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SHORT CONTRIBUTION

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Host-vector system for phenol-degrading *Rhodococcus erythropolis* based on *Corynebacterium* plasmids

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Abstract The strain *Rhodococcus erythropolis* CCM2595, which was shown to degrade phenol, was chosen for genetic studies. To facilitate strain improvement using the methods of gene manipulation, the technique of genetic transfer was introduced and cloning vectors were constructed. Using the plasmid pFAJ2574, an electrotransformation procedure yielding up to 7×10^4 transformants/μg DNA was optimized. *Escherichia coli-R. erythropolis* shuttle vectors were constructed using the replicons pSR1 and pGA1 from *Corynebacterium glutamicum*. The small vector pSRK21 (5.8 kb) provides six unique cloning sites and selection of recombinant clones using α-complementation of β-galactosidase in *E. coli*. This vector, exhibiting high segregational stability under non-selective conditions in *R. erythropolis* CCM2595, was applied to cloning and efficient expression of the gene coding for green fluorescent protein (*gfp*uv).

Introduction

Species of the Gram-positive genus *Rhodococcus* exhibit not only a wide range of catabolic activities (Hughes et al. 1998), but also some cell properties required for the technological application of microbial degraders (Lang and Philip 1998). Bacteria often suffer from growth inhibition at higher concentrations of various aromatic compounds during the course of their biodegradation (Prieto et al. 2002). To overcome such inhibition, cell immobilization, which enhances the ability of the cell to tolerate the cytotoxic effects of pollutants (Lazarova and Manem 1995), or genetically engineered microorganisms have been applied (Soda et al. 1998). Comparisons of biodegradation of aromatic compounds by suspended and attached bacteria proved that bacteria forming a biofilm were protected against cytotoxic effects and their ability to degrade phenol was enhanced (Prieto et al. 2002). In a preliminary cell attachment study, the strain *Rhodococcus erythropolis* CCM2595 was shown to form a phenol-resistant biofilm on inorganic surfaces and to degrade phenol efficiently. This strain was therefore chosen as a host for the development of a gene transfer system that would enable us to combine physiological and genetic improvements of this phenol degrader.

Genes coding for enzymes involved in degradation of various aromatic compounds have been found to be located on bacterial chromosomes or on plasmids (van der Meer et al. 1992). A number of large plasmids, of which only some were suitable for the construction of cloning vectors, has been isolated from *Rhodococcus* strains (Desomer et al. 1990; Denis-Larose et al. 1998). A few small plasmids, e.g., pFAJ2600 from *R. erythropolis* (De Mot et al. 1997), pNC903 from *R. rhodochrous* (Kalscheuer et al. 1999), pRC3 from *R. rhodochrous* (Hashimoto et al. 1992), and pMVS300 from *Rhodococcus* sp. (Singer and Finnerty 1988) have been used for the construction of shuttle vectors replicating in *Escherichia coli* and in various *Rhodococcus* strains. However, there is a general lack of information on the host range, classification into incompatibility groups, mechanism of replication and copy number control in the plasmids indigenous to *Rhodococcus* strains. As a basis for vectors replicating in *Rhodococcus* strains, some broad-host-range plasmids have also been used. The broad-host-range plasmid pNG2 from *Corynebacterium diphtheriae* also replicates in *C. glutamicum* and in *E. coli* (Serwold-Davis et al. 1987). Lessard et al. (1999) used the pNG2-based vector for transformation of *Rhodococcus* strains. The plasmid derived from the pNG2-related replicon pSR1 (Archer and Sinskey 1993) replicated in *Rhodococcus* sp. R312 (formerly *Brevibacterium* sp. R312) (Chion et al. 1991) and pJC1, also based on pSR1, replicated in

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Table 1 Strains and plasmids. *Km* Kanamycin, *Cm* chloramphenicol

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
Escherichia coli DH5α	Host for cloning	Gibco-BRL
Rhodococcus erythropolis, CCM2595	Wild type, phenol-degrading	CCM[a]
Corynebacterium glutamicum ATCC13058	Wild type, host of pSR1	Archer and Sinskey 1993
Plasmids		
pSR1	Cryptic plasmid from *C. glutamicum* ATCC13058, 3.0 kb	Archer and Sinskey 1993
pK19	*E. coli* vector, Km^R, 2.7 kb	Pridmore 1987
pEKplCmP45	Source of the promoter P-45, 7.6 kb	Pátek et al. 1996
pGFPuv	*E. coli* vector, Ap^R, *gfp*uv, 3.3 kb	Clontech
pK45	*E. coli* vector, Km^R 2.8 kb	This work
pEC6	*E. coli-C. glutamicum* vector, Cm^R, 7.2 kb	Eikmanns et al. 1991
pET2	*E. coli-C. glutamicum* vector, Km^R, 7.5 kb	Vašicová et al. 1998
pKG43	*E. coli-C. glutamicum* vector, Km^R, 7.0 kb	Nešvera et al. 1997
pSRK1	*E. coli-R. erythropolis* vector, Km^R, 5.7 kb	This work
pSRK21	*E. coli-R. erythropolis* vector, Km^R, 5.8 kb	This work
pSRK211	*E. coli-R. erythropolis* vector, *gfp*uv, Km^R, 6.5 kb	This work
pSRKgfp	*E. coli-R. erythropolis* vector, *gfp*uv, Km^R, 6.7 kb	This work
pFAJ2574	*E. coli-Rhodococcus* vector, Cm^R, 10.4 kb	De Mot et al. 1997
pNC9503	*E. coli-Rhodococcus* vector, Km^R, 6.3 kb	Kalscheuer et al. 1999

[a] Czech Collection of Microorganisms, Masaryk University Brno, Czech Republic

Rhodococcus opacus (Kalscheuer et al. 1999). The plasmids pNG2, pSR1 and pGA1 (Nešvera et al. 1997), indigenous to corynebacteria, are members of a new family of plasmids replicating by a rolling-circle mechanism (Osborn et al. 2000). These plasmids might also be utilized for the construction of vectors replicating in other *Rhodococcus* strains. The present paper describes optimization of an electrotransformation procedure of the studied strain with the plasmid vector pFAJ2574, construction of vectors based on *C. glutamicum* plasmids pSR1 and pGA1, and use of the constructed vector for expression of the gene coding for green fluorescent protein (*gfp*uv), which represents a new useful marker for *R. erythropolis*.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used are listed in Table 1. *E. coli* was grown in LB medium at 37°C. *R. erythropolis* CCM2529 was grown in 2×YT medium (Sambrook et al. 1989) with 0.5% glucose and 1.5% Tween 80 (used against cell clumping) at 30°C. Selection media contained kanamycin (Km, 30 μg/ml) or chloramphenicol (Cm, 50 μg/ml) for *E. coli* and Km (200 μg/ml) or Cm (50 μg/ml) for *R. erythropolis*.

DNA isolation and manipulation

Isolation of plasmid DNA from *E. coli* and DNA manipulations were carried out using standard techniques (Sambrook et al. 1989). Plasmid DNA from *R. erythropolis* was isolated by the same method as from *E. coli* with some modifications. Before lysis, cells were incubated for 2 h with lysozyme (5 mg/ml). For the lysis, a higher concentration of SDS (5%) was used. For PCR amplification of the promoter P-45, primers P45H (GCGGATCCTCATTCGCCGTGGC) and CM4 (GAAAATCTCGTCGAAGCTCG) and the plasmid pEKplCmP45 as a template (Pátek et al. 1996) were used. Primers KmTF1 (TCGGTACCTCATTCGCCGTGGCA) and KmTF2 (GCGGTACCTCTTGTTCAATCATGCGAAAC) and the plasmid pK45 as a template were used for PCR amplification of the regulatory sequences and the initial six codons of the Km resistance gene from pK45.

Electrotransformation

The optimized electrotransformation procedure was as follows: an overnight culture of *R. erythropolis* was used to inoculate 100 ml 2×YT medium in a 500-ml flask to OD_{600} =0.1. The medium was supplemented with 1.5% Tween 80 and 0.5% glucose. Cells were cultivated at 30°C with vigorous shaking (200 rpm). At OD_{600} =0.6 the cells were harvested by centrifugation, washed three times with 50 ml 10% glycerol and resuspended in 2 ml 10% glycerol. For electroporation, 200 μl cell suspension was mixed with 0.5 μg plasmid DNA, placed into an 0.2-cm cuvette and subjected to an electric pulse using a Gene Pulser (Bio-Rad, Richmond, Calif.) with the settings: 12.5 kV cm^{-1}, 600 Ω and 25 μF. Time constants of 14–16 ms were observed. Medium (1 ml 2×YT with 0.5% glucose but without Tween 80) was added immediately and the cells were cultivated for 4 h at 30°C. They were plated on 2×YT with Km (200 μg/ml) or Cm (50 μg/ml). Colonies appeared after 3–4 days and transformants were verified by detection of the relevant plasmid.

Results

Optimization of *R. erythropolis* electrotransformation

For initial transformation of *R. erythropolis* CCM2595, plasmids pFAJ2574 and pNC9503, previously described to replicate in several *Rhodococcus* strains (De Mot et al. 1997) and in *R. opacus* (Kalscheuer et al. 1999), respectively, were used. Initially, the electrotransformation procedure for *C. glutamicum* (Liebl et al. 1989) was applied. In both cases transformants of *R. erythropolis* CCM2595 were obtained. To optimize the electrotransformation technique in the strain studied, the plasmid

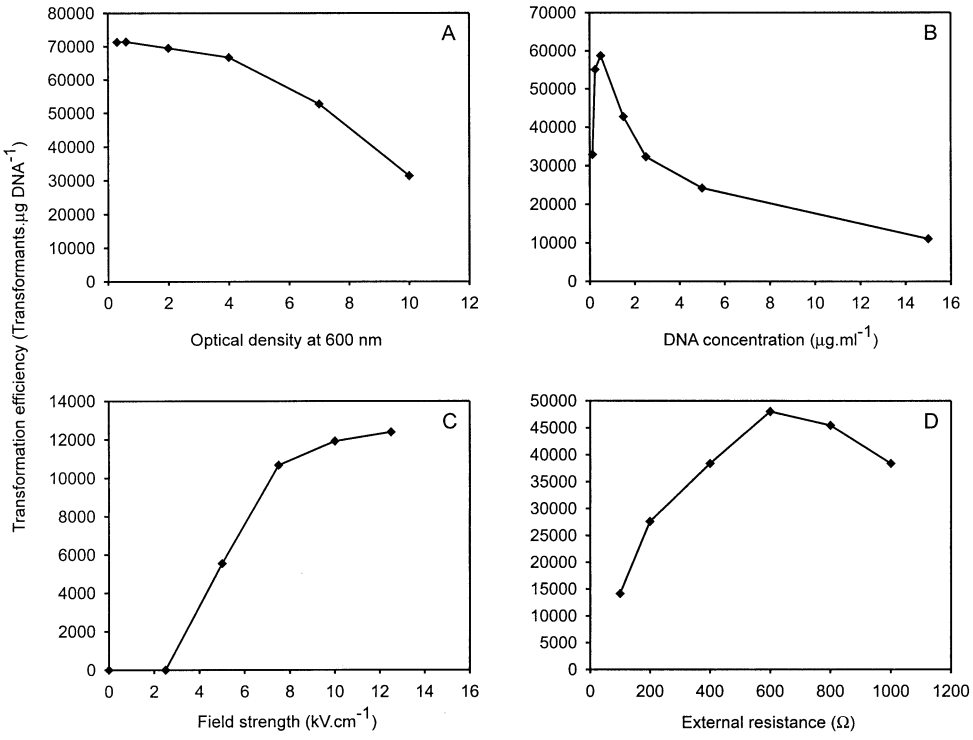

Fig. 1 Dependence of electrotransformation efficiency on **A** optical density of the cell culture, **B** DNA concentration, **C** field strength and **D** external resistance. The data represents the average of three independent experiments

pFAJ2574 (10.4 kb, Cm^R) was utilized. Different growth phase, DNA concentration, field strength, external resistance and cultivation time before plating were tested. Effects of the experimental variables on the transformation efficiency are shown in Fig. 1. The transformation efficiency increased with the increasing final concentration of the cells in the electroporation cuvette. However, when the cells were concentrated to OD_{600} =100, the probability of electric arc increased. Cells were concentrated to OD_{600} =30 in all cases shown in Fig. 1A. The highest efficiency was achieved using cells from the early growth stage (OD_{600} =0.3–0.7). At late stationary phase (OD_{600} >15,) no transformants were obtained. The optimum DNA concentration was found to be 0.25–1.0 µg/ml (Fig. 1B). Highest transformation efficiency was observed at a field strength of 10–12.5 kV/cm and external resistance of 600 Ω. At these settings, time constants of 14–16 ms were reached, and a 4-h cultivation before plating was found to be most favorable for the transformation yield. The optimized procedure was used for electrotransformation of *R. erythropolis* with plasmids derived from *Corynebacterium* replicons.

Transformation of *R. erythropolis* with plasmid vectors based on *Corynebacterium* replicons

The vectors based on the *C. glutamicum* plasmid pSR1 and related *C. diphtheriae* plasmid pNG2 have been reported to replicate in some *Rhodococcus* strains (Chion et al. 1991; Lessard et al. 1999). We therefore tested the constructs pSRK1 and pKG43 (based on the *C. glutamicum* indigenous cryptic plasmids pSR1 and pGA1, respectively) using the optimized electrotransformation procedure. Plasmid pSRK1 was constructed by cloning pSR1 linearized with *Bcl*I into the *Bam*HI site of pK19. Plasmid pKG43 (Nešvera et al. 1997) was created by cloning the large *Pst*I fragment (4.3 kb) of pGA1 into pK19. In addition to these constructs, *E. coli-C. glutamicum* shuttle vectors pET2 and pEC6, derived from the *C. glutamicum* ssp. *lactofermentum* replicon pBL1, were used for the electrotransformation of *R. erythropolis* CCM2595. Transformants harboring pSRK1 and pKG43 were obtained, while no transformants were observed with either pET2 or pEC6. The plasmid pSR1 was used for the construction of further vectors. First, the strong promoter P-45 (originally randomly cloned from the *C. glutamicum* chromosome) (Pátek et al. 1996) on a 120-bp PCR-amplified *Bam*HI fragment was inserted into the *Bcl*I site of pK19 upstream of the Km^R gene, giving rise to pK45. This step allowed pSR1 linearized with *Bcl*I to be cloned into the *Bgl*II site of pK45. The resulting vector pSRK21 (5.8 kb, Km^R; Fig. 2) provided the multiple cloning site of pK19 with six unique sites (*Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I), α-complementation of β-galactosidase for cloning in *E. coli*, and a strong selectable marker. After transfer into *R. erythropolis*, the plasmid was found to be structurally stable in the transformants. The minimal inhibitory concentration of Km in this host was higher than 3,000 µg/ml. Segregational stability of the plasmids in *R. erythropolis* was determined. After 30 generations of cultivation under non-selective conditions, 100% *R. erythropolis* cells harbored pFAJ2574 and 90% cells harbored pSRK21.

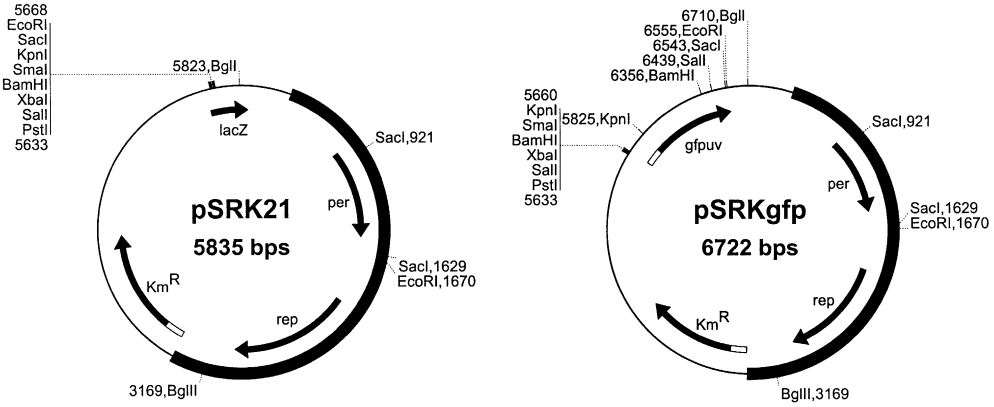

Fig. 2 Restriction and genetic maps of the *Escherichia coli-Rhodococcus erythropolis* shuttle vectors pSRK21 and pSRKgfp. The part originating from pSR1 is shown as a *thick black line*. The *empty box* at the 5'-end of the genes depicts a segment containing the regulatory sequences (promoter and Shine-Dalgarno site) and initial six codons of the kanamycin resistance (Km^R) gene used in translational fusion with *gfp*uv for efficient expression of the green fluorescent marker. The *per* gene of pSR1 is homologous to the *per* gene from pGA1, which positively influences the plasmid copy number and segregational stability (Nešvera et al. 1997)

The plasmid pKG43 was maintained in *R. erythropolis* cells during growth in the presence of Km, while it was unstable under non-selective conditions and was lost from the cells after 20 generations.

Expression of the *gfp*uv gene using vector pSRK21

The *gfp*uv gene, coding for green fluorescent protein, was used to demonstrate the usefulness of the vector pSRK21. The *Kpn*I-*Eco*RI fragment (730 bp) carrying the *gfp*uv coding sequence from the plasmid pGFPuv was cloned into pSRK21. In the resulting construct, pSRK211, *lacZ-gfp*uv fusion ensured a strong fluorescence of colonies of the *E. coli* host on plates illuminated with UV radiation (360 nm). However, no fluorescence of *R. erythropolis* transformants was detected. Therefore, the fragment containing the strong promoter P-45, a Shine-Dalgarno sequence and the initial six codons of the Km resistance gene was inserted upstream of the *gfp*uv gene in pSRK211, resulting in another translational fusion with *gfp*uv. The resulting plasmid, pSRKgfp (Fig. 2), conferred a reliable green fluorescent marker to *R. erythropolis* cells.

Discussion

The strain *R. erythropolis* CCM2595, which has convenient properties for application as a phenol-degrading organism, was chosen for genetic studies. To facilitate genetic analysis and manipulation of this strain, we have optimized conditions for genetic transformation and constructed plasmid vectors for gene transfer.

Starting with the conditions for electrotransformation of *C. glutamicum*, a gene transfer system for *R. erythropolis* was optimized. It was necessary to add Tween 80 to the cultivation medium to prevent clumping of the cells. Moreover, it has been shown that addition of Tween 80 to the cultivation medium increases transformation efficiency in some corynebacteria (Haynes and Britz 1989) and rhodococci (Sekizaki et al. 1998). The highest transformation efficiency in *R. erythropolis* CCM2595 reached (7×10^4 transformants/μg DNA) is comparable to that reported in *R. opacus* (Kalscheuer et al. 1999) and *R. fascians* (Desomer et al. 1990) and will allow further genetic tools for gene manipulations in the strain studied to be developed.

R. erythropolis falls into a distinct group of rhodococci with *R. globerulus*, *R. opacus* and *R. fascians* (Goodfellow et al. 1998). Construction of a few vectors replicating in some of these species has already been reported (Desomer et al. 1990; De Mot et al. 1997; Kalscheuer et al. 1999). However, knowledge of the basic functions of the *Rhodococcus* replicons used is scarce. According to the homologies of 16S rRNA sequences, *R. erythropolis* is related to other mycolic acid-containing genera like *Corynebacterium*. We therefore tested several *Corynebacterium* plasmid replicons as the basis for plasmid vectors for gene transfer to *R. erythropolis* CCM2595. The vectors based on the plasmids pSR1 and pGA1, classified into a new fifth group of rolling-circle-replicating plasmids (Osborn et al. 2000), were found to replicate in *R. erythropolis*, while no transformants were obtained with the pBL1 replicon, belonging to the pC194 family of rolling-circle-replicating plasmids. Plasmid replication and regulation of copy number have been studied in the plasmids pSR1 and pGA1 from *C. glutamicum* (Archer and Sinskey 1993; Nešvera et al. 1997). The product of the *per* gene of the plasmid pGA1 was found to positively influence the copy number of this plasmid and, consequently, its segregational stability (Nešvera et al. 1997). The N-terminal part of the pSR1-encoded ORF1 gene product (Archer and Sinskey 1993), whose amino acid sequence is identical with nearly the whole sequence of the pGA1-encoded Per protein,

appears to fulfill the same function (Nešvera et al. 1997). We have recently described negative control of pGA1 copy number by a small antisense RNA complementary to the *rep* gene leader region (Venkova-Canova et al. 2003). On the basis of sequence homology, we suppose that a similar mechanism functions also in pSR1. Since stable maintenance of small rolling-circle-replicating plasmids correlates with their copy number, vector molecules exhibiting higher segregational stability could be constructed by introducing sequence alterations alleviating the negative control of copy number of plasmids pSR1 and pGA1. These plasmids thus seem to represent a suitable basis for development of further tools for genetic analysis of *R. erythropolis* and for practical use in cloning the genes involved in degradation pathways. The *gfp*uv gene was efficiently expressed in *R. erythropolis* when cloned into the constructed vector pSRK21 using a suitable transcriptional and translational system. The *gfp*uv gene will be tested as a marker in a single copy after integration into the *R. erythropolis* chromosome and for construction of a promoter-probe vector for rhodococci.

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References

Archer JA, Sinskey AJ (1993) The DNA sequence and minimal replicon of the *Corynebacterium glutamicum* plasmid pSR1: evidence of a common ancestry with plasmids from *C. diphtheriae*. J Gen Microbiol 139:1753–1759

Chion CK, Duran R, Arnaud A, Galzy P (1991) Cloning vectors and antibiotic-resistance markers for *Brevibacterium* sp. R312. Gene 105:119–124

De Mot R, Nagy I, De Schrijver A, Pattanapipitpaisal P, Schoofs G, Vanderleyden J (1997) Structural analysis of the 6 kb cryptic plasmid pFAJ2600 from *Rhodococcus erythropolis* NI86/21 and construction of *Escherichia coli-Rhodococcus erythropolis* shuttle vectors. Microbiology 143:3137–3147

Denis-Larose C, Bergeron H, Labbe D, Greer CW, Hawari J, Grossman MJ, Sankey BM, Lau PC (1998) Characterization of the basic replicon of *Rhodococcus* plasmid pSOX and development of a *Rhodococcus-Escherichia coli* shuttle vector. Appl Environ Microbiol 64:4363–4367

Desomer J, Dhaese P, van Montagu M (1990) Transformation of *Rhodococcus fascians* by high-voltage electroporation and development of *R. fascians* cloning vectors. Appl Environ Microbiol 56:2818–2825

Eikmanns BJ, Kleinertz E, Liebl W, Sahm H (1991) A family of *Corynebacterium glutamicum/Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. Gene 102:93–98

Goodfellow M, Alderson G, Chun J (1998) Rhodococcal systematics: problems and developments. Antonie Van Leeuwenhoek 74:3–20

Hashimoto Y, Nishiyama M, Yu F, Watanabe I, Horinouchi S, Beppu T (1992) Development of a host-vector system in a *Rhodococcus* strain and its use for expression of the cloned nitrile hydratase gene cluster. J Gen Microbiol 138:1003–1010

Haynes JA, Britz ML (1989) Electrotransformation of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*: growth in Tween 80 increases transformation frequencies. FEMS Microbiol Lett 61:329–334

Hughes J, Armitage YC, Symes KC (1998) Application of whole cell rhodococcal biocatalysts in acrylic polymer manufacture. Antonie Van Leeuwenhoek 74:107–118

Kalscheuer R, Arenskotter M, Steinbüchel A (1999) Establishment of a gene transfer system for *Rhodococcus opacus* PD630 based on electroporation and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acids). Appl Microbiol Biotechnol 52:508–515

Lang S, Philip JC (1998) Surface-active lipids in rhodococci. Antonie Van Leeuwenhoek 74:59–70

Lazarova V, Manem J (1995) Biofilm characterization and activity analysis in water and wastewater treatment. Water Res 29:2227–2245

Lessard PA, O'Brien XM, Ahlgren NA, Ribich SA, Sinskey AJ (1999) Characterization of IS1676 from *Rhodococcus erythropolis* SQ1. Appl Microbiol Biotechnol 52:811–819

Liebl W, Bayerl A, Schein B, Stillner U, Schleifer KH (1989) High efficiency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiol Lett 53:299–303

Meer JR van der, de Vos WM, Harayama S, Zehnder AJ (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol Rev 56:677–694

Nešvera J, Pátek M, Hochmannová J, Abrhámová Z, Bečvářová V, Jelínková M, Vohradský J (1997) Plasmid pGA1 from *Corynebacterium glutamicum* codes for a gene product that positively influences plasmid copy number. J Bacteriol 179:1525–1532

Osborn M, Bron S, Firth N, Holsappel, Huddleson A, Kiewiet R, Meijer W, Seegers J, Skurray R, Terpstra P, Thomas CM, Thorsted P, Tietze E, Turner SL (2000). The evolution of bacterial plasmids. In: Thomas CM (ed) The horizontal gene pool. Harwood, Amsterdam, pp 301–361

Pátek M, Eikmanns BJ, Pátek J, Sahm H (1996) Promoters from *Corynebacterium glutamicum*: cloning, molecular analysis and search for a consensus motif. Microbiology 142:1297–1309

Pridmore RD (1987) New and versatile cloning vectors with kanamycin-resistance marker. Gene 56:309–312

Prieto MB, Hidalgo A, Rodriguez-Fernandez C, Serra JL, Llama MJ (2002) Biodegradation of phenol in synthetic and industrial wastewater by *Rhodococcus erythropolis* UPV-1 immobilized in an air-stirred reactor with clarifier. Appl Microbiol Biotechnol 58:853–859

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sekizaki T, Tanoue T, Osaki M, Shimoji Y, Tsubaki S, Takai S (1998) Improved electroporation of *Rhodococcus equi*. J Vet Med Sci 60:277–279

Serwold-Davis TM, Groman N, Rabin M (1987) Transformation of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, *Corynebacterium glutamicum*, and *Escherichia coli* with the *C. diphtheriae* plasmid pNG2. Proc Natl Acad Sci USA 84:4964–4968

Singer ME, Finnerty WR (1988) Construction of an *Escherichia coli-Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* spp. J Bacteriol 170:638–645

Soda S, Ike M, Fujita M (1998) Effects of inoculation of a genetically engineered bacterium on performance and indigenous bacteria of a sequencing batch activated sludge process treating phenol. J Ferment Bioeng 86:90–96

Vašicová P, Abrhámová Z, Nešvera J, Pátek M, Sahm H, Eikmanns B (1998) Integrating and autonomously replicating vectors for analysis of promoters in *Corynebacterium glutamicum*. Biotechnol Tech 12:743–746

Venkova-Canova T, Pátek M, Nešvera J (2003) Control of *rep* gene expression in the plasmid pGA1 from *Corynebacterium glutamicum.* J Bacteriol (in press)