with the cell side *down* on new agar plates. This yielded a new...
master plate plus colony replica filters suitable for immunological analysis [6].

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth media

*Acholeplasma laidlawii* strains A-EF22, JA1 and 8195 were grown in a bovine serum albumin-tryp- tose medium supplemented with 75 μM palmitic acid and 75 μM oleic acid. Strain 8195 lacks a restriction and modification system and is derived from JA1 [7]. Cells were assayed as colony forming units (cfu) on agar plates. *Escherichia coli* MC1061 and DH1 were grown in Luria-Bertani medium with 0.2 (w/v) glucose. Replica filters (cut to 6 cm²) were from Amersham (Hybond C and N).

3.2. Plasmid purification

Plasmid pAM120 is 21.4 kb and contains the transposon Tn916 which encodes tetracycline (tet) resistance. pNZ18 is 5.7 kb and contains the replicon from *Streptococcus lactis* plasmid pSH71, antibiotic resistance genes from *Staphylococcus aureus* plasmids pUB110 (kanamycin (kan)/ neomycin (neo)) and pC194 (chloramphenicol (cml)) and a cloning cassette from pJR158 (W.M. de Vos, personal communication and ref. 8). pAM120 and pNZ18 were isolated from amplified cultures of *E. coli* (strains DH1 and MC1061, respectively), using the alkaline lysis method [9]. Plasmids from *A. laidlawii* were isolated with Qiagen tip-20 (Qiagen GmbH), 15 ml culture yielded approximately 1 μg plasmid.

3.3. Transformations

Transformation of *A. laidlawii* with PEG was done as described by Sladek and Maniloff [10] with some minor modifications. Following the transformation, cells were incubated 2 h at 37°C and aliquots were thereafter plated on agar plates containing 2 μg/ml tet, 10 μg/ml cam, 70 μg/ml kan or 40 μg/ml neo, respectively. The plates were incubated for 5 days at 37°C. Controls were processed identically, but without plasmid DNA. Colonies were picked with pasteur pipettes and the agar plugs were placed in 2 ml prewarmed growth medium containing appropriate antibiotics. After 2–3 days, when turbidity was observed, transfers were made to growth media which contained stepwise higher concentrations of antibiotics.

3.4. Protein and lipid analysis

The protein composition of membranes and cytoplasm from different clones were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [11]. Proteins were identified by Coomassie staining and immunoblotting with anti-membrane antibodies [11]. Lipids were extracted from fatty acid labelled membranes and separated by thin layer chromatography. Lipid amounts were determined by liquid scintillation counting [11].

4. RESULTS AND DISCUSSION

4.1. Transformation and colony morphology

Plasmid pNZ18 (Fig. 1) is a variant of the promiscuous lactic streptococcal derived vector pNZ12, which can replicate in several Gram-positive bacteria as well as in *E. coli* (W.M. de Vos, personal communication and ref. 8). Since mycoplasmas are related to streptococci and bacilli [1], it was of interest to investigate if pNZ18 could replicate in *A. laidlawii*. The transposon containing plasmid pAM120, previously shown to be able...
to transform *A. laidlawii*, was used as a control for the efficiency of the transformation procedure used. pAM120 and the PEG-method yielded approximately $10^{-6}$ transformants per cfu of *A. laidlawii* 8195 on plates with 2 $\mu$g/ml tet. These transformants could also be grown in media containing 40 $\mu$g/ml tet. A similar transformation frequency has been observed by Dybvig [3] when transforming *A. laidlawii* with pAM120. With pNZ18 *A. laidlawii* 8195 could be transformed at approximately $10^{-7}$ transformants/cfu on plates with 40 $\mu$g/ml kan. This frequency could not be improved by the presence of RNA or chromosomal DNA in the transformation mixture, nor by CaCl$_2$ and heat shock treatment (cf. ref. 12) or by using pNZ18 from different hosts, i.e. *E. coli* and *A. laidlawii*. Strains A-EF22 and JA1 could not be transformed by pNZ18. Transformed 8195 cells could be transferred stepwise to liquid media containing 800 $\mu$g/ml kan or 80 $\mu$g/ml neo in which rapid growth was still maintained. Due to the long incubation times (i.e. $\geq$ 5 days at 37°C) a number of non-transformed cells could grow on plates with 20 to 70 $\mu$g kan/ml. This resistance has also been recently observed for *Mycoplasma pulmonis* [4]. However, colonies of cells transformed with pNZ18 were larger and grew substantially faster when compared to non-transformed colonies. Colonies of transformed cells also had a different morphology. This was easily detected by light microscopy (Fig. 2). *A. laidlawii* colonies usually exhibit a smooth, fried-egg-like morphology as seen at the top of Fig. 2, but on the agar plates with transformed cells there were many colonies with a rough or granular morphology. The granular morphology was most apparent on the primary plates after transformation. All granular colonies tested contained pNZ18, see below. *A. laidlawii* grows in clusters, with a dozen or so cells in each cluster. As a result of the low transformation frequency not all cells in a cluster can be transformed, so granular colonies could consist of both transformed and untransformed cells, growing at different rates (cf. *lac* phenotypes in *E. coli* colonies). Upon a number of transfers in liquid media containing antibiotics kan or neo, all colonies converted to the smooth type but still contained pNZ18 (cf. below). These different mot.

![Fig. 2. Different colony morphology on the primary agar plates (with kan or neo), after PEG transformation of *A. laidlawii*. The granular colonies contain the plasmid pNZ18. Bar = 0.3 mm.](https://academic.oup.com/femsle/article-abstract/72/1-2/147/454345)
Phyllosomes represent a possible characteristic to initially distinguish transformed from nontransformed *A. laidlawii* cells.

4.2. Structural and segregational stability of pNZ18

pNZ18 isolated from *A. laidlawii* and from *E. coli* were cut with 5 different restriction enzymes (SalI, SacI, PstI, BglII and PvuII), one by one or pairwise. There were no differences in cleavage patterns between the two isolates, and the sizes of the fragments were in accordance with the restriction map of pNZ18 (Fig. 1). These results show that there have not been any deletions or rearrangements of pNZ18 in *A. laidlawii*.

*A. laidlawii* transformed with pNZ18 could not grow in a higher concentration of *cam* than could the untransformed *A. laidlawii* (≤ 5 μg/ml). *E. coli* transformed with pNZ18 isolated from this *A. laidlawii* could grow on agar plates with 10 μg/ml *cam*, whereas untransformed *E. coli* could not. This indicates that the *cam* resistance gene could not be expressed in *A. laidlawii*.

A selected clone of 8195, transformed with pNZ18 (called 2500) was maintained for 10 daily transfers in growth medium without antibiotic. This transformed clone was compared to strain 2500 maintained in growth medium with *kan* and with untransformed 8195 (Table 1). *A. laidlawii* with pNZ18 grown in 220 μg/ml *kan* (strain 2500 + ) yielded large (smooth) colonies on plates with and without *kan* whereas for cells maintained without selection pressure (strain 2500 - ) or without plasmid (strain 8195), the dominating colony morphology was small on plates containing *kan* (Table 1). For 2500 +, all colonies tested contained pNZ18 whereas for 2500 - colonies only one out of five on 0 μg *kan* plates, most of the large colonies and a minority of the small colonies on 20 μg/ml *kan* plates contained pNZ18. Hence, pNZ18 is lost from the cells in the absence of antibiotics. Furthermore, on *kan* plates a correlation existed between colony size and the presence of pNZ18. An isolate from 2500 - (called 2501) shown to have lost pNZ18 (see above) could repeatedly be transformed by pNZ18 to *kan* or *neo* resistance with a frequency of $4 \times 10^{-4}$/cfu or $4 \times 10^3$/μg plasmid DNA. pNZ18 could be isolated from all these transformants tested, but pNZ18 could not be isolated from colonies on the control plates (frequency < $10^{-7}$/cfu).

4.3. Replica filter plating

A genetic analysis of transformant colonies is usually improved by replica plating techniques. However, so far these techniques have not worked with mycoplasmas (cf. ref. 13). Here we have used pNZ18 as a selectable marker to improve the method. Nitrocellulose filters (prewetted) were put on *A. laidlawii* colony plates. After 1 min the filters were transferred to new agar plates with the cell side up or down against the agar. The plates with filters were incubated for 4 days at 37°C. Nylon filters could also be used (without prewetting). For filters with the cell side down, but not up, colonies could be seen both on the down-side of the filter and on the new agar plate. The same master plate could be used for several replica filter transfers. Table 1 shows that *A. laidlawii* could be replica transferred to *kan* plates at percentages similar to the occurrence of pNZ18 in the strains used, cf. results above. Colonies smaller than approximately 0.5 mm were difficult to transfer. These filters should be useful for immunobinding [6] and enzymatic assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Kanamycin concentration (μg/ml)</th>
<th>Colony size</th>
<th>Filter colony transfer (%)</th>
<th>Filter colony transfer (%)</th>
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</thead>
<tbody>
<tr>
<td>8195</td>
<td>0</td>
<td>large</td>
<td>100</td>
<td>0</td>
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<tr>
<td>20</td>
<td>small</td>
<td>100</td>
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<td>2500 +</td>
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<td>20</td>
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<tr>
<td>2500 -</td>
<td>0</td>
<td>large</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>20% large</td>
<td>100</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>80% small</td>
<td>100</td>
<td>20</td>
<td>20</td>
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</table>

* Strain 2500 (with pNZ18) maintained for at least 20 transfers (≥ 140 generations) in the presence of kanamycin.

* Strain 2500 (with pNZ18) maintained for 10 daily consecutive transfers in growth medium without kanamycin.

* The percentage of colonies which could be transferred with replica filter plating to new agar plates. The number of colonies were about 30 per replica filter.
4.4. Protein and lipid composition in transformed cells

SDS-PAGE plus immunoblotting analysis of membrane and cytoplasmic proteins of transformed (strain 2500 in kan and neo) and untransformed A. laidlawii (strains 8195 and 2501) revealed great similarities between transformed and untransformed clones (data not shown). Protein migrating like the kan/neo nucleotidytransferase or the cam acetyltransferase could not be seen in the Coomassie stained gels. Thin layer chromatography and scintillation analysis of the membrane lipids in transformed and untransformed 8195 showed no differences in lipid composition between the clones.

4.5. Conclusions

pNZ18 is a plasmid vector that can transform A. laidlawii at usable frequencies and is not modified. Furthermore, pNZ18 can shuttle between A. laidlawii, E. coli and Bacillus subtilis. The replication of pNZ18 in A. laidlawii is corroborated by recent findings showing great sequence similarities of the ori and repA regions of pNZ18 (Fig. 1) with the corresponding regions of the cryptic plasmid pADB201 [14] from Mycoplasma mycoides (ref. 15 and W.M. de Vos, personal communication).

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REFERENCES