Utilization of Virus φCh1 Elements To Establish a Shuttle Vector System for Halo(alkali)philic Archaea via Transformation of Natrialba magadii

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In the study described here, we successfully developed a transformation system for halo(alkali)philic members of the Archaea. This transformation system comprises a series of Natrialba magadii/Escherichia coli shuttle vectors based on a modified method to transform halophilic members of the Archaea and genomic elements of the N. magadii virus φCh1. The shuttle vector pRo-5, based on the repH-containing region of φCh1, stably replicated in E. coli and N. magadii and in several halophilic and haloalkaliphilic members of the Archaea not transformable so far. The φCh1 operon ORF53/ORF54 (repH) was essential for pRo-5 replication and was thus identified as the minimal replication origin. The plasmid allowed homologous and heterologous gene expression, as exemplified by the expression of φCh1 ORF34,22, which encodes a structural protein, and the reporter gene bgaH of Haloferax lucenterse in N. magadii. The new transformation/vector system will facilitate genetic studies within N. magadii and other haloalkaliphilic archaea and will allow the detailed characterization of the gene functions of N. magadii virus φCh1 in their extreme environments.

Natrialba magadii belongs to the haloalkaliphilic group of the Halobacteriaceae. In contrast to the neutrophilic haloarchaea, the halophilic holoarchaea require a high pH (8.5 to 11) and high salt (4 to 5 M NaCl) for growth and thus are considered a distinct physiological group (1). Although there is limited information on the biology of this group, the extremophilic properties of the haloalkaliphiles with respect to salinity and pH suggest that these microbes and their enzymes represent an underutilized resource for basic research and industrial applications.

A wide range of extracellular enzymes, such as alkaline proteases, cellulases, and amylases, have been isolated from the haloalkaliphilic Bacteria (mostly Bacillus spp.) and used for industrial production (2). The members of these classes of enzymes are also encoded by the haloalkaliphilic Archaea, as exemplified by the haloarchaeal amylase of Natronococcus occultus, which exhibits extracellular activity (2). N. magadii has been phyllogenetically classified within the order Halobacterales, which includes the intensively studied Halobacterium salinarum and Haloferax volcanii (3). For a variety of neutrophilic members of the order Halobacteria, data about the methods for transformation and genetic manipulation are available (4). To our knowledge, there are no reports about the genetic manipulation of some haloalkaliphilic members of the Archaea. Although a plasmid, pNB101, of the Natronobacterium sp. strain AS7091 (5, 6) was isolated, attempts to transform haloalkaliphilic members of the Archaea with this vector failed (5). Nevertheless, a shuttle vector created by fusion of pNB101 with the Escherichia coli plasmid pKS11+ and additional incorporation of a mevinolin resistance cassette was successfully used to transform the halophiles H. salinarum and H. volcanii (6). This indicated that pNB101-based plasmids are, in principle, useful vectors but that standard transformation methods for the haloalkaliphilic Archaea (7, 8, 9, 10) are not sufficient to efficiently transform the haloalkaliphilic Archaea.

φCh1 was the first virus isolated from a member of the haloalkaliphilic branch of the Archaea (11). φCh1 is a head-tail virus belonging to the family Myoviridae. It infects the species N. magadii, thereby eventually causing cell lysis. Two strains of N. magadii are available: the lysogenic strain L11 and strain L13, which has been cured of the virus (11). φCh1 shows remarkable sequence similarity to the halophilic archaeal virus φH, which infects H. salinarum (12), and in general the same organization of functional modules, at least for those parts of the φH genome that have been sequenced (13, 14). This is evident for the module-containing genes that encode structural proteins. Moreover, the central part of the φCh1 genome, nucleotides (nt) 30,000 to 42,000 (accession no. AF440695), shares sequence identity ranging from 50% to 97% with the so-called L fragment of φH. A comparison of the central parts of the 2 viruses revealed that most of the φCh1 open reading frames (ORFs) can also be found on the invertible L segment of φH and vice versa. Since the L fragment of φH is able to replicate as an autonomous plasmid (pφHL) in H. salinarum cells
(14), it is likely that the respective region of ϕCh1 harbors the viral origin of replication.

Genetic analysis of ϕCh1 has been hampered so far by the inability to transform its host strain, namely, N. magadii. Here we present a method for the transformation of N. magadii. The method is based on a technique used for the transformation of halophilic members of the Archaea, but it includes additional steps for the efficient generation of spheroplasts. We demonstrate the functionality of the method by employing a series of N. magadii E. coli shuttle vectors comprising genetic elements of the virus ϕCh1. Furthermore, we demonstrate the applicability of these vectors for the transformation of not only N. magadii but also a series of other, previously untransformable haloalkaliophilic members of the Archaea. In addition, we also define the origin of replication of ϕCh1.

MATERIALS AND METHODS

Strains, plasmids, and primers. All strains, plasmids, and primers used are listed in Table S1 in the supplemental material.

Media and growth conditions. N. magadii and Natronomonas gregoryi were incubated in a nutrient-rich medium as described previously (11). Cells were incubated at 37°C in sealed plastic bags for 1 to 2 weeks. Natrtilaba asiatica and H. salinarum R1 were grown in 5 g yeast extract, 5 g Casamino Acids, 1 g Na-glutamate, 2 g KCl, 3 g Na3-citrate, 20 g MgSO4, 7 H2O, 200 g NaCl, 36 mg FeCl2·4 H2O, and 0.36 mg MnCl2·4 H2O per liter at pH 7. E. coli strains were incubated in Luria-Bertani medium at 37°C as previously described (15). Ampicillin and tetracycline were added to the LB medium to a final concentration of 100 μg/ml or 10 μg/ml, as required. Halorubrum saccharovorum, Halorubrum coriense, and Halorubrum lacusprofundi were incubated in 18% MGM medium, as described before (16). Transformation of H. volcanii was performed as described previously (7, 9). Novobiocin or mevinolin was added to a final concentration of 3 μg/ml or 4 μg/ml when required.

Transfection and transformation of N. magadii L13. Cells were incubated in nutrient-rich medium containing 75 μg/ml bacitracin at 37°C to an optical density of 0.6 (600 nm). The culture was collected by centrifugation (6,000 rpm); the pellet was resuspended in high-salt-buffered solution (http://www.halolaurea.com/resources/halohandbook/) with 20 μg/ml proteinase K and incubated for 48 h at 42°C with agitation. Cells after treatment with bacitracin and/or proteinase K were used to prepare spheroplasts as described previously (7, 9). A 1.5-ml volume of the sample was collected by centrifugation and resuspended in 150 μl high-salt solution. After the addition of EDTA (50 mM) and incubation for 10 min at room temperature, DNA was added and incubated for 5 min at room temperature. A 150-μl volume of 60% polyethylene glycol (PEG) 600 in high-salt solution was added and incubated for 30 min at room temperature. The cells were washed with nutrient-rich medium. After regeneration of the cells at 37°C for 16 h, the transfection samples were mixed with N. magadii L13 cells and poured on plates with top agar. Transformation samples were plated on agar plates containing novobiocin (3 μg/ml) or mevinolin (7.5 μg/ml) and incubated for 10 to 15 days at 37°C before determination of plaque forming or numbers of CFU, respectively.

DNA isolation from halophilic members of the Archaea and retransformation. Plasmid DNA (50 ng) prepared from N. magadii L13 by a modified alkaline lysis procedure (http://www.halolaurea.com/resources/halohandbook/) was transformed into E. coli XL1-Blue cells. Plasmid integrity was verified by restriction analysis. Plasmids containing the cloned ϕCh1 origin of replication were verified by PCR analysis using primers TR-1/TR-4. The integrity of plasmids carrying the origin of replication derived from plasmid pNB101 (5) was assessed using primers NB-3/MeVR-4. Chromosomal DNA of N. magadii L13 was isolated as described before (11).

Plasmid copy number determination. Copy numbers of the different shuttle vector constructs were determined as already described (17). Numbers of CFU were determined by plating.

Isolation and purification of virus particles. Virus particles and virus nucleic acids were isolated as previously described (11).

Plasmid construction. A description of the constructed plasmids is given in Text S3 of the supplemental material.

Hybridization techniques. Hybridization techniques were performed as described previously (18) by using the NEBlot Phototope-STAR labeling and detection kit (New England Biolabs).

Protein manipulations. Western blot analyses were performed as described previously (19), and immune detection was performed using polyclonal antibodies against protein gp36. As a secondary antibody, antirabbit IgG linked to horseradish peroxidase was used. Detection of the antigen-antibody complex was performed using the SuperSignal West Pico chemiluminescent substrate (Pierce).

β-Galactosidase assays. β-Galactosidase (βGal) activities in N. magadii L13 were determined as described previously (20). The protein concentrations were quantified using the Bradford method (21).

Periodic acid-Schiff’s reagent staining. Periodic acid-Schiff’s reagent (PAS) staining was performed as described previously (22). SDS-PAGE gels were incubated in 7.5% acetic acid (30 min, room temperature [RT]) and transferred to 0.2% periodate (1 h, 4°C) and then to Schiff reagent (1 h, 4°C). The stained gels were returned to 7.5% acetic acid (30 min, RT) and extensively washed in water.

RESULTS AND DISCUSSION

Spheroplast formation and transfection of N. magadii L13 with ϕCh1 DNA. Because archaeal genetic markers were unavailable at the time, the first efficient transformations of a halophilic archeon, namely, Halobacterium salinarum, were performed using DNA isolated from the virus ϕH7 (7). The polyethylene glycol-mediated transformation method described was quickly adapted for the transformation of Haloferax volcanii (8) and various other members of the Archaea, including Methanococcus maripaludis and Pyrococcus abyssi (23, 24). However, this method is effective only in species for which spheroplasts can readily be generated (i.e., H. volcanii and H. salinarum), usually by removing the paracrystalline glycoprotein surface layer (S layer) by EDTA treatment of the cells (4). Using this method, transformation rates as high as 1 × 106 CFU per microgram of plasmid DNA can be obtained (25).

To evaluate the efficacy of N. magadii transformation, we tried to introduce purified ϕCh1 DNA into N. magadii cells. Since ϕCh1 virus particles are able to infect the cured N. magadii L13 strain (11), plaque formation on an agar lawn was used as an indication of successful transformation. In a first attempt, the polyethylene glycol-mediated transformation method was used, and the morphology of the cells was monitored by phase-contrast microscopy (Fig. 1). Using this transformation method, neither spheroplast nor plaque formation because of successful transfection was observed (Fig. 2). As shown earlier, the antibiotic bacitracin interferes with the glycosylation of the H. salinarum S layer and thereby inhibits the growth of H. salinarum, but it also causes a morphological change of the cells from rod-shaped to spherical (26, 27). In H. salinarum, bacitracin is thought to interfere with the processing of the dolichol pyrophosphate carrier used for glycosylation of the S-layer glycoprotein (28). Growth of N. magadii L13 with bacitracin (75 μg/ml) did not produce spheroplasts but rather cells with pleiotropic morphology (Fig. 1b). Using cells of N. magadii L13 grown in 75 μg/ml bacitracin, successful transfection was not observed (Fig. 2). The less-pronounced effect of bac-
itracin on *N. magadii* L13 cell morphology can be explained by the lower stability of bacitracin in alkaline solutions (29). Since *N. magadii* growth requires a high pH of 9.5, bacitracin may quickly become inactivated in the *N. magadii* growth medium.

Methods to transform halophilic members of the *Archaea* are based on the removal of the outermost S layer, which eventually leads to spheroplast formation and uptake of foreign DNA. As shown by Mescher and Strominger, incubation of *H. salinarum* cells with insoluble protease changed the morphology of the cells to spheroplasts and led to the removal of the S-layer protein (27). Since the addition of bacitracin or EDTA did not lead to the formation of spheroplasts of *N. magadii* and therefore did not lead to transfection with φCh1 DNA, we tried to remove the *N. magadii* S layer by proteinase K treatment. Proteinase K retains activity at high salt concentrations (11). Treatment of *N. magadii* cells with proteinase K led to the formation of spheroplasts (Fig. 1c), and these cells could be transfected with φCh1 DNA (Fig. 2). Here, a transfection rate of 10^4 PFU/μg DNA was obtained. Compared to the transfection rates of *H. halobium* with φH DNA (7), this method is significantly less effective. To improve the efficacy of transfection, a combined method was used: cells were grown in nutrient-rich medium in the presence of bacitracin (75 μg/ml) to an optical density of 0.6 (measured at 600 nm) before treatment with proteinase K (20 μg/ml) for an additional 48 h at 42°C. Treatment of *N. magadii* L13 with bacitracin/proteinase K caused a morphological change from the normal rod shape to spherical (Fig. 1a and c). The cells remained viable, and their rod-shaped morphology was obtained by incubation in nutrient-rich medium lacking proteinase K (Fig. 1d). These observations indicated that a cell wall protein, probably the putative S-layer protein, had been removed from the cell surface of *N. magadii*. SDS-PAGE gels containing extracts of *N. magadii* cells before and after incubation with proteinase K were stained for glycoproteins with periodic acid-Schiff’s reagent (PAS staining) (22). A specific band of approximately 110 kDa, degraded by the protease after 48 h of incubation, was detected (data not shown). Other detectable proteins did not change (data not shown). Incubation of the cells with proteinase K led to the formation of spheroplasts concomitant with the disappearance of the major glycosylated protein. These results suggest that the glycoprotein removed by combined treatment with bacitracin/proteinase K is a major envelope structural component responsible for the maintenance of cell shape in *N. magadii* L13.

When bacitracin/proteinase K-treated cells were incubated with φCh1 DNA, we were able to transfecit with φCh1 DNA at a rate of 10^5 PFU/μg DNA (Fig. 2). Although the transfection rate was lower than the transfection/transformation rates of other halophilic strains, this method enables genetic manipulation of *N. magadii* and functional analysis of φCh1 elements in their natural host. We therefore used this method to develop an *E. coli/N. magadii* shuttle vector system based on elements of the φCh1 genome.

**Construction and efficiency of a shuttle vector for *N. magadii* L13.** Sequence analysis of the minimal replication origin of the plasmids of halophilic *Archaea* showed that a unique gene, repH, and an AT-rich region located upstream of the gene were required (accession number AF440695) (30). Elimination of either the AT sequence or the repH gene abolished the autonomous replication ability of the plasmids (30). Sequence similarity analysis revealed an open reading frame (ORF54) within the φCh1 genome with similarities to the protein-encoding sequences of halo philic plasmids (12). ORF54 encodes a large 581-amino-acid (aa) protein with a calculated size of 65.2 kDa and an isoelectric point of 4.8. The predicted protein shares similarities (highest similarity using a BLASTP alignment, 5 × 10^-46) with archaeal proteins encoded by Haloarcula marismortui plasmid pNRC100, *H. salinarum* plasmid pH1, *H. salinarum* φH1, *Halobacterium* sp. (strain NRC-1) plasmid pNRC100, and *Haloferax volcanii* plasmid pHV2. Some of the proteins are essential components of the minimal repilons of the plasmids in halophilic members of the *Archaea* (30, 31). Analysis of the deduced amino acid sequence of ORF54 using the software program COILS revealed a putative coiled-coil domain located in the central part of the protein (aa 254 to 282; data not shown). However, ORF54 is smaller than its homologs, and sequence similarities were found only in the C-ter-
minal parts of these proteins. ORF53, located upstream of ORF54, had a lower but significant similarity to the RepH protein encoded by *H. marismortui*, namely, pNRCl00 (Fig. 3a). This similarity is restricted to the N-terminal part of RepH. The same arrangement was found in the genome of the closest relative of *ChlCh1*, i.e., *H. salinarum* virus ψH. Both genes are part of the autonomously replicating plasmid ψHL-1 (13, 14). In analogy to regions found upstream of genes involved in the replication of plasmids of halophilic *Archaea*, an AT-rich region was also detected upstream of ORF53, and a second one was identified downstream of ORF54. No such area was seen in the 5′ region of ORF54 (Fig. 3a). Taken together, it is reasonable that the above-described sequence comprises the minimal replicon of *ChlCh1*. Therefore, this region was investigated for its capability to promote autonomous replication in *N. magadii* and ultimately for its usability to develop a shuttle vector system for *E. coli/N. magadii*.

Because of the lack of a selectable marker for haloalkaliphilic members of the *Archaea*, the cloned and mutated *gyrB* gene of halophilic *Haloflexus alicantei*, which confers resistance toward novobiocin (17), was used as a selection marker. The gene was isolated from the plasmid pMD511 (25) and cloned into the vector pKS10, giving rise to the plasmid pNov-1. In a second step, different parts of the region containing ORF53 and ORF54 (Fig. 3a) were cloned into pNov-1, and the resulting plasmids were tested for their ability to autonomously replicate upon transformation of *N. magadii* L13. As shown in Fig. 3b, the plasmids pRo-1 and pRo-2, lacking the promoter region upstream of ORF49 as well as the AT-rich region of the ORF53 upstream region, were not able to replicate in *N. magadii* L13. The AT-rich region downstream of ORF54 (pRo-2) alone was not able to maintain the plasmid in *N. magadii* L13. In contrast, replication in *N. magadii* L13 was achieved with the plasmid pRo-4, containing the entire region described above. Only the plasmids pRo-3, pRo-5, pRo-6, pRo-7, and pRo-10 were able to transform *N. magadii* with satisfactory transformation rates of 10^5 CFU/μg DNA. The transformation rate of the plasmid pRo-9 was only slightly reduced, to 10^3 CFU/μg DNA. The plasmids pRo-3 and pRo-6 contain open reading frames that encode possible transcriptional repressors: ORF49 seems to influence the infection cycle of *ChlCh1*, as demonstrated by the earlier onset of lysis in a *ChlCh1* mutant harboring a mutated ORF49 (19), and ORF55 displays similarities to known transcriptional regulators (data not shown). To avoid interference with ORF49 or ORF55 in upcoming genetic studies, all subsequent experiments were performed with pRo-5. It lacks the start codons of ORF49 and ORF55. A schematic representation of pRo-5 is shown in Fig. 3c. A detailed characterization of strain *N. magadii* (pRo-5) can be found in Text S1 of the supplemental material.

Previously, higher transformation efficiency for unmethylated DNA than for methylated DNA was reported for *H. volcanii* (18). This is due to a restriction-modification system in *H. volcanii* that seems to degrade DNA methylated in a Dam- and/or Dcm-like fashion. To investigate such a possible effect for *N. magadii* L13, unmethylated and methylated pRo-5 DNA was used for transformations. Plasmid DNA was isolated from *E. coli* strain JM110 (dam dcm), and *N. magadii* L13 was transformed with this DNA. No increase in the transformation rates was detected when unmethylated plasmid DNA was used (Fig. 3b). This suggests that *N. magadii* lacks or has a restriction-modification system different from that of *H. volcanii* and that transformation is independent of the methylation status of the plasmid DNA.

Palindromic sequences were found adjacent to the AT-rich sequences of the upstream region of ORF53 and the downstream region of ORF54 (Fig. 3a). To analyze the importance of these sequences, one (pRo-7 and pRo-9) or both (pRo-10) of them were deleted. Transformation assays revealed that these palindromic sequences are nonessential for plasmid replication in *N. magadii* L13 (Fig. 3b). The importance of ORF53 and ORF54 was proven by the introduction of frame shifts into both genes (pRo-8 and pRo-11). This created a stop codon in both cases, and both plasmids were unable to replicate in *N. magadii* L13. Therefore, both genes, ORF53 and ORF54, seem to be essential for replication in *N. magadii*.

Suitability of the shuttle vector for functional analysis of *ChlCh1* genes or establishment of reporter gene assays. In a former study, we investigated the putative function of ORF48 (32). By similarity searches and by analyzing the mutant strain *ChlCh1*-1, -2 genes, ORF48 (rep) and ORF49, were identified as encoding possible regulators of the *ChlCh1* life cycle. Both genes are arranged head-to-head. Promoter consensus sequences (AT-rich sequences typical for halophilic members of the *Archaea* [33]) are present in the intergenic region of the 2 genes. Investigation of the ORF48-ORF49 intergenic region in *H. volcanii* revealed promoter activity. Data indicated that ORF48 could act as a repressor via binding to 2 direct repeats located in its coding region (32). Expression of the repressor gene rep dramatically decreased the expression level of the reporter gene bgaH when transcribed from the ORF49 promoter. These results suggested that Rep, the gene product of ORF48, shuts down expression of ORF49 and thus probably acts as a transcriptional repressor. This is in accordance with protein structure predictions suggesting that Rep belongs to the family of winged helix repressor proteins (32). To verify the function of ORF48 as a repressor, the rep gene and its upstream region were cloned into pRo-5. *N. magadii* L13 was transformed with the plasmid and subsequently infected with *ChlCh1*. The relative plating efficiency of *ChlCh1* on the strain *N. magadii* L13 (pRo-5/2) carrying the repressor gene ORF48 was significantly reduced, by 3 or-

FIG 3 Presentation of the putative origin of replication of *ChlCh1*. (a) Part of the *ChlCh1* sequence is shown (nt 33701 to 38000) (12). Large arrows indicate ORF53 and ORF54, as well as ORF49 and ORF55. Sequence similarities of ORF53 and ORF54 to the pNRCl00 replication protein H-like open reading frame of *H. marismortui* are indicated (3 × 10^-2 and 3 × 10^-1, respectively), as well as the open reading frame. The promoter within the 5′ region of ORF49 is indicated with an arrow and marked with “P.” Arrows mark the palindrome sequences. Lanes indicate the parts of the sequence cloned, and the names of the different constructs are given on the right. The asterisk and the vertical lane of the clones pRo-8 and pRo-11 indicate the sides where frame shifts were introduced into ORF53 and ORF54, respectively. The interruptions in the lanes indicate the introduced deletions. (b) Transformation efficiency of *N. magadii* L13 with different constructs. The efficiency is given in CFU per microgram of plasmid (CFU/μg) on the left side, and the clones used in this study are indicated at the bottom. pRo-5, the plasmid DNA of pRo-5 was isolated from the dam- and dcm-lacking *E. coli* strain JM110 and transformed into *N. magadii* L13. Transformation assays were performed in triplicate. Error bars are indicated, ± 1 SD. (c) Physical map of the shuttle vector pRo-5. Black area shows ColEl origin of replication; dark gray arrow indicates β-lactamase (bla); gray arrow indicates novobiocin resistance cassette (gyrB); light gray arrows indicate putative origin of *ChlCh1*. Positions of the restriction sites are (clockwise from the top) NotI (3385), EcoRV (3841), HindIII (7065), and KpnI (7105). The positions of the sites are given in parentheses.
The bgaH gene of *H. lucenense* is a suitable reporter gene for the halophilic *Archaea* (36, 37). It has also been used by us to investigate the promoter activity of the integenic region between rep and ORF49 of φCh1, as well as the influence of rep on transcription in *H. volcanii* (31). To establish the bgaH gene as a reporter for gene expression in *N. magadii*, the gene, including its own promoter region, was cloned into pRo-5, resulting in pRo-5/BgaH. BgaH activity in *N. magadii* L13 (pRo-5/BgaH) was low (13 mU/μg protein) compared to the activity in *H. volcanii* (500 mU/μg protein) when the gene was expressed from pMLH32 (20). One possible explanation for this result could be that the promoter of bgaH was not as efficiently recognized in *N. magadii* as in *H. volcanii*. To circumvent a possible dysfunctionality of the bgaH promoter in *N. magadii*, we tried to express the bgaH gene from the φCh1 promoter of the ORF48-ORF49 integenic region (31). The integenic region of φCh1 and the bgaH gene were isolated from plasmid pM1 (31) and introduced into the shuttle vector pRo-5, resulting in pRo-5/BgaH. BgaH activities in *N. magadii* L13 transformed with pRo-5/1/BgaH were in the range of 100 mU/μg protein (data not shown), which is comparable to the promoter activity of this region when used to express the bgaH gene in *H. volcanii* (31). Thus, combining the bgaH gene with the ORF48-ORF49 integenic region of φCh1 allowed us to establish a functional reporter system for future genetic analyses of *N. magadii* or its virus φCh1.

In a third approach to evaluate the efficacy of the *N. magadii* transformation system, we tried to express the φCh1 tail fiber-encoding gene ORF3452 (38, 39) in *N. magadii*. The gene, as well as its upstream region, was cloned into pRo-5, creating pRo-5/3452. After transformation of *N. magadii* L13 with the plasmid, crude extracts were prepared and separated by SDS-PAGE. The presence of gp3452, the gene product of ORF ORF3452, was investigated by Western blotting using anti-ORF36 antibodies. The cross-reactivity of the anti-ORF36 antisera with ORF34 and ORF36 results from the occurrence of the peptide repeats MDAV within both proteins (38). As shown in Fig. 4, a gp3452 signal could be detected only with crude extracts of strain *N. magadii* L13 (pRo-5/3452) (Fig. 4, lane 3), not with crude extracts of the control strain *N. magadii* L13 (pRo-5) (Fig. 4, lane 2). The results demonstrate the successful expression of ORF3452 in *N. magadii* after transformation with the plasmid pRo-5/3452.

**TABLE 1** Transformation of halo(alkali)philic members of the *Archaea* with pRo-5

| Strain                  | Transformation with pRo-5 | Plasmid stability
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<tr>
<td><em>N. magadii</em> L13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>H. saccharovorum</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>H. coriense</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>H. lacusprofundi</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>H. volcanii</em> WF11</td>
<td>+</td>
<td>+ /-</td>
</tr>
<tr>
<td><em>H. volcanii</em> WF11(pMDS24)</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><em>H. salinarum</em> R1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. asiatica</em></td>
<td>+</td>
<td>+</td>
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<td><em>N. gregoryi</em></td>
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* Plasmid stability was determined by isolation of plasmid DNA from transformed cells, retransformation of the DNA into *E. coli* cells, reisolation, and restriction of the plasmid DNA with SacI (performed in triplicate). ND, not determined; + /- , 50% of the plasmid samples were stable.
efit and allows for more complex cotransformation experiments (for details, see Text S2 in the supplemental material).

Conclusions. We have developed a method for the transformation of the haloalkaliphilic archaean *N. magadii*. Stable plasmid replication was achieved with pNB102, an engineered version of a plasmid originally isolated from the haloalkaliphilic *Natronobacterium* sp. strain AS7091, but also with a series of plasmids constructed in this study that are based on the genetic elements of *N. magadii* virus ϕCh1. Thereby, we were also able to experimentally determine the origin of replication of ϕCh1. The plasmids were stably maintained within the cells, did not integrate into the host chromosome, and did not interfere with the growth rate of the cells. As demonstrated, the plasmids can be used for expression of homologous and heterologous genes in *N. magadii*. The ϕCh1-derived plasmid pRo-5 replicated not only in *N. magadii* but also in a series of other halo(alkali)philic Archaea, making it a universal tool for simple (comparative) genetic studies in a broad range of the Archaea. The plasmid was compatible with other plasmids containing either a *H. volcanii* pVH2- or *Natronobacterium* sp. strain AS7091 pNB101-derived origin of replication, thus also allowing cotransformation experiments and simple expression of sets of genes. Taken together, the development of the described transformation method and construction of plasmids for replication in *N. magadii* make this organism accessible to genetic manipulation and open the path for a detailed characterization of gene function of *N. magadii* virus ϕCh1.

REFERENCES


