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First report of linear megaplasmids in the genus *Micrococcus*

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ABSTRACT

High-altitude wetlands (above 4200 m) in the northwest of Argentina are considered pristine and extreme environments. *Micrococcus* sp. A1, H5, and V7, isolated from such environments, were shown to contain linear megaplasmids, designated pLMA1, pLMH5, and pLMV7, respectively. As known from linear plasmids of other actinomycetes, all three plasmids were resistant to λ exonuclease treatment, which is consistent with having terminal proteins covalently attached to their 5′ DNA ends. Electrophoretic mobility, Southern analysis, and restriction endonuclease patterns revealed pLMA1 and pLMH5 being indistinguishable plasmids, even though they were found in different strains isolated from two distant wetlands – Laguna Azul and Laguna Huaca Huasi. Analysis of 16S rDNA sequences of *Micrococcus* sp. A1, H5, and V7 suggested a close relationship to *Micrococcus luteus*. Typing of isolates was performed using fingerprint patterns generated by BOX-PCR. Plasmid-deficient strains, generated from *Micrococcus* sp. A1, showed a significantly decreased resistance level for erythromycin.

1. Introduction

Micrococci are Gram-positive, strictly aerobic bacteria with a high G+C content, spherical in shape and usually found in tetrad forms. They are nonmotile and nonspore-forming. The genus *Micrococcus* consists of several species that differ in various criteria from the second group of cocci, frequently isolated from skin, the staphylococci. *Micrococcus* is routinely isolated from skin, but can also be found in soil, sediments and marine and fresh water (Kocur et al., 1992). There are even reports on the isolation of *Micrococcus* species from ancient materials, such as amber (Greenblatt et al., 1999, 2004).

Descriptions of plasmid profiles of *Micrococcus* exist, but with rather limited characterization (Mathis and Kloos, 1969). The largest extrachromosomal element reported and characterized so far is a 50-kb-circular plasmid, rich in putative mobile genetic elements (Zhong et al., 2002). However, up to the present no linear extrachromosomal DNA elements – so-called linear plasmids – have been reported for the genus *Micrococcus*.

Linear plasmids in Gram-positive bacteria were originally described in *Streptomyces rochei* (Hayakawa et al., 1979). Meanwhile, they have been reported for a number of *Streptomyces* spp., several rhodococci and mycobacteria, *Planobispora rosea*, the plant pathogen *Clavibacter michiganensis*, *Arthrobacter nitroguajacolicus* Rü61a, and a *Terrabacter* sp. (Meinhardt et al., 1997; Overhage et al., 2005; Rose and Fetzner, 2006, and references therein; for a compilation of microbial linear plasmids, see Meinhardt and Klassen, 2007). Linear replicons of the above actinobacteria belong to a class of genetic elements called invertrons, which are characterized by terminal proteins covalently attached to each 5′ end and terminal inverted repeats (TIRs) (Sakaguchi, 1990).

Another class of linear replicons possess covalently closed single-stranded hairpin loops at their ends and short TIRs (Barbour and Garon, 1987; Hinnebusch and Barbour, 1991). Such linear structures are common in *Borrelia*

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(Kobryn, 2007), but are also found in prophages of Gram-negative bacteria, a few eukaryotic viruses, and some mitochondria (Hinnebusch and Tilly, 1993; Hertwig, 2007).

Linear plasmids are known to confer several valuable qualities, such as heavy metal resistance (Ravel et al., 1998), catabolic traits (Overhage et al., 2005; König et al., 2004), hydrogen autotrophy (Kalkus et al., 1990) or the production of secondary metabolites (Suwa et al., 2000). Up to the present, only linear plasmids of streptomycetes were reported to confer resistance to antibiotics, such as methylenomycin (Chater and Bruton, 1985).

We isolated *Micrococcus* strains from high-altitude wetlands (4200–4600 m) in the northwest of Argentina, which are considered extreme and pristine environments. Such strains unexpectedly displayed multiple resistance to antibiotics, UV radiation and heavy metals (Dib et al., 2008), however, it remained uncertain whether those qualities are conferred by extrachromosomal DNA elements.

In the present study, we analyzed these above *Micrococcus* strains by pulsed-field gel electrophoresis (PFGE) and were able to isolate and characterize three linear megaplasmids, designated pLMA1, pLMH5, and pLMV7, respectively. By making use of a cured plasmid-deficient strain of *Micrococcus* sp. A1 we obtained evidence suggesting that resistance to erythromycin may be encoded by the linear plasmid pLMA1. This is the first report on the presence of linear plasmids in the genus *Micrococcus*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Strains carrying linear plasmids were isolated from Argentinean high-altitude wetlands. Sequencing of the 16S rRNA gene, performed in this work, suggests their assignment to the species *Micrococcus luteus*. *Micrococcus* sp. A1 was isolated from Laguna Azul, a high-altitude wetland located in the province Catamarca in the Northwest of Argentina at 4560 m. *Micrococcus* sp. H5 was isolated from Laguna Huaca Huasi (4250 m), a flat with wetlands of glacier origin, which is located at the top of the Calchaquíes summit in the north of the province Tucumán. In a region called Laguna Vilama (4600 m) *Micrococcus* sp. V7 was isolated. For details concerning the extreme environmental conditions of above geographic areas, see Dib et al. (2008) and Ordonez et al. (2009). (Illustrations of Argentinean high-altitude wetlands are available upon request.) All strains were isolated from water samples. Sampling and isolation procedures were performed as previously described by Dib et al., 2008. As a reference strain *M. luteus* 20030^T from the DSMZ (German Collection of Microorganisms and Cell Cultures) was used. Strains were grown in Luria–Bertani (LB) medium (Sambrook and Russell, 2001) on a rotary shaker (Innova®44, New Brunswick Scientific GmbH, Nürtingen, Germany) at 160 rpm or on solid LB agar at 30 °C.

For cloning purposes, *Escherichia coli* DH5α (Bethesda Research Laboratories) was used. Plasmid pUC18 (Vieira and Messing, 1982) was applied for cloning and sequencing of chromosomal fragments and fragments of linear plasmids generated by PCR or restriction digests, respectively.

2.2. DNA techniques

Total DNA of *Micrococcus* strains was isolated according to the method of Rainey et al. (1996). For isolation of plasmid DNA from *E. coli* cells the Jetquick Plasmid Miniprep SpinKit (Genomed GmbH, Löhne, Germany) was used.

Agarose gel electrophoresis, DNA restriction, and DNA cloning were carried out as described in Sambrook et al. (1989). GeneRuler™ 1 kb DNA ladder (Fermentas GmbH, St. Leon-Rot, Germany) was used as size standard. Restriction-enzyme-digested DNA fragments were obtained from agarose gels by the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany).

Genotypic characterization of isolated strains was performed with single colonies. Universal primers 27_f and 1492_r were used to amplify 16S rRNA genes (Martin-Laurent et al., 2001). Resulting sequences were compared with published 16S rRNA gene sequences from the National Center for Biotechnology Information (NCBI) database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the approximate phylogenetic affiliation. Sequence alignment with closely related and representative *Micrococcus* species and construction of a phylogenetic tree was done with MEGA4.1 (http://www.megasoftware.net) using the neighbor-joining method. Gaps at the 5′ and 3′ ends of the alignment were omitted from further analyses.

2.3. Labeling of DNA probes and Southern hybridization

DNA separated on pulsed-field (PF) gels was transferred to Amersham Hybond-N nylon membranes (GE Healthcare Europe GmbH, Freiburg, Germany) by capillary blotting overnight as described by Sambrook et al. (1989) and subsequently fixed to the membrane by cross-linking under UV light. Probes were labeled using the DIG-High Prime DNA Labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany). The sequence of the labeled 321-bp-*Bfu*AI fragment of pLMA1, used as a probe, was deposited under GenBank Accession No. FN395369. Prehybridization, hybridization and chemiluminescent detection followed the DIG Application Manual for Filter Hybridization (Roche Diagnostics GmbH, 2003).

2.4. Preparation of cell plugs, PFGE, and isolation of linear plasmid DNA

Micrococcus cells were grown in LB medium to an OD_{600} of 2–3. After washing, bacterial cell pellets were used for preparation of cell plugs for PFGE following the procedure of Ravel et al. (1998). PFGE was carried out in an electric field apparatus (Rotaphor Type VI; Biometra, Göttingen, Germany) or in a clamped homogenous electric field system (CHEF DR-III; Bio-Rad, Melville, NY) using 1% broad range agarose (Carl Roth GmbH, Karlsruhe, Germany). Detailed running conditions are provided in the appropriate figure legend. Concatemers of λ DNA were used as size standard (New England Biolabs GmbH, Frankfurt/Main, Germany). For precise estimation of plasmid sizes Mid-Range I PFG Marker (New England Biolabs GmbH) was used. Given plasmid sizes are values determined from

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three independent pulsed-field (PF) gels. After electrophoresis, the gels were stained with ethidium bromide.

To isolate plasmid DNA, the plasmid DNA bands were excised from the PF agarose gels and the DNA was obtained by electroelution using dialysis bags (ZelluTrans, V series, MWCO 10,000, Carl Roth GmbH) or the Elutrap System (Whatman Group, Dassel, Germany) followed by ethanol precipitation.

2.5. Exonuclease treatment of linear plasmid DNA

Treatment of isolated plasmid DNA with exonuclease III and λ exonuclease was performed as described by Meinhardt et al. (1986) with modifications introduced by Rose and Fetzner (2006). *Sma*I-linearized cloning vector BlueScribe KS Minus (pKS−) (Stratagene, GenBank Accession No. L08784.1) was used as reference in reaction controls.

2.6. Genomic fingerprinting

The box element BOX1A was amplified using BOXA1R primer 5′-CTACGGCAAGGCGACGCTGACG-3′ and total DNA as template (Versalovic et al., 1994). PCR and electrophoresis conditions were according to Clark et al. (1998). PCR was repeated 3 times to check reproducibility. DNA fingerprint patterns were evaluated by comparison of band sizes of PCR products after separation by agarose gel electrophoresis.

2.7. Plasmid curing

To generate a plasmid-deficient strain, *Micrococcus* sp. A1 was grown in LB medium on a rotary shaker (Innova®44, New Brunswick Scientific GmbH) at 160 rpm at 42 °C. After 48 h, cells were diluted 1:200 in fresh LB medium and cultivation was continued. Following four repeated rounds of cultivation cells were diluted to yield single colonies (a dilution factor of 10^5 was appropriate) and grown on LB agar plates. Fifty single colonies were subsequently transferred to a grid plate and screened by colony hybridization using a cloned 321-bp-*Bfu*AI fragment of pLMA1 as probe and Amersham Hybond-N nylon membranes (GE Healthcare Europe GmbH) according to manufacturer's instructions. Three colonies lacking hybridization signals were checked for the loss of plasmid pLMA1 by PFGE and Southern hybridization, applying the above probe.

2.8. Determination of minimal inhibitory concentration (MIC)

To check the antibiotic resistance phenotype, MIC of erythromycin was determined on Mueller–Hinton plates using Etest® stripes (bioMérieux Deutschland GmbH, Nürtingen, Germany) according to the manufacturer's instructions.

3. Results and discussion

3.1. Characterization of Micrococcus strains isolated from high-altitude wetlands

Micrococcus sp. A1 and V7 were previously isolated from water samples from Laguna Azul (4560 m) and Laguna Vilama (4600 m), respectively. As described previously (Dib et al., 2008), strains A1 and V7 were able to grow under high arsenic concentrations and resist high levels of UV radiation. They showed multiple resistance to antibiotics, particularly to macrolides. *Micrococcus* sp. H5 was isolated from Laguna Huaca Huasi, another high-altitude wetland at 4250 m, with similar conditions. As for A1 and V7, strain H5 displayed multiple antibiotic resistances (data not shown). As characteristic for members of the genus *Micrococcus*, such strains are Gram-positive cocci arranged in tetrads, nonmotile and do not form spores. All of them produce a yellow insoluble pigment.

Analysis of the 16S rRNA gene sequences suggested that all three strains belong to the genus *Micrococcus*, as highest similarity was found to several *M. luteus* strains. Fig. 1S (see Supplementary material) shows the phylogenetic positions of the isolates in comparison to related *Micrococcus* strains and *Citricoccus muralis* 4-0^T as the outgroup. Thus, *Micrococcus* sp. A1, H5, and V7 are apparently representatives of the species *M. luteus* or at least are very closely related. The exact species determination requires further physiological tests.

3.2. Micrococcus strains harbor megaplasmids

Nucleic acids isolated from *M. luteus* A1, H5, and V7 were analyzed by PFGE. PFGE of lysed and proteinase-K-treated cells revealed a discrete DNA band of approximately 100 kb (Fig. 1A), which was assumed to correspond to a megaplasmid. The plasmids from *M. luteus* A1, H5, and V7 were designated pLMA1, pLMH5, and pLMV7, respectively. By comparing their sizes to appropriate linear size standards (Fig. 1A), lengths of the plasmids pLMA1, pLMH5, and pLMV7 were estimated to be 110, 110, and 90 kb, respectively. Since different pulse times revealed constant migration distances of plasmid bands in comparison to the linear size standards (Fig. 1A), such megaplasmids were considered to be linear rather than circular.

As pLMA1 and pLMH5 migrated identically in PFGE and shared the same size, we checked whether pLMA1 and pLMH5 are closely related plasmids in two different *Micrococcus* strains. We used a randomly cloned 321-bp-*Bfu*AI fragment of pLMA1 as the probe and hybridized it with total DNA of *M. luteus* A1, H5, and V7, separated by PFGE; pLMA1 and pLMH5 reacted similarly, whereas no signal was to be observed for pLMV7 (Fig. 1B).

Furthermore, plasmid DNA, isolated by electroelution from a preparative PF gel, was cleaved with different restriction endonucleases and subsequently separated by agarose gel electrophoresis. The uniform restriction patterns for pLMA1 and pLMH5 – restriction patterns observed for pLMV7 clearly differed (Fig. 1C) – along with results from the Southern analysis revealed that pLMA1 and pLMH5 are very similar elements.

From an ecological point of view, the presence of indistinguishable plasmids in *Micrococcus* strains isolated from two different wetlands, separated by about 300 km, including mountain regions in between, may be taken as an indication that the respective genetic information is beneficial for survival in these pristine and extreme environmental conditions. Whether horizontal transfer of the element is

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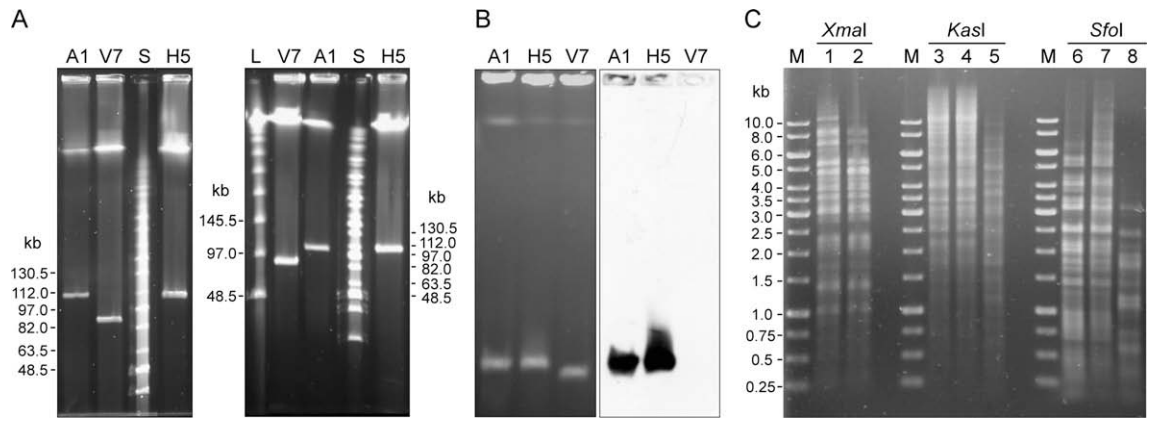

Fig. 1. Identification, Southern analysis, and restriction analysis of large plasmids in three *Micrococcus* strains. (A) PFGE of total DNA of the three *Micrococcus* strains under different running conditions. The plug-embedded and lysed cells were electrophoresed in a CHEF DR-III apparatus, at 6 V cm^{-1}, in 0.5× TBE buffer, at 15 °C, for 24 h. Pulse times were 10–30 s (left) and 1–25 s (right), respectively. Each figure is compiled from lanes of the same gel. A1, *M. luteus* A1; H5, *M. luteus* H5; V7, *M. luteus* V7; L, λ DNA concatemers; S, MidRange I PFG Marker. (B) PFGE separation of total DNA of the three *Micrococcus* strains (left) and Southern hybridization (right) using a 321-bp-*Bfu*AI fragment, obtained from plasmid pLMA1, as the probe. The plug-embedded and lysed cells of the three *Micrococcus* strains were electrophoresed in a Rotaphor Type VI apparatus, at 6 V cm^{-1} (decreasing logarithmically), in 0.25× TBE buffer, at 15 °C, for 24 h, at a pulse time of 60 ± 10 s (changing logarithmically), with a rotor angle of 120 ± 110° (decreasing linearly). Abbreviations same as in (A). (C) Restriction patterns of pLMA1, pLMH5, and pLMV7. Plasmids pLMA1, pLMH5, and pLMV7, isolated by electroelution from a preparative PF gel, were digested using restriction endonucleases *Xma*I, *Kas*I, and *Sfo*I, respectively, and separated on a 0.7% agarose gel. Lanes 1, 3, and 6, pLMA1; 2, 4, and 7, pLMH5; 5 and 8, pLMV7; M, GeneRuler™ 1 kb DNA ladder.

possible, as for pAL1 in different *Arthrobacter* species (Overhage et al., 2005), needs to be elucidated.

3.3. 5′ ends of pLMA1, pLMH5, and pLMV7 are protected from degradation by λ exonuclease

Linear replicons of streptomycetes, other actinomycetes and also those of eukaryotes were shown to possess terminal proteins covalently bound to the 5′ ends (Polo et al., 1998; Ravel et al., 1998; Yang et al., 2002; Stecker et al., 2003; Overhage et al., 2005; Huang et al., 2007; Klassen and Meinhardt, 2007), making such genetic elements insensitive for 5′–3′ exonucleases, but amenable to degradation by 3′–5′ exonucleases.

To verify our assumption that pLMA1, pLMH5, and pLMV7 are linear plasmids, which was based on results of PFGE, respective bands were isolated from a preparative PF gel and tested for exonuclease sensitivity. *Sma*I-linearized pKS− served as the control to demonstrate functionality of exonuclease III and λ exonuclease (Fig. 2, lanes 2 and 3). As shown in Fig. 2 (lanes 6, 9, and 12), all three plasmids were degraded by *E. coli* exonuclease III, which hydrolyses DNA in 3′–5′ direction. However, no degradation was to be observed for λ exonuclease, which acts in 5′–3′ direction (Fig. 2, lanes 5, 8, and 11), indicating that the 5′ ends of pLMA1, pLMH5, and pLMV7 are protected.

Plasmids were protected from degradation by λ exonuclease although DNA was treated with proteinase K. As the covalent linkage of the terminal protein to the 5′ end of the plasmid cannot be cleaved by proteinase K (Yang et al., 2002), plasmid DNA is not accessible to λ exonuclease digestion. Since terminal proteins of *Streptomyces* linear plasmids play an essential role in replication (Bao and Cohen, 2001, 2003; Yang et al., 2002; Huang et al., 2007), the proteins covalently bound to the 5′ ends of pLMA1, pLMH5, and pLMV7 are likely to be instrumental in replication as well.

3.4. Typing of Micrococcus strains by BOX-PCR

Among genomic fingerprinting techniques applied for classifying and typing of various prokaryotic microorganisms, BOX-PCR was effectively used to generate highly specific fingerprint patterns (Freitas et al., 2008). In Fig. 3, analysis of BOX-PCR fingerprinting showed similar PCR product profiles for isolated *Micrococcus* strains. Strains A1 and H5 revealed almost identical profiles, however, in the profile of H5 a band of about 950 bp was missing. The most dissimilar profile corresponded to strain V7, which not only was missing of a few bands compared to A1 and H5, but mainly for the presence of a band of about 1450 bp, clearly absent in the other *Micrococcus* amplification patterns. BOX-PCR was performed three times, each time yielding identical results. *M. luteus* DSM 20030^T was

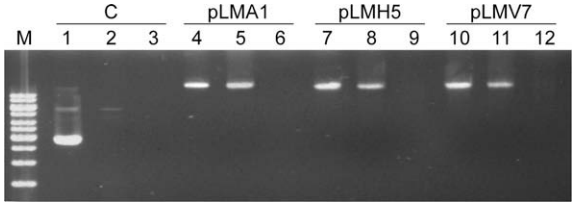

Fig. 2. Exonuclease treatment of pLMA1, pLMH5, and pLMV7. Proteinase-K-treated cells of the three *Micrococcus* strains were applied to a preparative PFGE. Plasmids pLMA1, pLMH5, and pLMV7 were isolated by electroelution, treated with λ exonuclease and exonuclease III, respectively, and analyzed on a 1.5% agarose gel. M, GeneRuler™ 1 kb DNA ladder, fragment sizes in kb from the bottom up: 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0; C, control DNA (*Sma*I-linearized plasmid pKS−); 1, 4, 7, and 10, plasmids untreated; 2, 5, 8, and 11, plasmids treated with λ exonuclease; 3, 6, 9, and 12, plasmids treated with exonuclease III.

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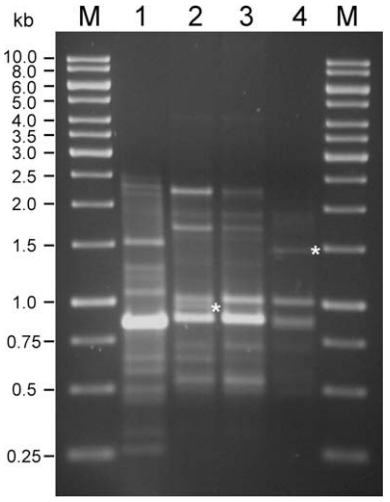

Fig. 3. Typing of *Micrococcus* strains by BOX-PCR. Amplification of the box element BOX1A using BOXA1R as primer and isolated total DNA of *Micrococcus* strains as template. Obtained BOX-PCR products were analyzed on a 0.7% agarose gel. Asterisks label bands that show main differences in PCR product patterns. M, GeneRuler™ 1 kb DNA ladder; 1, *M. luteus* DSM 20030^T (reference strain); 2, *M. luteus* A1; 3, *M. luteus* H5; 4, *M. luteus* V7.

used as the reference strain. Hence, we were able to demonstrate that *M. luteus* A1 isolated from Laguna Azul, *M. luteus* H5 isolated from Laguna Huaca Huasi, and *M. luteus* V7 from Laguna Vilama are different strains even though isolates A1 and H5 harbor indistinguishable linear plasmids. Not only was BOX-PCR affirmed to be a useful tool for typing of *Micrococcus* ssp., but also cross-contamination was excluded.

3.5. Plasmid-deficient strains of *M. luteus* A1 are sensitive to erythromycin

To check whether the antibiotic resistance of *M. luteus* A1 and V7 (Dib et al., 2008) is conferred by linear plasmids, we generated a plasmid-deficient *M. luteus* A1 strain.

Curing of pLMA1 was achieved by heat treatment. *M. luteus* A1 grew satisfactorily at the sublethal temperature of 42 °C, which was therefore chosen as the growth temperature in the curing process. After several rounds of cultivation, cells were diluted and spread on LB agar plates, as described in Material and methods. From 50 single colonies analyzed by colony hybridization using the randomly cloned 321-bp-*Bfu*AI fragment of pLMA1 as probe, three colonies did not show signals and were subjected to further analysis by PFGE and Southern analysis. All three colonies had no plasmid band and also Southern hybridization, using the pLMA1 probe, confirmed the loss of pLMA1 (data not shown). BOX-PCR profiles of the plasmid-deficient strains, designated *M. luteus* A1-M1, A1-M2, and A1-M3, were generated and compared to the profile of *M. luteus* A1 wild-type, which ensured identity and purity of the generated plasmid-deficient strains (data not shown).

Curing of plasmids from *M. luteus* A1 worked efficiently using the temperature method; it was not necessary to use other classical but mutagenic methods, such as incubation with ethidium bromide or exposure to UV radiation (Gunge et al., 1994; Brown et al., 2005). For *Streptomyces* spp. and *Actinoplanes missouriensis* harboring linear plasmids, plasmid curing by growth at elevated temperatures was attempted, however, plasmids were refractory for curing or the procedure resulted only in strains with a reduced copy number as judged from the intensity of plasmid bands in PF gels (Ravel et al., 2000; Rose and Fetzner, 2006).

To check whether the loss of pLMA1 affects resistance to macrolides, erythromycin sensitivity was investigated. The MIC of erythromycin observed for *M. luteus* A1 wild-type is >256 μg/ml, as no inhibition zone was detected for this strain (Fig. 2S, Supplementary material). However, MICs of the plasmid-deficient strains *M. luteus* A1-M1, A1-M2, and A1-M3 were ca. three orders of magnitude less at 0.25–0.5 μg/ml (Fig. 2S, Supplementary material), proving a considerably reduced resistance level. This suggests the intriguing possibility of a link between pLMA1 and erythromycin resistance that needs to be further investigated.

We are currently investigating whether other qualities, such as arsenic resistance, UV tolerance, other antibiotic resistances (Dib et al., 2008), and further genetic determinants for survival in these oligotrophic, pristine and extreme environments, are provided by such plasmids, as well as sequencing of the plasmid DNA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plasmid.2009.10.001.

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