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Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria

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Full exploitation of the information available in bacterial genome sequences requires the availability of facile tools for rapid genetic manipulation. One bacterium for which new genetic tools are needed is the methylotroph *Methylobacterium extorquens* AM1. IncQ and small IncP vectors were shown to be unsuitable for use in this bacterium, but a spontaneous mutant of a small IncP plasmid was isolated that functioned efficiently in *M. extorquens* AM1. This plasmid was sequenced and used as a base for developing improved broad-host-range cloning vectors. These vectors were found to replicate in a wide variety of bacterial species and have the following advantages: (1) high copy number in *Escherichia coli*; (2) small size (7·2 and 8·0 kb); (3) complete sequences; (4) variety of unique restriction sites; (5) blue–white screening via *lacZ*α; (6) conjugative mobilization between bacterial species; and (7) readily adaptable into species-specific promoter-probe and expression vectors. Two low-background promoter-probe vectors were constructed based on these cloning vectors with either *lacZ* or *xylE* as reporter genes; these were shown to report gene expression effectively in *M. extorquens* AM1. Specific expression vectors were developed for use in *M. extorquens* AM1, which were shown to express foreign genes at significant levels, and a simple strategy is outlined to develop specific expression vectors for other bacteria. The strong *mxaF* promoter was used for expression, since *E. coli lac*-derived promoters were expressed at very low levels. This suite of genetic tools will enable a more sophisticated analysis of the physiology of *M. extorquens* AM1, and these vectors should also be valuable tools in the study of a variety of bacterial species.

Keywords: plasmids, cloning vectors, expression vectors, promoter-probe vectors, *Methylobacterium extorquens* AM1

INTRODUCTION

In the past six years, the complete nucleotide sequences for dozens of prokaryotic genomes have been determined and published, and nearly 200 more are currently being sequenced (listed at http://216.190.101.28/GOLD/). Advances in bioinformatics have permitted phylogenetic classification of up to 80% of the putative ORFs in these organisms (Tatusov *et al.*, 2000). Biochemical and genetic experiments are required to test the myriad of biological hypotheses that have been generated. A major hurdle for studying the majority of these organisms is the paucity of sophisticated genetic tools. The capabilities and user-friendliness of broad-host-range (bhr) vector tools lag far behind those of the plasmids available for use in model organisms such as *Escherichia coli*. In order to take full advantage of the wealth of information available from bacterial genome sequencing, significant advances in the quality of bhr vectors are required.

Abbreviations: Ap, ampicillin; βGal, β-galactosidase; bhr, broad-host-range; Km, kanamycin; LB, Luria–Bertani (medium); oligo, oligodeoxyribonucleotide; *ori*, origin(s) of DNA replication; *P*, promoter; Rif, rifamycin; Sm, streptomycin; *t*, terminator of transcription; Tc, tetracycline; wt, wild-type.

The GenBank accession numbers for the sequences reported in this paper are AF327711, AF327712, AF327713, AF327714, AF327715, AF327716, AF327717, AF327718, AF327719 and AF327720.

The majority of bhr vectors are based upon IncP or IncQ replicons. Most IncP plasmids are derived from RK2, a 60·1 kb self-transmissible plasmid (Pansegrau *et al*., 1994). RK2 has an estimated copy number in *E. coli* of five to seven per chromosome (Figurski *et al*., 1979), and plasmids containing the IncP origin of transfer, *oriT*, can be mobilized by IncP transfer proteins provided *in trans* (Figurski & Helinski, 1979). Currently available vector tools based on IncP replicons are low- to medium-copy in *E. coli*, and few are fully sequenced or have a large number of available restriction sites.

Methylobacterium extorquens AM1 is an example of an organism for which improved genetic tools are needed. The α-proteobacterium *M. extorquens* AM1 is the best-studied representative of the methylotrophic bacteria, which are those capable of using single-carbon (C_1) substrates as their sole source of carbon and energy (Lidstrom, 1991). Methylotrophs play a critical role in the biogeochemical cycling of C_1 compounds (Hanson & Hanson, 1996) and have the potential to convert alternate feedstocks, such as methanol or methane, into fine chemicals and/or biopolymers. *M. extorquens* AM1 is a facultative methylotroph capable of growth on C_1 substrates, such as methanol and methylamine, as well as on a limited number of multi-carbon compounds, such as succinate and pyruvate. The ability to grow on nonmethylotrophic compounds and the ease of DNA transfer by either conjugation (Windass *et al*., 1980) or electroporation (Toyama *et al*., 1998) have made *M. extorquens* AM1 a model organism for the genetic dissection of the methylotrophic metabolism. In addition, the availability of genome-level sequence data for this strain (http://faculty.washington.edu/lidstrom/genome/genome/genome.html) is providing a rich database of information on metabolic potential. Thus far, only large (19–23 kb) IncP vectors with limited cloning sites available have been used with success in *M. extorquens* AM1. These include the cloning vectors pRK310 (Ditta *et al*., 1985) and pVK100 (Knauf & Nester, 1982), and the promoter-probe vectors pHX200 (Xu *et al*., 1993) and pGD500 (Ditta *et al*., 1985). No IncQ vectors or smaller IncP vectors have been found to be maintained in this strain (Lidstrom, 1992). Here we present the isolation of a spontaneous mutant of a small IncP plasmid that functions efficiently in *M. extorquens* AM1, the development of improved bhr cloning vectors based upon this plasmid, and their modification into host-specific promoter-probe and expression tools.

METHODS

Bacterial strains, plasmids and media. Wild-type (wt) *M. extorquens* AM1 (Peel & Quayle, 1961) and the UV-induced *mxaF* mutant UV26 (Nunn & Lidstrom, 1986) were used for the experiments described. The *E. coli* strains JM109 (Promega) and TOP10 (Invitrogen) were used as the hosts for construction and amplification of all plasmids used in this study. The plasmids used in this study are presented in Table 1. *M. extorquens* AM1 was cultured at 30 °C in a minimal salts medium described previously (Harder *et al*., 1973) containing 50 μg rifamycin (Rif) ml^{-1} and supplemented with either 125 mM methanol or 15 mM succinate. *E. coli* strains were grown in LB medium at 37 °C (Sambrook *et al*., 1989). Additional antibiotics were used when relevant at the following concentrations (μg ml^{-1}): 100 ampicillin (Ap), 50 kanamycin (Km), 35 streptomycin (Sm) and 10 (for *M. extorquens* AM1) or 12·5 (for *E. coli*) tetracycline (Tc).

Genetic procedures and recombinant DNA techniques. All DNA manipulations were performed according to standard techniques (Sambrook *et al*., 1989). Plasmids were transferred between *E. coli* and *M. extorquens* AM1 by conjugative transfer using an *E. coli* helper strain bearing pRK2013 or pRK2073 as described previously (Chistoserdov *et al*., 1994). DNA sequencing was performed at the Biochemistry DNA Sequencing Facility of the University of Washington.

Construction of the minimal transferable and selectable replicon for use in *M. extorquens* AM1. The 2·0 kb *Nde*I–*Not*I region of pDN19X containing the *traJ′* allele that allowed efficient replication and/or transfer in *M. extorquens* AM1 was transferred into the corresponding region of pTJS75 (Schmidhauser & Helinski, 1985), a parent vector of pDN19 (Nunn *et al*., 1990), to create pCM48. The plasmid pCM48 was then cut with *Mun*I and *Bln*I, and the DNA ends were blunted and self-ligated to produce pCM50. pCM50 was then digested with *Hin*dIII and *Xmn*I, and the DNA ends were blunted and self-ligated to produce the 5·3 kb minimal transferable and selectable replicon pCM51. Two additional plasmids were created to confirm that the *traJ′* allele present in pDN19X is sufficient to maintain and/or transfer the plasmid into *M. extorquens* AM1. The 2·0 kb *Nde*I–*Not*I region surrounding the substitution in pDN19X was swapped into the corresponding region of pDN19 to create the plasmid pCM46. Similarly, the 2·0 kb *Nde*I–*Not*I region from pDN19 encoding the full-length TraJ was cloned into pDN19X to create the plasmid pCM47.

To compare the reduced gene complement of the minimal selectable and transferable replicon pCM51 to the cloning vector previously used with *M. extorquens* AM1, pRK310 (Ditta *et al*., 1985), we have pieced together the complete sequence of this large IncP vector by determining the sequence over the junctions created during the partial digestion steps in its cloning history (Fig. 1).

Construction of improved bhr cloning vectors. The improved bhr vector pCM62 was created by combining the functions present in the minimal transferable and selectable replicon pCM51 with the polylinker and ColE1 origin of replication (*ori*) of pUC19 (Fig. 1). This was accomplished by ligating the blunted 5·3 kb *Hin*dIII–*Xmn*I region of pCM50 (used to make pCM51) with the blunted 1·9 kb *Aat*II–*Ava*II region of pUC19 (Yanisch-Perron *et al*., 1985). During the construction of pCM62 an additional 356 bp inward from the site of *Xmn*I cleavage of pCM50 was lost, presumably caused by either non-specific cleavage or by the exonuclease activity of T4 DNA polymerase. Loss of this region leads to a small alteration of the C-terminus of the TetR protein, from ·GDD-COOH to ·GAKKPLLS-COOH, but this mutation does not adversely affect the tetracycline resistance conferred by this plasmid in either *E. coli* or *M. extorquens* AM1. A derivative of pCM62 conferring resistance to kanamycin, pCM66, was constructed by inserting the 1·3 kb *Hin*cII fragment of pUC4K (Veira & Messing, 1982) between the *Eco*RV and *Nru*I sites of pCM62 (Fig. 2).

Construction of plasmids containing the reporter genes *xylE* and *gfp*, and the P_{mxaF} and *mxaF* gene of *M. extorquens* AM1. *xylE* was amplified by PCR from the promoter-probe vector

Table 1. Plasmids used in this study

	Description	Source
pBBR1MCS-2	Cloning vector developed from *Bordetella bronchiseptica*	Kovach *et al.* (1995)
pCM20	pCR2.1 with *xylE* PCR product	This study
pCM21	pCR2.1 with *gfp* PCR product	This study
pCM22	pMTL23 with *xylE*	This study
pCM23	pMTL23 with *gfp*	This study
pCM27	pCR2.1 with P_{mxaF} PCR product	This study
pCM46	pDN19 with *traJ′* allele from pDN19X	This study
pCM47	pDN19X with wt *traJ* from pDN19	This study
pCM48	pTJS75 with *traJ′* allele from pDN19X	This study
pCM50	Self-ligation of pCM48 *Mun*I–*Bln*I	This study
pCM51	Self-ligation of pCM50 *Hin*dIII–*Xmn*I, minimal transferable and selectable replicon	This study
pCM60	pCM50 derivative	C. J. M. and M. E. L. (unpublished)
pCM62	Hybrid of pUC19 and pCM51, improved bhr cloning vector (Tc^R)	This study
pCM63	pCM62 with *xylE*	This study
pCM64	pCM62 with P_{mxaF}	This study
pCM66	Kanamycin cassette inserted into *tetA* of pCM62, improved bhr cloning vector (Km^R)	This study
pCM66LacZ	pCM66 with *lacZ* (opposite orientation to P_{lac})	This study
pCM74	pCR2.1 with *mxaF* PCR product	This study
pCM75	pMTL23 with *xylE*	This study
pCM76	pCM62 with *xylE* replacing P_{lac}	This study
pCM79	pCM64 with *Hin*dIII filled-in	This study
pCM80	pCM79 with linker, *M. extorquens* AM1 expression vector (Tc^R)	This study
pCM81	pCM80 with *xylE*	This study
pCM85	pCM62 with *mxaF*	This study
pCM86	pCM80 with *mxaF*	This study
pCM87	pCM62 with *gfp*	This study
pCM88	pCM80 with *gfp*	This study
pCM110	pCM60 with P_{mxaF} replacing P_{lac}, *M. extorquens* AM1 expression vector (Tc^R) with basal expression in *E. coli*	This study
pCM111	pCM110 with *xylE*	This study
pCM130	pCM76 with t_{rrnB} of *E. coli*, low-background bhr *xylE* promoter-probe vector (Tc^R)	This study
pCM131	pCM130 with P_{mxaF}	This study
pCM132	pCM66lacZ with t_{rrnB} from *E. coli*, low-background bhr *lacZ* promoter-probe vector (Km^R)	This study
pCM133	pCM132 with P_{mxaF}	This study
pCM160	kanamycin cassette inserted into *tetA* of pCM80, *M. extorquens* AM1 expression vector (Km^R)	This study
pCR2.1	Commercial vector for cloning PCR products	Invitrogen
pDN19	small IncP cloning vector	Nunn *et al.* (1990)
pDN19X	Spontaneous mutant of pDN19 (in *traJ*), efficiently maintained/transferred in *M. extorquens* AM1	This study
pDN411	Cosmid containing 8·6 kb *Hin*dIII region of *mxa* operon	Nunn & Lidstrom (1986)
pGFPuv	Commercial vector containing *gfp*	Clontech
pHX200	Large IncP *xylE* promoter-probe vector	Xu *et al.* (1993)
pJB656	Small IncP expression vector with $xylS/P_m$	Blatny *et al.* (1997)
pLacZ2.1+	pCR2.1 with *lacZ* PCR fragment	R. Meima & M. E. L. (unpublished)
pMMB67HE.tet	IncQ expression vector with $lacI^q/P_{tac}$	Jiang & Howard (1992)
$pMTL20T_1T_2$	Cloning vector with t_{rrnB} from *E. coli*	Cordes *et al.* (1996)
pMTL23	Cloning vector with an extensive polylinker	Chambers *et al.* (1988)
pRK310	large IncP cloning vector	Ditta *et al.* (1985)
pRK2013	Helper plasmid expressing IncP *tra* functions (Km^R)	Figurski & Helinski (1979)
pRK2073	Helper plasmid expressing IncP *tra* functions (Sm^R)	Figurski & Helinski (1979)
pTJS75	Small IncP plasmid	Schmidhauser & Helinski (1985)
pUC4K	Vector with kanamycin cassette	Veira & Messing (1982)
pUC19	Universal cloning vector	Yanisch-Perron *et al.* (1985)

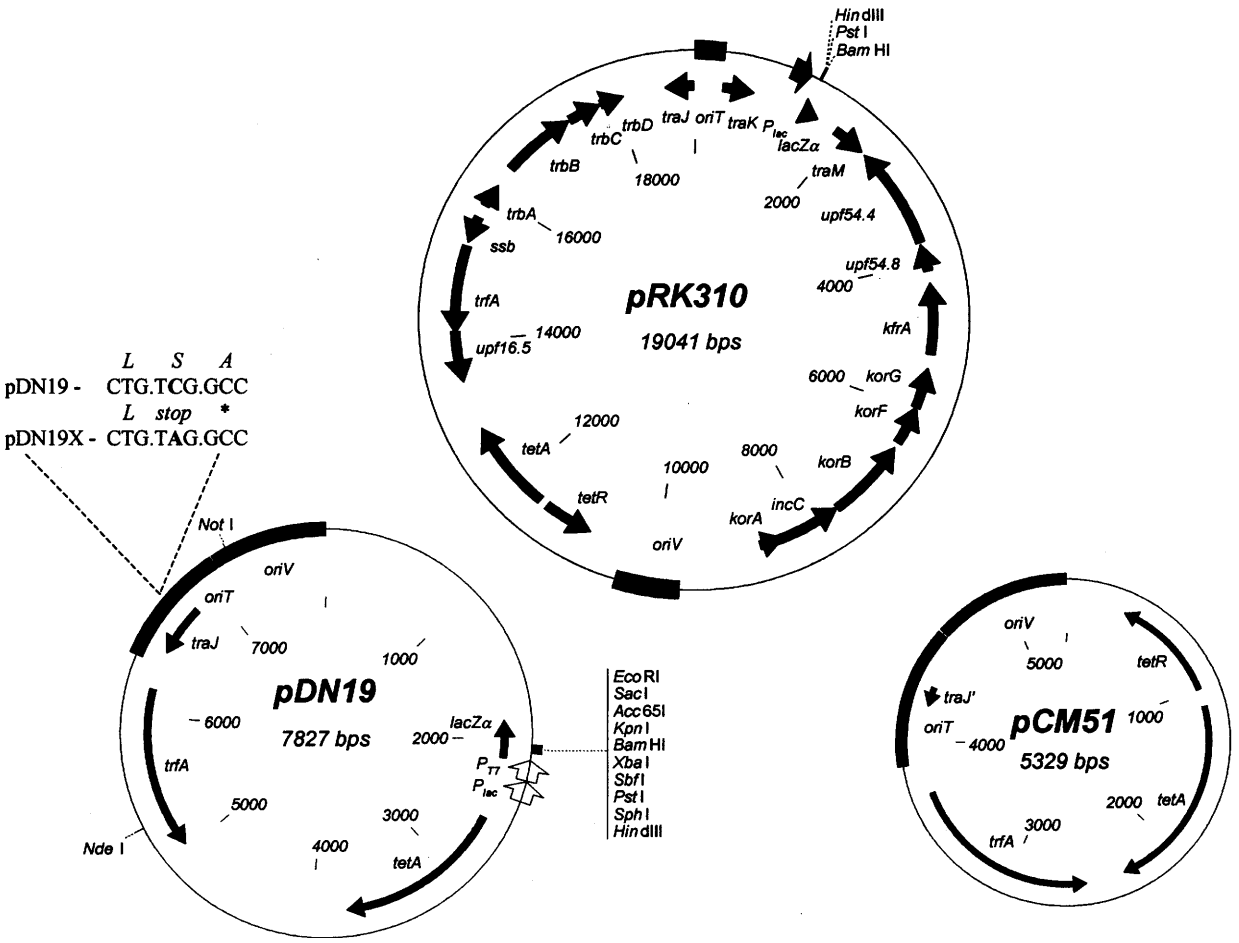

Fig. 1. Plasmid maps depicting the relevant features of the large IncP cloning vector pRK310, the small IncP cloning vector pDN19, and the minimal transferable and selectable IncP replicon pCM51. The C to A transversion present in pDN19X that created the truncated *traJ'* allele is diagrammed. The GenBank accession numbers for these plasmids are AF327712, AF327711 and AF327713, respectively.

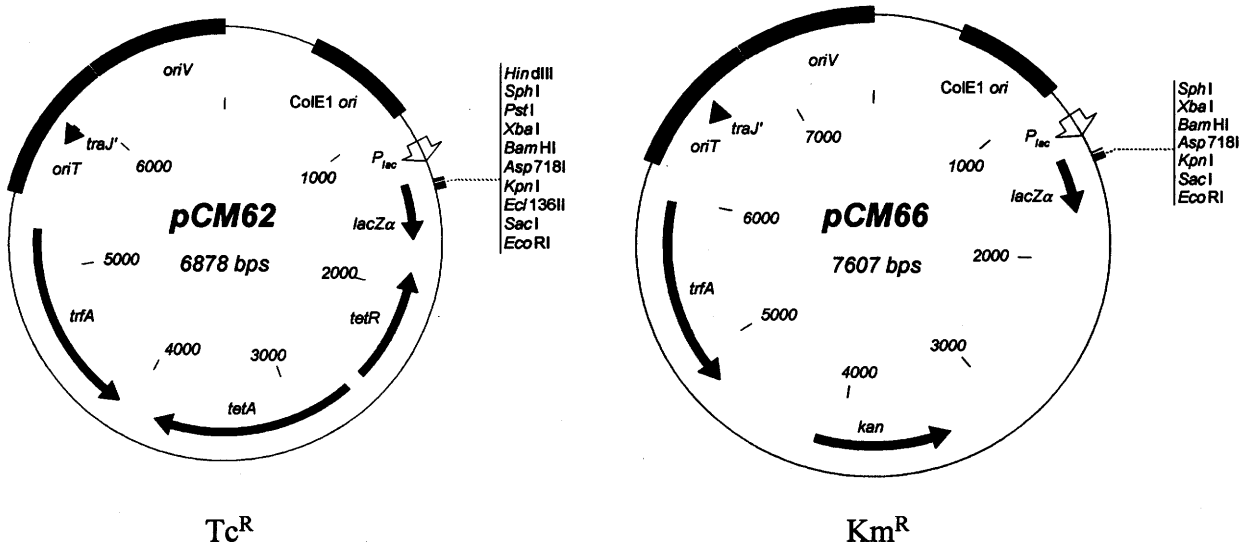

Fig. 2. Plasmid maps depicting the relevant features of the improved bhr cloning vectors pCM62 and pCM66. The GenBank accession numbers for these plasmids are AF327714 and AF327715.

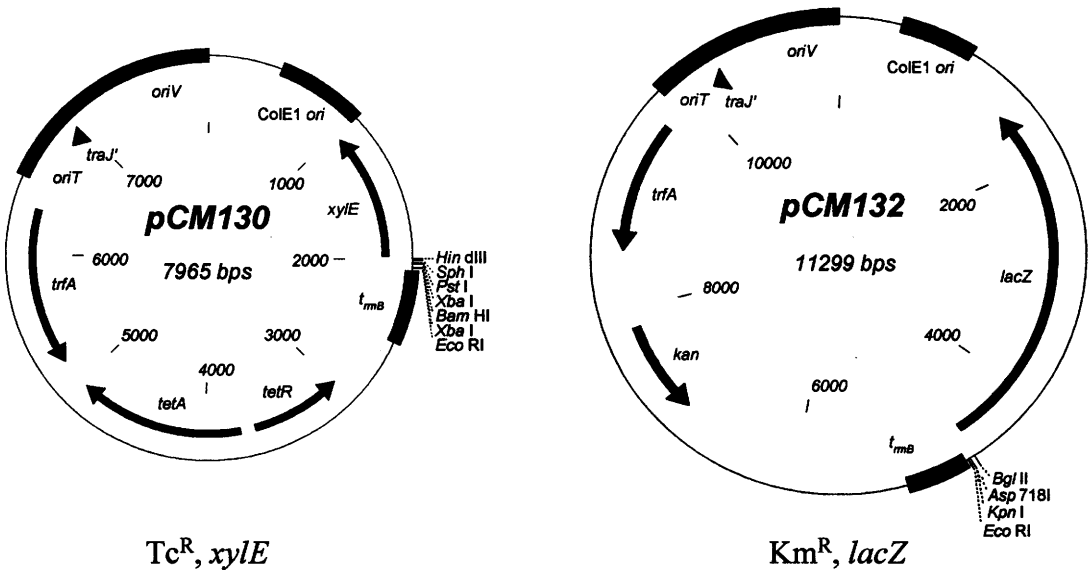

Fig. 3. Plasmid maps depicting the relevant features of the low-background bhr promoter-probe vectors pCM130 and pCM132. The GenBank accession numbers for these plasmids are AF327719 and AF327720.

pHX200 (Xu *et al*., 1993) using the following primers: CM-xylEf, 5′-TAGCTGCAGTAAGCTTCAGGAGGTGACG-TC-3′, and CM-xylEr, 5′-AGGGATCCGAGCTCCATCAG-GTGAGCACGGTC-3′. The resulting PCR product was cloned into pCR2.1 (Invitrogen) to create pCM20. Two other plasmids were created to allow the introduction of *xylE* into various plasmids. The 1·0 kb *Hin*dIII–*Bam*HI region of pCM20 containing *xylE* was cloned between the *Hin*dIII and *Bam*HI sites of the cloning vector pMTL23 (Chambers *et al*., 1988) to produce pCM22. Finally, the 1·0 kb *Hin*dIII–*Sac*I fragment of pCM22 containing *xylE* was inserted into pMTL23 cut with *Hin*dIII and *Sac*I to create pCM75. *lacZ* was amplified by PCR from pMUTIN2 (Vagner *et al*., 1998) and cloned into pCR2.1 (Invitrogen) to create pLacZ2.1+ (R. Meima & M. E. Lidstrom, unpublished results). *gfp* was amplified by PCR from pGFPuv (Clontech) using the following primers: CM-gfpf, 5′-TAGCTGCAGTAAGCTTGTCGAC-TCTAGAGGATCCCC-3′, and CM-gfpr, 5′-AGGGGATCC-GAGCTCGGCGCTCAGTTGGAAT-3′. The resulting PCR product was cloned into pCR2.1 (Invitrogen) to create pCM21. An additional plasmid bearing *gfp*, pCM23, was created by inserting the 0·8 kb *Hin*dIII–*Nsi*I fragment of pCM21 into pMTL23 cut with *Hin*dIII and *Nsi*I. The P_{mxaF} of *M. extorquens* AM1 was amplified by PCR from pDN411 (Nunn & Lidstrom, 1986) using the following primers: CM-PmxaFf, 5′-TAGATCTCGACAAGCTTCCCGCTTGG-3′, and CM-PmxaFr, 5′-AGGATCCGCGGTATCTCTCAGA-CG-3′. The resulting 324 bp PCR product was cloned into pCR2.1 (Invitrogen) to create pCM27. Similarly, a 1·9 kb region bearing the *mxaF* gene without its promoter was amplified by PCR from pDN411 (Nunn & Lidstrom, 1986) using the following primer pair: CM-mxaFf, 5′-GGCATG-CGAGGAGACGCAGGATG-3′, and CM-mxaFr, 5′-CGAA-TTCCGGCTTCAGACGTTAC-3′. This product was intro-duced into pCR2.1 (Invitrogen) to create pCM74.

Construction of low-background promoter-probe vectors. Two low-background bhr promoter-probe vectors with different reporters were created using the cloning vectors pCM62 and pCM66 as their vector backbone. The 1·0 kb *Hin*dIII–*Nco*I fragment from pCM75 containing *xylE* was inserted into pCM62 cut with *Afl*III and *Hin*dIII to create pCM76. Similarly, the 3·2 kb *Eco*RI fragment of pLacZ2.1+ (R. Meima & M. E. Lidstrom, unpublished) containing *lacZ* was blunted and cloned into the *Bam*HI site of pCM66 which had been similarly blunted to create pCM66LacZ. pCM76 and pCM66LacZ were each found to have a high background reporter gene activity in *M. extorquens* AM1 (data not shown). To reduce this background activity, an *E. coli* terminator, t_{rrnB}, was introduced upstream of the multiple cloning sites on the two vectors. The 0·5 kb *Xba*I–*Xmn*I fragment of pMTL20T$_1$T$_2$ (Cordes *et al*., 1996) containing t_{rrnB} was excised, blunted and ligated into the blunted *Eco*RI site of pCM76 to create pCM130 (Fig. 3). The 1·0 kb *Eco*RI–*Rsr*II fragment of pCM130 containing t_{rrnB} was then transferred into pCM66LacZ, cut with *Eco*RI and *Rsr*II, to create pCM132 (Fig. 3). In order to test the ability of these vectors to detect promoter activity, the P_{mxaF} of *M. extorquens* AM1 was introduced into both plasmids. The 0·4 kb *Eco*RI–*Bam*HI fragment from pCM27 was cloned into pCM130 cut with *Eco*RI and *Bam*HI to create pCM131. Similarly, the 0·4 kb *Eco*RI fragment from pCM27 was inserted into the *Eco*RI site of pCM132 to produce pCM133.

Construction of facile expression vectors specifically developed for use in *M. extorquens* AM1. The first step toward the creation of the expression vector pCM80 was the introduction of the P_{mxaF} of *M. extorquens* AM1 into pCM62. This was accomplished by ligating the 0·4 kb *Hin*dIII–*Bam*HI fragment of pCM27 into pCM62 cut with *Hin*dIII and *Bam*HI to produce pCM64. To keep the *Hin*dIII site in the pCM80 polylinker unique, pCM64 was cut with *Hin*dIII, blunted with T4 DNA polymerase, and self-ligated to produce pCM79. Finally, the polylinker present in pCM62, including an intact *lacZα*, was restored through the introduction of a 64 bp linker (Fig. 4). A pair of overlapping oligos with *Bam*HI compatible 5′ overhangs were designed for this purpose: CM-L64f, 5′-GATCTCACACAGGAAACAGCTATGACCATGATTACG-CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAG-3′, and CM-L64r, 5′-GATCCTCTAGAGTCGACCTGCAGG-CATGCAAGCTTGGCGTAATCATGGTCATAGCTGTT-

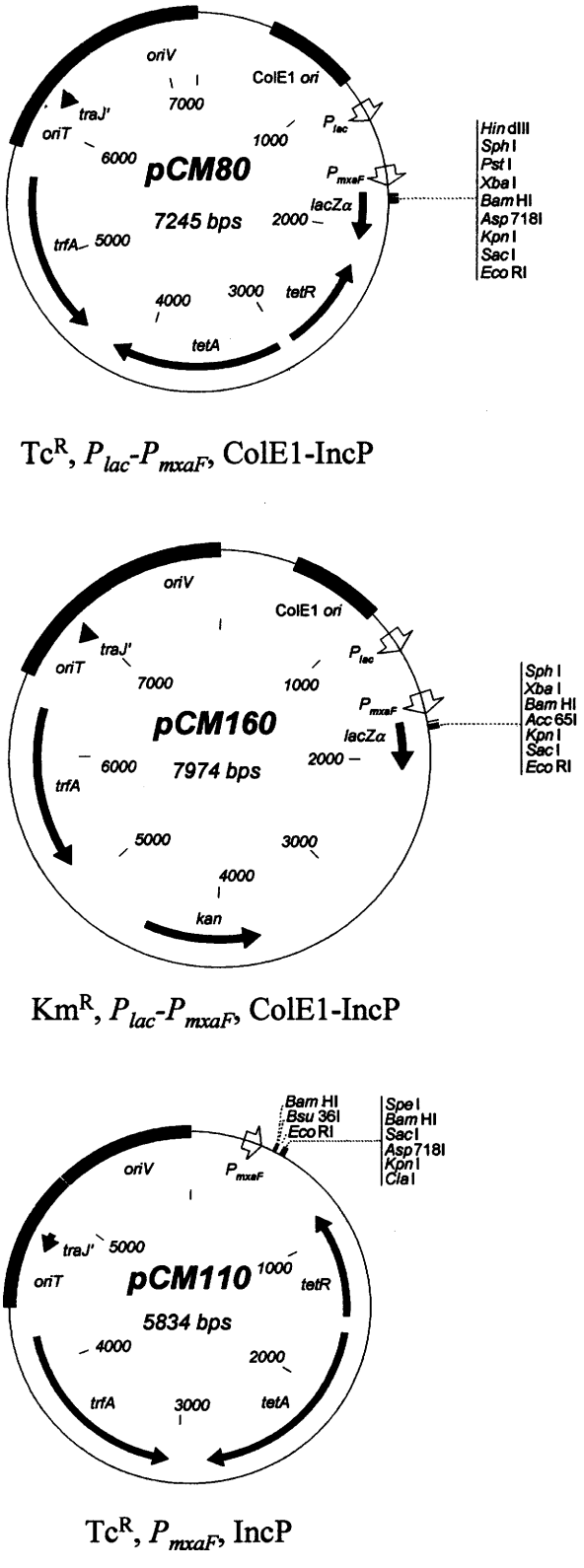

Fig. 4. Plasmid maps depicting the relevant features of the *M. extorquens* AM1 expression vectors pCM80, pCM110 and pCM160. The GenBank accession numbers for these plasmids are AF327716, AF327718 and AF327717, respectively.

TCCTGTGTGA-3′. These oligos were mixed to a final concentration of 100 μM each in distilled water, heated to 95 °C for 5 min, and then allowed to cool at 70 °C for 5 min followed by 50 °C for 5 min. The linker was then added directly to a ligation mix containing pCM79 cut with *Bam*HI. Plates containing X-Gal were used to screen for clones containing a linker region inserted in the desired orientation. Restriction analysis and sequence data across this region from one such clone, pCM80, confirmed the genuine insertion of a single linker in the desired orientation (Fig. 4). A kanamycin-resistant derivative, pCM160, was created by ligating together the 2·4 kb *Not*I–*Eco*RI region of pCM80 containing P_{mxaF} with the 5·6 kb *Not*I–*Eco*RI region of pCM66 containing the kanamycin-resistance cassette (Fig. 4).

A number of genes were introduced into pCM80 and pCM62, in order to demonstrate the utility of pCM80 as an expression vector. The 0·8 kb *Hin*dIII–*Bgl*II fragment of pCM23 bearing *gfp* was inserted into both pCM62 and pCM80 cut with *Hin*dIII and *Bam*HI to create pCM87 and pCM88. Similarly, the 1·0 kb *Hin*dIII–*Bam*HI fragment of pCM22 containing *xylE* was introduced into both pCM62 and pCM80 cut with *Hin*dIII and *Bam*HI to produce pCM63 and pCM81, respectively. Finally, the 2·0 kb *Hin*dIII–*Xba*I fragment of pCM74 containing *mxaF* was cloned into both pCM62 and pCM80 cut with *Hin*dIII and *Xba*I, generating pCM85 and pCM86.

In addition to pCM80 and pCM160, an additional expression vector, pCM110, was created that would provide minimal expression in *E. coli* to allow toxic genes to be introduced into *M. extorquens* AM1 (Fig. 4). pCM110 was constructed by inserting the 0·4 kb *Hin*dIII–*Nsi*I fragment of pCM27 containing P_{mxaF} into pCM60 (C. J. Marx & M. E. Lidstrom, unpublished results) which had been cut with *Hin*dIII and *Nsi*I. To compare expression from the P_{mxaF} in pCM80 to that in pCM110, the 1·0 kb *Eco*RI fragment from pCM20 containing *xylE* was inserted into the *Eco*RI site in pCM110 to create pCM111.

Reporter gene assays and SDS-PAGE analysis of vector expression. Cell extracts for enzyme assays and SDS-PAGE gels were prepared from mid-exponential cultures of *M. extorquens* AM1 harvested at an OD_{600} of 0·7–0·8, as determined using 1·0 cm cuvettes in a Beckmann DU 640B spectrophotometer. XylE and β-galactosidase (βGal) assays are reported as the mean and standard deviation of three replicates and were performed as described previously (Zukowski *et al.*, 1983; Kataeva & Golovleva, 1990; Miller, 1972). XylE activities in *E. coli* JM109 were assayed in extracts prepared from cultures grown to an OD_{600} of 0·6–1·0 in LB. The total protein was estimated either by direct spectrophotometric methods (Kalb & Bernlohr, 1977; Whitaker & Granum, 1980) for enzyme assays, or by the Bradford method using the Protein Assay Kit (Bio-Rad), using BSA as a standard, for SDS-PAGE analysis on 15 % gels. The relative intensities of selected bands on Coomassie-blue-stained SDS-PAGE gels were determined using Kodak 1D Image Analysis Software (Eastman Kodak).

RESULTS AND DISCUSSION

Isolation of a spontaneous mutant of the small IncP plasmid pDN19 that could be maintained efficiently in *M. extorquens* AM1

The initial step toward the construction of improved genetic tools for use in *M. extorquens* AM1 was to examine a few small bhr vectors for their ability to be

transferred by conjugation and maintained efficiently relative to the large IncP cloning vector pRK310 (Ditta *et al*., 1985). The plasmids tested included the small IncP vectors pDN19 (Nunn *et al*., 1990) and pJB656 (Blatny *et al*., 1997), the IncQ vector pMMB67HE.tet (Jiang & Howard, 1992), and pBBR1MCS-2 from *Bordetella bronchiseptica* (Kovach *et al*., 1995). Tetracycline-resistant colonies were not obtained using pMMB67HE.tet, consistent with the previous observation that IncQ plasmids serve as suicide plasmids in *Methylobacterium* strains (Biville *et al*., 1989). The vectors pJB656 and pBBR1MCS-2 could be established in *M. extorquens* AM1. However, both plasmids were transferred at a low efficiency relative to pRK310 and caused *M. extorquens* AM1 to grow at a significantly reduced growth rate. Only the small IncP vector pDN19 was maintained at a growth rate comparable to strains carrying pRK310 (data not shown), and was thus chosen as the basis for further vector development.

Initially, it appeared that pDN19 was transferred from *E. coli* to *M. extorquens* AM1 with an efficiency 1000 times less than that observed for pRK310. Following transfer of pDN19 from *M. extorquens* AM1 back into *E. coli*, however, the plasmid could be reintroduced into *M. extorquens* AM1 at the same high transfer efficiency as pRK310 (data not shown). This result suggested that the original pDN19 plasmid may have acquired a mutation that increased its transfer efficiency or the ability of *M. extorquens* AM1 to maintain this plasmid. No difference was observed in the maintenance of this plasmid derivative in *E. coli* (data not shown). We designated this pDN19 derivative as pDN19X.

Sequencing of pDN19 and the identification of the spontaneous mutation present in pDN19X

The exact sequence of the RK2-derived vector pDN19 (Nunn *et al*., 1990) was not known. Therefore, single-strand sequence was obtained for pDN19. A map of pDN19 and the features present on this plasmid are presented in Fig. 1. Seven single-nucleotide differences were observed relative to the reported sequence of RK2. These included single-nucleotide deletions and insertions, and nucleotide replacements. The only changes present in coding regions disrupt *upf-16.5*, a putative ORF of unknown function (Pansegrau *et al*., 1994). The single-strand sequence of pDN19X revealed a single nucleotide difference relative to the parent plasmid, pDN19, which was located within *traJ*. This C to A transversion results in an early stop codon in *traJ*, whose gene product recognizes the origin of transfer, *oriT*, initiating the DNA-processing events required for conjugal transfer (Ziegelin *et al*., 1989). This early stop codon creates a TraJ′ polypeptide that is missing the final 85 of 123 amino acids.

To determine whether the *traJ′* allele of pDN19X is sufficient to increase the maintenance and/or transfer of this plasmid in *M. extorquens* AM1, a 2·0 kb region encompassing *traJ* was swapped between pDN19X and the corresponding region of pDN19 to create pCM46. Similarly, the region from pDN19 encoding the full-length TraJ was cloned into pDN19X to create pCM47. The plasmid pCM46 bearing the mutation was maintained and/or transferred as had been observed for pDN19X, whereas pCM47 lacking the mutation lost this capacity. This confirmed that the ability of pDN19X to be efficiently maintained and/or transferred in *M. extorquens* AM1 was due to the region containing the *traJ′* allele, and not due to a distal region of the plasmid. The plasmid pRK310 is transferred and maintained in *M. extorquens* efficiently, and it has the same *traJ* allele as pDN19. In addition, all necessary *tra* functions including *traJ* are provided by the helper plasmid (pRK2013 or pRK2073) during triparental matings. Therefore, it is not clear why an alteration in TraJ would be required for efficient maintenance and/or transfer of pDN19 into *M. extorquens* AM1. It is possible that the effect may be due to altered expression of downstream genes, such as the essential initiator gene *trfA*. TrfA has been shown to affect copy number and host range (Haugan *et al*., 1995). In addition, our sequencing has shown that pDN19 lacks the native *trfA* promoter and the only known upstream promoter is that for *traJ*. It was not possible to compare expression of *trfA* or copy number between pDN19 and pDN19X, because pDN19 is apparently not maintained in *M. extorquens* AM1.

Reduction of pDN19X to the minimal transferable and selectable replicon pCM51

The nucleotide sequence of pDN19X was utilized to guide the removal of potentially nonessential plasmid regions in order to create a minimal transferable and selectable bhr replicon that could be utilized as the backbone for further vector development. Three regions of the 7·8 kb pDN19X plasmid were sequentially excised to create the 5·3 kb minimal transferable and selectable replicon pCM51 (Fig. 1). The plasmid pCM51 consists solely of the IncP *oriV* and *oriT*, *traJ′*, *trfA* and *tetA* and *tetR*. It behaved identically to pDN19X with regard to transfer and maintenance (data not shown), indicating that the regions of the parent plasmid that had been removed did not affect plasmid transfer or maintenance in *M. extorquens* AM1. Furthermore, pCM51 possesses a considerably reduced gene complement relative to the 19·1 kb IncP cloning vector previously used with *M. extorquens* AM1, pRK310 (Ditta *et al*., 1985) (Fig. 1). Achieving a small, functional replicon was critical in the development of improved bhr cloning vectors, and the subsequent elaboration of these vectors into more sophisticated genetic tools.

Development of facile bhr cloning vectors pCM62 and pCM66

The first goal of this study was to develop improved bhr cloning vectors that could be maintained in *M. extorquens* AM1 and other bacterial species, based on the minimal transferable and selectable replicon pCM51. This was accomplished by fusing a portion of pUC19 containing its multiple cloning site and the ColE1 *ori* to

Table 2. XylE or βGal activity [nmol min^{-1} (mg protein)$^{-1}$] present in cell extracts prepared from wt *M. extorquens* AM1 and *E. coli* JM109

Reporter	Plasmid	Features	*M. extorquens* AM1 grown in:	
			Methanol	Succinate
XylE	pCM63	pCM62 (P_{lac}) with *xylE*	32 ± 12	37 ± 5
	pCM81	pCM80 (P_{lac}–P_{mxaF}) with *xylE*	1180 ± 60	680 ± 30
	pCM111	pCM110 (P_{mxaF}) with *xylE*	1580 ± 210	1170 ± 10
	pCM130	*xylE* promoter-probe vector	1·6 ± 0.4	3.4 ± 0.4
	pCM131	pCM130 with P_{mxaF}	580 ± 100	320 ± 10
	pHX200	Large *xylE* promoter-probe vector	10 ± 1	10 ± 3
βGal	pCM132	*lacZ* promoter-probe vector	2·4 ± 0·1	9.4 ± 0.2
	pCM133	pCM132 with P_{mxaF}	2920 ± 110	4140 ± 210
			E. coli grown in LB	
XylE	pCM63	pCM62 (P_{lac}) with *xylE*	2300 ± 600	
	pCM81	pCM80 (P_{lac}–P_{mxaF}) with *xylE*	1270 ± 65	
	pCM111	pCM110 (P_{mxaF}) with *xylE*	1·1	

the region of pCM50 present in the small IncP replicon pCM51, creating pCM62 (Fig. 2). A kanamycin-resistant derivative, pCM66, was constructed by inserting the kanamycin resistance cassette from pUC4K for use in bacteria such as methanotrophs in which tetracycline is a poor marker (Fig. 2). Both of these plasmids were maintained in and transferred into *M. extorquens* AM1 with efficiency equal to pCM51 (data not shown). Routine alkaline lysis plasmid minipreps of pCM62 and pCM66 from *E. coli* cloning strains, however, indicated that the presence of the ColE1 *ori* raised their copy number in *E. coli* to that typical of pUC19 and related plasmids (data not shown).

The bhr cloning vectors pCM62 and pCM66 have a number of features that simplify their routine use: (1) high copy number in *E. coli*; (2) small size (7·2 and 8·0 kb, respectively); (3) complete sequences; (4) variety of unique restriction sites; (5) blue–white screening via *lacZα*; (6) conjugative mobilization between bacterial species; and (7) readily adaptable into species-specific promoter-probe and expression vectors.

A number of proteobacterial species other than *E. coli* and *M. extorquens* AM1 have been found to maintain pCM62 and/or pCM66. These include the α-proteobacteria *Agrobacterium tumefaciens* (L. Chen & E. Nester, personal communication), *Methylobacterium* strains CM4 and DM4 (S. Vuillemaier & T. Leisinger, personal communication), and *Rhodobacter sphaeroides* (J. Hickman & T. Donohue, personal communication), the β-proteobacterium *Ralstonia eutropha* (O. Lenz & B. Friedrich, personal communication) and the γ-proteobacteria *Methylococcus capsulatus* Bath (S. Stolyar & M. E. Lidstrom, unpublished) and *Pseudomonas aeruginosa* (T. Motley & S. Lory, personal communication). These reports suggest that the improved bhr cloning vectors and the promoter-probe vectors described below will be generally applicable to a wide variety of Gram-negative bacteria.

Conversion of bhr cloning vectors into low-background promoter-probe vectors pCM130 and pCM132 and their demonstration in *M. extorquens* AM1

The second goal of this work was to construct facile promoter-probe vectors for use in *M. extorquens* AM1, as well as other bacterial species. Two large IncP promoter-probe vectors have been used in *M. extorquens* AM1, pHX200 bearing *xylE* as a reporter (Xu *et al*., 1993) and the *lacZ*-containing pGD500 (Ditta *et al*., 1985). These plasmids are each greater than 20 kb in size, are not fully sequenced, and have limited cloning sites available upstream of their reporter genes. In addition, pGD500 has a high background reporter activity in *M. extorquens* AM1 (Morris & Lidstrom, 1992). In order to facilitate the identification and dissection of promoter regions in *M. extorquens* AM1, we created two promoter-probe vectors, pCM130 and pCM132, that are based upon the cloning vectors pCM62 and pCM66, respectively (Fig. 3). These promoter-probe vectors were created from their respective cloning vectors in two cloning steps. The reporter genes *xylE* and *lacZ* were first introduced into one side of the polylinker. This was followed by the introduction of a transcriptional terminator into the opposite side of the polylinker, in order to reduce background activity. The resulting bhr promoter-probe vectors have low background reporter gene activity in *M. extorquens* AM1 (Table 2) as compared to pHX200, facilitating their use in analysing weak promoters as well as strong promoters.

To test the utility of pCM130 and pCM132 as promoter-probe vectors, we introduced the strong promoter upstream of the methanol dehydrogenase operon of *M. extorquens* AM1, P_{mxaF} (Morris & Lidstrom, 1992), to produce pCM131 and pCM133, respectively. High reporter activities were obtained with both constructs (Table 2). pCM131 showed an approximately twofold

increase in XylE activity in methanol-grown cells as compared to succinate-grown cells, similar to previously reported results (Springer *et al*., 1998). An analogous increase in βGal activity was not observed with pCM133. This result supports previous data suggesting the inability to use *lacZ* as a plasmid-borne reporter gene to monitor P_{mxaF} regulation (Morris & Lidstrom, 1992). These results demonstrate the utility of pCM130 and pCM132 to locate, and potentially dissect, promoter regions. We expect that these low-background bhr promoter-probe vectors, and variants developed from them, will prove similarly useful in locating promoter regions in a wide variety of bacterial species.

Conversion of bhr cloning vectors into expression vectors pCM80 and pCM160 specifically designed for use in *M. extorquens* AM1

The final goal of this work was to create expression tools appropriate for use in *M. extorquens* AM1 based on these improved bhr cloning vectors. The three-step approach we outline below can be readily adapted to generate expression tools specific for any bacterial species found to maintain these plasmids. Firstly, amplify by PCR a known promoter region and insert this fragment into the P_{lac}-proximal side of the pCM62 or pCM66 polylinker, taking advantage of sites introduced on the primers, if necessary. Secondly, fill in the upstream site used for the insertion of the promoter fragment to preserve it for eventual use as a unique site in the polylinker. Thirdly, design and insert a linker with ends compatible to the downstream site used for insertion that can reconstitute the original polylinker within *lacZ*. This strategy allows the development of an expression vector that retains all of the advantages outlined for these cloning vectors.

For the conversion of pCM62 into an expression vector specifically designed for use in *M. extorquens* AM1, we chose to utilize P_{mxaF} (Morris & Lidstrom, 1992). In the promoter-probe vectors described above, this promoter provides high-level expression during growth on both methylotrophic and nonmethylotrophic substrates. The expression vectors pCM80 and the kanamycin-resistant derivative, pCM160 (Fig. 4), were constructed using the strategy outlined above.

Demonstrations of the utility of the *M. extorquens* AM1 expression vector pCM80

As a first test of the ability of pCM80 to express cloned genes in *M. extorquens* AM1, we used the gene encoding GFP from pGFPuv (Clontech). *gfp* was cloned into the cloning vector pCM62 (containing the P_{lac} promoter) and into pCM80 to produce pCM87 and pCM88, respectively. *In vivo* GFP activity of *M. extorquens* AM1 bearing pCM87, pCM88, or the 'empty' vector pCM80 was assessed on plates by observing the fluorescence of colonies during a brief exposure to UV light from a UV transilluminator (TFX-35M, Life Technologies). *M. extorquens* AM1 bearing pCM88 exhibited substantial fluorescence upon UV exposure, whereas colonies with pCM87 were indistinguishable from those carrying the 'empty' vector pCM80. These results suggested that substantial expression of a heterologous gene could be achieved in *M. extorquens* AM1 using pCM80, while the P_{lac} promoter of pCM62 did not appear to direct significant expression.

To quantify gene expression in *M. extorquens* AM1 using pCM80, two constructs, pCM63 and pCM81, were made that contain *xylE* inserted into the polylinker of either pCM62 or pCM80, respectively. Only low-level XylE expression in *M. extorquens* AM1 was observed from the P_{lac} of pCM62 (Table 2). Similar results using plasmids bearing the P_{lac} derivatives P_{tac} and P_{spac} further suggest that P_{lac}-derived expression vectors will not be useful for high-level expression in *M. extorquens* AM1 (C. J. Marx & M. E. Lidstrom, unpublished results). In contrast, a high level of XylE activity was detected in extracts prepared from *M. extorquens* AM1 bearing pCM81, which expresses XylE from the P_{mxaF} of pCM80 (Table 2). Similar results were found for a pRK310-derived expression vector that bears P_{mxaF} (C. J. Marx & M. E. Lidstrom, unpublished results). The modest induction observed using pCM81 during growth on methanol is similar to that observed from cells bearing the plasmid pCM131; however, the magnitude of XylE activity is roughly twofold higher. pCM131 differs from pCM81 by the presence of the t_{rrnB} upstream of P_{mxaF} in pCM131 and the orientation of P_{mxaF}::*xylE* relative to the remainder of the plasmid (Figs 3 and 4).

One concern about the use of pCM80 is its high copy number in *E. coli* and the presence of P_{lac} upstream of P_{mxaF}. This could lead to significant expression of cloned genes in *E. coli*, preventing the use of this vector for the expression of genes in *M. extorquens* AM1 that are toxic in *E. coli*. Accordingly, XylE activity was measured in cell extracts prepared from *E. coli* strain JM109 bearing various plasmids that had been grown in LB and harvested at an OD_{600} of 0·6–1·0 (Table 2). Significant expression was observed from both pCM63 and pCM81 in JM109. An additional expression vector, pCM110, was constructed from an IncP plasmid that lacks both the ColE1 *ori* and P_{lac}. A construct containing *xylE* cloned into pCM110 (pCM111) was created to compare its XylE expression level to that of pCM81 in both organisms. pCM111 provided a high level of expression in *M. extorquens* AM1, 1.5–2-fold higher than that achieved with pCM81; however, extracts from *E. coli* JM109 bearing pCM111 had a basal level of XylE activity, nearly at the background. These results indicate that the P_{mxaF} is expressed at very low levels in *E. coli*. Therefore, while facile expression vectors such as pCM80 are useful for the expression of most cloned genes, an alternative expression vector, such as pCM110, may be required to express genes that are toxic in *E. coli*.

To determine the percentage of the total cell protein that could be obtained using pCM80, SDS-PAGE was used to estimate the relative content of the XylE and GFP

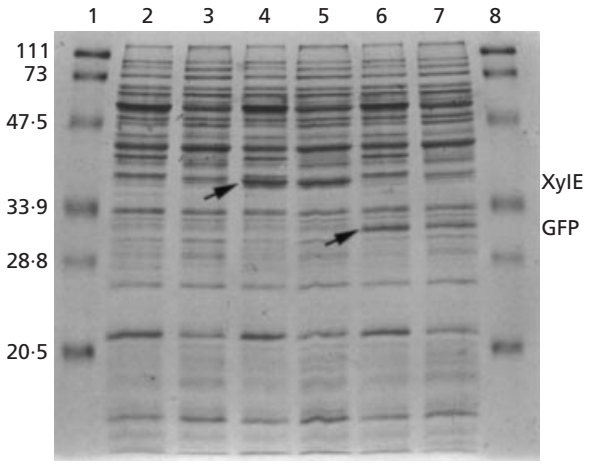

Fig. 5. SDS-PAGE of cell extracts prepared from *M. extorquens* AM1 bearing various plasmids. Lanes 1 and 8, molecular mass markers (Bio-Rad), masses in kDa indicated to the left; lanes 2 and 3, pCM80; lanes 4 and 5, pCM81; lanes 6 and 7, pCM88. Lanes 2, 4 and 6 were loaded with extracts prepared from methanol-grown cultures; lanes 3, 5 and 7 contain extracts from succinate-grown cells. Arrows indicate the position of XylE and GFP (predicted masses of 35·1 and 26·8 kDa, respectively).

polypeptides expressed from pCM81 and pCM88, respectively (Fig. 5). Protein bands could be identified specifically in the extracts from cells containing pCM81 and pCM88 that corresponded to the 35·1 and 26·8 kDa molecular masses predicted for XylE and GFP, respectively. Image analysis of the Coomassie-blue-stained SDS-PAGE gel indicated that pCM80 can express heterologous proteins at 5–9% of the total cell protein, with a modest increase in protein levels during growth on methanol relative to succinate. This level of induction corroborates the XylE activity data obtained from *M. extorquens* AM1 containing pCM81.

Our final test of the utility of the expression vector pCM80 was to determine whether the expression level achieved is sufficient to complement a mutant of a highly expressed gene. For this, we chose to introduce a promoterless *mxaF* into pCM80 and examine the growth of an *mxaF* mutant strain bearing this plasmid on methanol. *mxaF* was amplified by PCR and cloned into both pCM62 and pCM80 to produce pCM85 and pCM86. These two plasmids were then introduced into the *mxaF* mutant UV26 (Nunn & Lidstrom, 1986) by conjugation. UV26 carrying pCM86, which expresses *mxaF* from the P_{mxaF} of pCM80, grew at the same rate on methanol plates as wt *M. extorquens* AM1 with the empty vector, pCM80. UV26 carrying pCM85, which expresses *mxaF* from the P_{lac} of pCM62, however, grew very slowly on methanol plates and was indistinguishable from UV26 carrying the 'empty' vector pCM62. These results indicate that overexpression of MxaF from pCM80, but not the low-level expression from the cloning vector pCM62, is sufficient to replace that from P_{mxaF} on the chromosome.

Conclusions

We report the development of improved bhr cloning vectors and low-background promoter-probe vectors, and demonstrate a simple strategy to convert these plasmids into species-specific expression vectors. This suite of tools possesses a number of advantages over previously described bhr genetic tools in terms of their ease of use and their ability to be adapted for multiple purposes. These genetic tools will greatly facilitate the genetic dissection of the metabolism of *M. extorquens* AM1. The observation that these vectors can be maintained in a wide variety of bacterial species suggests that these vectors, and future elaborations upon them, will help fulfil the need for more sophisticated bhr genetic tools in a variety of bacteria. Such tools are critical for the facile genetic analysis of the wide spectrum of bacterial species for which genome-level sequence data are now available.

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