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Replication of plasmids with the p15A origin in *Shewanella putrefaciens* MR-1

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C.R. MYERS AND J.M. MYERS. 1997. The plasmid pACYC184 was introduced into *Shewanella putrefaciens* MR-1 by electroporation. In 100% of the transformants examined, the plasmid was maintained as a free replicon outside the chromosome. This was the case whether or not the plasmid contained a 224-bp DNA insert derived from an open-reading frame of MR-1 genomic DNA. Therefore, in contrast to a report in the literature, plasmids containing the p15A origin of replication can replicate freely in *S. putrefaciens* MR-1, and do not make convenient vectors for gene replacement in this bacterium. However, we found that plasmids with the pMB1 origin of replication (e.g. pBR322) cannot replicate in MR-1 and could therefore have potential as vectors for gene replacement.

INTRODUCTION

Shewanella putrefaciens MR-1 (Myers and Nealson 1988a,b; Myers and Myers 1992a,b, 1993a) is a Gram-negative facultative anaerobe that was originally isolated from the sediments of Lake Oneida, New York, as a direct reducer of manganese (Mn) oxides (Myers and Nealson 1988a,b). It exhibits remarkable respiratory plasticity, and can generate energy from the reduction of a variety of compounds, including fumarate, nitrate, TMAO, Mn(IV) oxides, iron (III) oxides, etc. (Myers and Nealson 1988a, 1990a,b; Lovley *et al.* 1989). Since many aquatic environments, sediments and soils are rich in Mn and iron (Fe) oxides, bacteria such as *S. putrefaciens* could play important roles in the environmental cycling of these metals (Myers and Nealson 1990a; Nealson and Myers 1990; Nealson *et al.* 1991). Understanding the mechanism(s) used to mediate the reduction of these insoluble metal oxides is therefore a necessary component of a thorough understanding of their role in the environment and their potential interaction with other micro-organisms.

Previous studies have implicated respiratory electron transport components in the reduction of Mn and Fe oxides by MR-1 (Myers and Nealson 1988a, 1990b; Myers and Myers 1993a,b, 1994). The generation of mutants deficient in various aspects of anaerobic electron transport has been used as one means to elucidate the mechanism(s) of Fe and Mn reduction by this bacterium (Myers and Myers 1993b; Saffarini and Nealson 1993). For example, gene replacement has been used to generate a putative mutant deficient in *etrA*, an *fnr* analogue (Saffarini and Nealson 1993); to accomplish this, plasmid pACYC184 was used in gene replacement experiments because 'this plasmid is not replicated in MR-1' (Saffarini and Nealson 1993). In contradiction to this, we report here that pACYC184 readily replicates in MR-1, indicating that it and other vectors with the p15A replication origin do not make convenient vectors for gene replacement in this bacterium.

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MATERIALS AND METHODS

Materials

Tris, SDS, dithiothreitol, boric acid, citric acid trisodium, fumaric acid disodium and EDTA disodium were purchased from Research Organics (Cleveland, OH). Acrylamide and formamide were from EM Science (Gibbstown, NJ), tryptone, yeast extract, and bacteriological agar were from Oxoid (Unipath Ltd, Hampshire, UK), agarose was from FMC BioProducts (Rockland, ME), NaCl and urea were from Fisher Scientific (Itasca, IL) and β-agarase I, Vent$_R$™ DNA polymerase, Phototope™ Detection Kit and biotinylated λ *Hin*dIII DNA markers were from New England BioLabs (Beverly, MA). Proteinase K, calf intestinal alkaline phosphatase and some restriction enzymes were from Promega (Madison, WI), DNA polymerase (Klenow fragment) and

some restriction enzymes were from Pharmacia Biotech (Piscataway, NJ), T4 DNA ligase was from Promega or Life Technologies (Gaithersburg, MD), deoxynucleotide mix was from Stratagene (La Jolla, CA) and Immobilon S nylon membranes were from Millipore (Bedford, MA). Ethidium bromide and phenol/chloroform were from Amresco (Solon, OH) and X-Omat AR and BioMax MR X-ray film were from Eastman Kodak (Rochester, NY). All other reagents and chemicals were from Sigma (St Louis, MO) or Aldrich Chemical (Milwaukee, WI). Custom oligonucleotides were synthesized by Genemed Biotechnologies (South San Francisco, CA).

Bacterial strains, plasmids, media and growth conditions

Shewanella putrefaciens MR-1 (Myers and Nealson 1988a) and *Escherichia coli* JM109 (Yanisch-Perron *et al*. 1985) were grown aerobically on LB medium (Sambrook *et al*. 1989) supplemented, as needed, with antibiotics at the following concentrations: tetracycline (Tc), 8 μg ml^{-1} and chloramphenicol (Cm), 35 μg ml^{-1}. MR-1 was grown at room temperature (23–25°C) and *E. coli* at 37°C.

Plasmid pACYC184, a 4·24-kb tetracycline- and chloramphenicol-resistant (Tc^r Cm^r) cloning vector with the p15A origin of replication (Chang and Cohen 1978), was obtained from New England BioLabs. A 249-bp *Nco*I-*Dra*I fragment corresponding to a portion of an MR-1 genomic open-reading frame (ORF) that complements a previously isolated electron transport mutant (Myers and Myers 1993b) was synthesized from MR-1 genomic DNA template using high-fidelity Vent$_R$™ DNA polymerase and the following custom oligonucleotide primers: 5′CAACAGGGCCATGGGGTAAGTG and 5′GGCGTGCACTATTTAAACCCAGC (the *Nco*I and *Dra*I sites, respectively, are underlined); the buffer and thermal cycling conditions were those recommended by New England BioLabs. The resulting 249-bp product was purified from an agarose gel using β-agarase I, and then digested with *Dra*I and *Nco*I; the resulting 224-bp fragment was gel-purified and ligated into the Cm^r gene of pACYC184, generating pCMDN-1. To accomplish this, pACYC184 was first digested with *Dra*I and *Nco*I, and the 3861-bp fragment was purified by gel electrophoresis and β-agarase I prior to ligation. The DNA sequence of the amplified *Dra*I-*Nco*I insert from MR-1 was obtained using the Circum Vent™ Phototope™ Thermal Cycle DNA Sequencing kit (New England BioLabs) to verify that the correct sequence had been amplified.

DNA manipulations

Restriction digests, ligation, cloning, DNA electrophoresis and Southern transfers were done according to standard techniques (Sambrook *et al*. 1989). Plasmid DNA was purified from *E. coli* using a commercial kit (QIAprep Spin Plasmid kit, Qiagen, Chatsworth, CA) and from *S. putrefaciens* using an alkaline lysis protocol (Zhou *et al*. 1990) with the addition of a standard phenol : chloroform extraction step (Sambrook *et al*. 1989). The size of DNA fragments was estimated based on their relative electrophoretic mobilities to known standards using a computer program kindly provided by G. Raghava (Raghava 1994). DNA probes were labelled with biotin using the Random Primed Images™ Biotin Labelling kit (USB, Cleveland, OH), and Southern blots were developed using the Phototope™ Detection system (New England BioLabs).

Electroporation

Aerobically grown mid-logarithmic phase *S. putrefaciens* cells were prepared for electroporation as follows: cells were centrifuged for 1 min at 12 000 *g* in 1·5-ml microfuge tubes, washed once in 0·33 volume of sterile 1 mol l^{-1} D-sorbitol (pH 7·59), and resuspended in 0·05 volume 1 mol l^{-1} sorbitol (approximately 10^9–10^{10} cells ml^{-1}) and placed on ice; they were used within 15 min. Plasmid DNA (approximately 0·1–0·5 μg) was introduced into MR-1 cells (40 μl) by electroporation using 0·1-cm cuvettes (BTX, Inc., San Diego, CA) and a BioRad Gene Pulser™ with Pulse Controller (resistance, 200 Ω; capacitance, 25 μFD; voltage, 0·55 kV). After electroporation, the cells were immediately suspended in 0·5 ml LB broth and were either plated immediately on LB agar with Tc, or were held at room temperature for 90 min prior to plating. Resulting colonies were tested for their ability to use reduced Fe(III) under anaerobic conditions as previously described (Myers and Myers 1992b) in defined medium (Myers and Nealson 1990b) supplemented with Tc, 15 mmol l^{-1} lactate, vitamin-free Casamino acids (0·1 g l^{-1}) and 10 mmol l^{-1} ferric citrate. Fe(II) was determined by a ferrozine extraction procedure (Lovley and Phillips 1986; Myers and Nealson 1988b). Colonies were screened for plasmid content, and the identity of the plasmids was verified by Southern blotting using biotinylated pACYC184 as a probe.

RESULTS AND DISCUSSION

A gene replacement strategy (Zimmerman *et al*. 1991) has been reported for the generation of a putative MR-1 mutant deficient in *etrA*, an *fnr* analogue (Saffarini and Nealson 1993). This strategy relies on the introduction of a plasmid containing a small (approximately 200-bp) fragment of a desired gene. If the plasmid cannot be maintained as a free replicon in the recipient cell, then the only cells that should survive antibiotic selection should be those in which the entire plasmid has been integrated into the chromosome through a single site-specific recombination between the vector insert

and the homologous region of the chromosome (Zimmerman *et al*. 1991). Thus, antibiotic selection should provide a means to select for the desired cells.

Plasmid pACYC184, which carries the p15A origin of replication, has been used for gene replacement in MR-1 because 'this plasmid is not replicated in MR-1' (Saffarini and Nealson 1993). We intended to use this vector for gene replacement in MR-1. Plasmid pACYC184, which was purified from *E. coli*, was electroporated into MR-1. While we expected no transformants, numerous Tc^r transformants were obtained. Plasmid minipreps of 44 of these Tc^r transformants chosen at random demonstrated that all of them maintained a plasmid of ~4·2 kbp, the expected size of pACYC184. A gel showing 10 of these transformants chosen at random is shown in Fig. 1a. A Southern blot of this gel probed with pACYC184 verifies the identity of this 4·2-kbp band (Fig. 1b).

In a previous report on the use of pACYC184 for gene replacement in MR-1, a 383-bp *Dra*I-*Nco*I fragment of the Cm^r gene of pACYC184 was replaced with a 165-bp *Dra*I-*Nco*I fragment of the *etrA* gene of MR-1 (Saffarini and Nealson 1993); this resulted in the generation of a putative *etrA* mutant through site-specific integration of this recombinant plasmid into the genome of MR-1. Even though we have shown here that pACYC184 can freely replicate outside the chromosome in MR-1, perhaps site-specific recombination still occurs with sufficient efficiency to allow for the use of pACYC184 in gene replacement in *S. putrefaciens*. To test this possibility, we similarly replaced the 383-bp *Dra*I-*Nco*I fragment of the Cm^r gene of pACYC184 with a 224-bp *Dra*I-*Nco*I fragment of an MR-1 genomic ORF that complements a previously isolated mutant deficient in the reduction of Fe(III), nitrate and fumarate (Myers and Myers 1993b). After purification of this resulting recombinant plasmid (pCMDN-1) from *E. coli*, it was electroporated into MR-1. We observed numerous Tc^r transformants. Ninety-four of these transformants were chosen at random; 100% of them tested positive for Fe(III) reduction. If gene replacement had occurred in any of them, we would have expected a complete loss of Fe(III) reduction (Myers and Myers 1993b). Plasmid minipreps of these Tc^r transformants demonstrated that they all maintained a plasmid of ~4·1-kbp, the expected size of pCMDN-1; a gel with *Hind*III-digested plasmid preparations from 10 of these transformants chosen at random is shown in Fig. 2a. Note that pCMDN-1 is slightly smaller than pACYC184 because a 383-bp *Dra*I-*Nco*I pACYC fragment has been replaced with a 224-bp *Dra*I-*Nco*I MR-1 insert. A Southern blot of this gel probed with pACYC184 verifies the identity of this 4·1-kbp band (Fig. 2b). Thus, in our hands, all Tc^r transformants maintained pCMDN-1 outside the chromosome. These results indicate that site-specific recombination does not occur with sufficient efficiency to allow for the convenient use of pACYC184 in gene replacement in *S. putrefaciens*. Very large numbers of transformants would probably have to be screened to identify a gene replacement event.

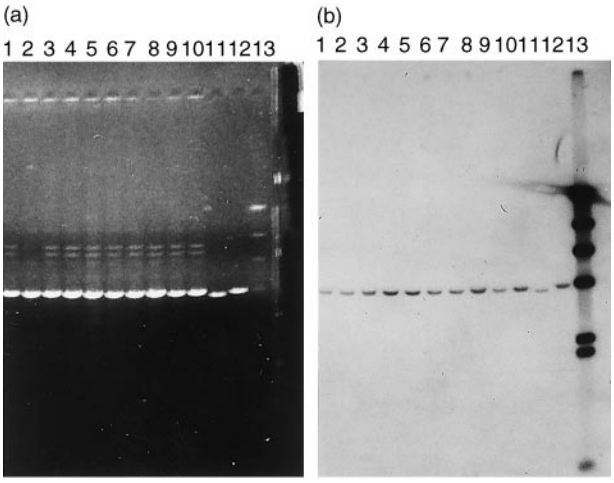

Fig. 1 (a) Ethidium bromide-stained agarose gel and (b) Southern blot of plasmid preparations from MR-1 transformed with pACYC184. Lanes 1–10, *Hind*III-digested plasmid preparations from MR-1 transformed to Tc^r with pACYC184. Other lanes are *Hind*III-digested pCMDN-1 (lane 11) and *Hind*III-digested pACYC184 (lane 12) as purified from *Escherichia coli*. The blot was hybridized with a biotin-labelled pACYC184 probe. Lane 13 contains biotinylated λ DNA/*Hind*III markers (23·1, 9·4, 6·6, 4·4, 2·3 and 2·0 kbp)

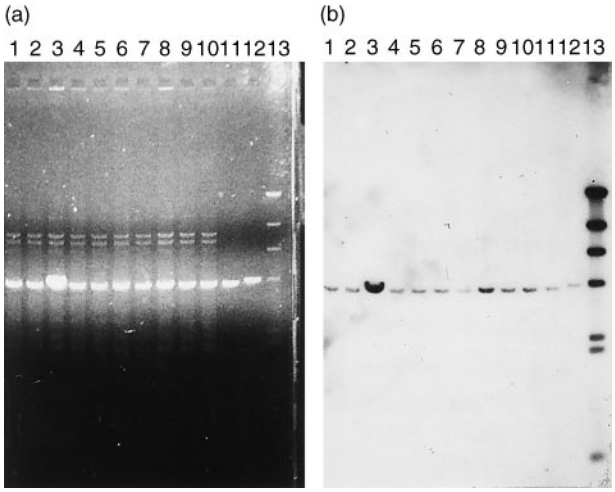

Fig. 2 (a) Ethidium bromide-stained agarose gel and (b) Southern blot of plasmid preparations from MR-1 transformed with pCMDN1. Lanes 1–10, *Hind*III-digested plasmid preparations from MR-1 transformed to Tc^r with pCMDN-1. Other lanes are *Hind*III-digested pCMDN-1 (lane 11) and *Hind*III-digested pACYC184 (lane 12) as purified from *Escherichia coli*. The blots were hybridized with a biotin-labelled pACYC184 probe. Lane 13 contains biotinylated λ DNA/*Hind*III markers (23·1, 9·4, 6·6, 4·4, 2·3 and 2·0 kbp)

Both pACYC184 and pCMDN1 were stably maintained in MR-1 through repeated passage into fresh medium containing Tc. We observed transformation efficiencies of 70–3600 Tc^r transformants per μg of pACYC184 or pCMDN1 DNA. While these efficiencies are low, our previous experience with electroporation of other vectors into MR-1 has resulted in similar low efficiencies (unpublished observations). Part of this may result from the restriction of plasmids in *S. putrefaciens* which have *E. coli* modification (i.e. which were recovered from *E. coli*). Transformation efficiency was increased approximately two- to fourfold by increasing the electroporation voltage from 5·5 to 7·5 kV cm^{-1}. Transformation efficiency also increased with increasing cell numbers over the range used (approximately 10^9–10^{10} cells ml^{-1}).

In recent studies involving repeated attempts, we were unable to isolate any Tc^r transformants after pBR322 (Bolivar *et al*. 1977) was electroporated into MR-1. Plasmids pBR322 and pACYC184 have a similar size and copy number and they have the identical class C tetracycline resistance gene which codes for an inner membrane tetracycline efflux protein (McMurry *et al*. 1980; Johnson and Adams 1992; Lenski *et al*. 1994). Hence, differences in the isolation of Tc^r transformants of MR-1 should not be related to these properties. A notable difference between these two plasmids is their replication origin, pMB1 for pBR322 versus p15A for pACYC184. Since most plasmids rely heavily on host cell replication machinery, it seems likely that the difference in ability of MR-1 to maintain pACYC184 *vs* pBR322 is related to their different replication origins. Hence, plasmids with the pMB1 origin of replication (e.g. pBR322) should be suitable vectors for gene replacement in MR-1, whereas those with the p15A replication origin (e.g. pACYC184) are not suitable.

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