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Multicopy Integration and Expression of Heterologous Genes in *Methylobacterium extorquens* ATCC 55366†

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High-level expression of chromosomally integrated genes in *Methylobacterium extorquens* ATCC 55366 was achieved under the control of the strong *M. extorquens* AM1 methanol dehydrogenase promoter (P_{mxaF}) using the mini-Tn*7* transposon system. Stable maintenance and expression of the integrated genes were obtained in the absence of antibiotic selective pressure. Furthermore, using this technology, a multicopy integration protocol for *M. extorquens* was also developed. Chromosomal integration of one to five copies of the gene encoding the green fluorescent protein (*gfp*) was achieved. The multicopy-based expression system permitted expression of a preset number of gene copies. A unique specific Tn*7* integration locus in the chromosome of *M. extorquens*, known as the Tn*7* attachment site (*att*Tn*7* site), was identified. This single *att*Tn*7* site was identified in an intergenic region between *glmS*, which encodes the essential enzyme glucosamine-6-phosphate synthetase, and *dhaT*, which encodes 1,3-propanediol dehydrogenase. The fact that the integration event is site specific and the fact that the *att*Tn*7* site is a noncoding region of the chromosome make the mini-Tn*7* transposon system very useful for insertion of target genes and subsequent expression. In all transformants tested, expression and segregation of the transforming gene were stable without generation of secondary mutations in the host. In this paper, we describe single and multicopy chromosome integration and stable expression of heterologous genes (*bgl* [β-galactosidase], *est* [esterase], and *gfp* [green fluorescent protein]) in *M. extorquens*.

Methylotrophic bacteria are a diverse group of microorganisms that are able to grow on single-carbon substrates, such as methane or methanol, as sole sources of organic carbon and energy. Methylotrophs are ubiquitous and inhabit different aquatic and terrestrial habitats, including the phyllosphere (21, 23, 32, 33, 37, 39). One of the best-characterized methylotrophs, both genetically and physiologically, is the methanol-utilizing organism *Methylobacterium extorquens* AM1 (9). This *Methylobacterium* species has received special attention as a potential source of industrially pertinent natural and recombinant proteins (5, 16). The industrial potential of *M. extorquens* as a producer of recombinant proteins is due to (i) the simplicity of its growth requirements and its utilization of a comparatively inexpensive substrate (methanol), (ii) the development and optimization of protocols for high-cell-density cultivation, (iii) the fact that the *M. extorquens* AM1 genome has been sequenced and annotated, and (iv) the development of genetic tools specifically for *M. extorquens* comprising novel cloning and expression vectors, efficient transposon mutagenesis, and simple and efficient electroporation protocols (5–7, 15, 28–30). The potential of *M. extorquens* and other pink-pigmented facultative methylotrophs as "cell factories" is further enhanced by their inherent abilities to produce natural products of great importance, including pyrroloquinoline quinone (2, 13), vitamin B_{12} (37), poly-β-hydroxybutyric acid (PHB) (6, 7, 24), carotenoids (38), and phytohormones (14, 23, 27, 31). By using the molecular tools and high-cell-density fermentation technologies mentioned above, it has been possible to overexpress a variety of recombinant proteins in *M. extorquens* strains ATCC 55366 and AM1. Representative proteins overexpressed in these two *M. extorquens* strains include the green fluorescent protein (GFP), esterase from *Lactobacillus casei*, catechol 2,3-dioxygenase from *Pseudomonas putida*, enterocin P from *Enterococcus faecium*, and haloalkane dehalogenase from *Xanthobacter autotrophicus* (5, 11, 16, 17). Production of several of these recombinant proteins was achieved in high-cell-density fed-batch 20-liter and 150-liter fermentation processes (5, 4, 11). Recombinant product yields in the range from 3 to 6 g liter^{-1} were easily obtained in the absence of selective pressure (tetracycline). However, Bélanger et al. (5) observed that fermentation bioprocesses requiring more than 15 generations of growth in the absence of antibiotic selection resulted in a marked decrease in expression levels. This loss of recombinant product yield was largely attributed to plasmid segregational instability. Generally, industrial fermentation bioprocesses tend to avoid the use of antibiotics primarily due to economic, downstream processing, and regulatory safety concerns (35, 36). Stable integration of expression cassettes into bacterial chromosomes would obviate the need to use antibiotic selection for the expression of recombinant proteins. Recently, Marx and Lidstrom developed a series of insertional expression vectors which use homologous recombination for integration as tools for the study of central methylotrophy metabolism (30). An alternate integration system, known as the Tn*7*-based broad-range bacterial cloning and expression system, has also been described recently (22). The Tn*7* system integrates recombinant DNA fragments into a specific site on the bacterial chromosome, known as the *att*Tn*7*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
M. extorquens strains		
ATCC 55366	Wild type	ATCC
BGL	One-copy integrant of the lactase cassette derived from pBRI-bgl	This study
EST	One-copy integrant of the esterase cassette derived from pBRI-est	This study
GFP1	One-copy integrant of the *gfp* cassette derived from pBRI-gfp1	This study
GFP2	Two-copy integrant of the *gfp* cassette derived from pBRI-gfp2	This study
GFP3	Three-copy integrant of the *gfp* cassette derived from pBRI-gfp3	This study
GFP5	Five-copy integrant of the *gfp* cassette derived from pBRI-gfp5	This study
E. coli strains		
Top10	Strain for cloning and propagating plasmid DNA	Invitrogen Inc.
S17-1/λ *pir*	Host strain for pUX-BF13	3
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen Inc.
pCR-bgl	pCR2.1-TOPO containing lactase expression cassette	This study
pCR-est	pCR2.1-TOPO containing esterase expression cassette	This study
pCR-gfp1	pCR2.1-TOPO containing one copy of *gfp* expression cassette	This study
pCR-gfp2	pCR2.1-TOPO containing two copies of *gfp* expression cassette	This study
pUC19	Multipurpose cloning vector	Invitrogen Inc.
pCM110	Wide-host-range cloning vector containing P_{mxaF}; Tet^r	29
pCM-bgl	pCM110 containing lactase expression cassette	This study
pCM-est	pCM110 containing esterase expression cassette	This study
pCM-gfp	pCM110 containing *gfp* expression cassette	This study
pBK-miniTn*7*-ΩSm2	pUC19-based delivery plasmid for a mini-Tn*7*-Km transposon; Km^r Sm^r	22
pBRI70	pUC19-based delivery plasmid for a mini-Tn*7*-Km transposon; Km^r	This study
pBRI-bgl	pBRI70 containing lactase expression cassette	This study
pBRI-est	pBRI70 containing esterase expression cassette	This study
pBRI-gfp1	pBRI70 containing one copy of *gfp* expression cassette	This study
pBRI-gfp2	pBRI70 containing two copies of *gfp* expression cassette	This study
pBRI-gfp3	pBRI70 containing three copies of *gfp* expression cassette	This study
pBRI-gfp5	pBRI70 containing five copies of *gfp* expression cassette	This study
pUX-BF13	R6K replicon-based helper plasmid	3
pCESTa	Esterase gene source	11
pBGLIII	Lactase gene source	19
pQBI63	GFP gene source	Qbiogene Inc.

site (10, 12). The *att*Tn*7* site has been localized in the intergenic region downstream of the *glmS* gene in several gram-negative bacteria, including, notably, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *P. putida*, and *Yersinia pestis* (10, 12, 25, 26). Unlike the Tn*5* transposon mutagenesis system, the Tn*7* system does not cause insertional inactivation of host genes and therefore permits construction of isogenic strains that differ only in the nature of the added DNA (22).

We have applied and modified this technology, and we report here the first application of Tn*7*-based single-copy and multicopy expression of recombinant genes (*bgl* [β-galactosidase], *est* [esterase], and *gfp* [green fluorescent protein]) under the control of the strong *M. extorquens* AM1 inducible methanol dehydrogenase promoter (29) in *M. extorquens* ATCC 55366. The multicopy Tn*7*-based expression system permits expression of a preset number of gene copies.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured in Luria-Bertani broth at 37°C. *M. extorquens* ATCC 55366 was grown in CHOI medium, as previously described (5, 7), and 1% (vol/vol) methanol was used as the sole carbon source. Both media were solidified with 1.8% agar (Difco) when appropriate. Antibiotics were used for *E. coli* and *M. extorquens* at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 40 μg/ml; and tetracycline, 35 μg/ml. The mini-Tn*7* recombinant plasmids and helper plasmid pUX-BF13 were purified from *E. coli* (18, 22).

DNA isolation and manipulations. Plasmids were prepared from *E. coli* by using a QIAGEN miniplasmid purification kit according to the manufacturer's instructions (QIAGEN Inc., Mississauga, Ontario, Canada). Recombinant plasmids were constructed and agarose gel electrophoresis was performed by using the methods of Sambrook and Russell (34). DNA fragments were isolated from agarose gels by using a QIAquick gel extraction kit (QIAGEN). T4 DNA ligase and other DNA-modifying enzymes were purchased from New England Biolabs Inc., GIBCO/BRL Life Technologies, Inc., or Pharmacia LKB Biotechnology and were used as recommended by the manufacturers. Electroporation was performed with a Gene-Pulser II electroporation apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

Construction of Tn7 vectors. The mini-Tn*7* base vector pBRI70 for *M. extorquens* was constructed as follows. The P_{mxaF} ribosome binding site (RBS) was amplified from pCESTc (11) using primers MDH-F-PstI (5′-GGCTGCAGGTTGACGACAACGGTGCGATG-3′) and MDH-R-MluI (5′-CCGACGCGTATGTATATCTCCTTCTTAAAG-3′) (restriction sites are underlined). The PCR fragment containing the P_{mxaF} RBS was cloned into pBK-min iTn*7*-KmΩSm2 (22), which was partially digested with PstI/MluI to delete the Sm^r Sp^r cassette in order to generate pBRI70 (Fig. 1A). (Restriction sites are underlined.)

The 2.1-kb fragment carrying the β-galactosidase gene (*bgl*) was amplified from pEGIG4 (19) using primers bgl-F-MluI (5′-CACGCGTATG GAACATAGAGCGTTCAAGTG-3′) and bgl-R-NotI (5′-GCGGCCGCTTACAGCTTGACGACGAGTACGCCG-3′). For amplification of the 1.8-kb esterase gene (*estI*), pCESTa (11) was used as a template with primers est-F-MluI (5′-GACGCGTATGGATCAATCTAAAACAAATC-3′) and est-R-KpnI (5′-CGGTACCTTATTTATTTGTAATACCGTCTGC-3′). (Restriction sites are underlined.)

The 0.8-kb fragment carrying the *gfp* gene was amplified with pCM110-gfp

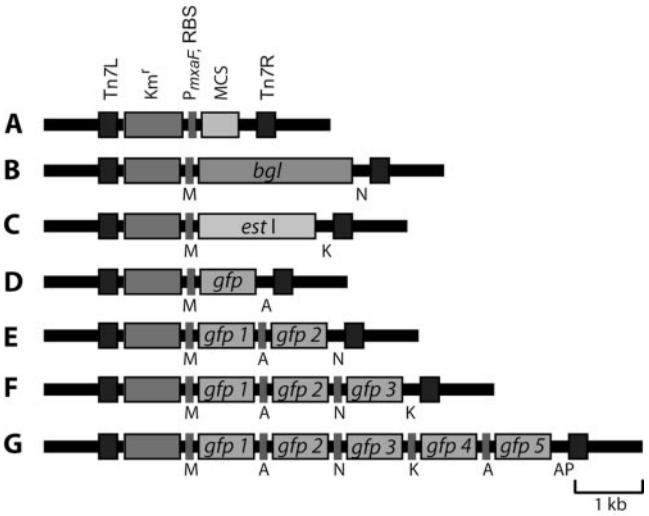

FIG. 1. Construction of mini-Tn*7* delivery plasmids. All genes are transcribed from left to right. Km^r, kanamycin resistance gene; Tn*7*L and Tn*7*R, Tn*7* left and right ends which are the minimal requirements for transposition; P_{mxaF}, methanol dehydrogenase promoter; MCS, multiple cloning site; *bgl*, β-galactosidase gene; *est*I, esterase gene; *gfp*, green fluorescent protein gene; M, MluI; A, AflII; N, NotI; K, KpnI; AP, ApaI.

using primers gfp-F-MluI (5′-GACGCGTATGGCTAGCAAAGGAGAAGAAC-3′) and gfp-R-AflII (5′-CCTTAAGTCAGTTGTACAGTTCATCCATGC-3′) (Restriction sites are underlined.) All PCR products were then cloned into the pCR2.1-TOPO vector, generating pCR-bgl, pCR-est, and pCR-gfp. The expression cassette was then cloned into the integration vector pBRI70 to form pBRI-bgl, pBRI-est, and pBRI-gfp, respectively (Fig. 1B, C, and D). Similarly, three recombinant plasmids, pBRI-gfp2, pBRI-gfp3, and pBRI-gfp5, containing two, three, and five copies of the *gfp* expression cassette, respectively, were constructed with different restriction enzyme sites available in the multiple cloning site of pBRI70. (Fig. 1E to G).

Chromosomal integration of constructs by electroporation. Preparation of electrocompetent cells and electroporation of *M. extorquens* were performed by using the methods described previously (15). Competent *M. extorquens* cells (100-μl suspension) were mixed in an Eppendorf tube with 0.5 μg of plasmid DNA (pBRI derivatives) and 0.5 μg of helper plasmid containing genes encoding the transposition proteins necessary for insertion of the Tn*7* cassette into the genomic target site (3). The mixture was transferred to an ice-cold electroporation cuvette and treated in a Bio-Rad electroporator (25 μF, 200 Ω, 5 ms, 2.5 kV/cm). Immediately after this, 1 ml of CHOI medium was added to the cuvette. The cell suspension was transferred to a 15-ml tube and incubated at 30°C for 5 h, and then 100 μl of culture was spread on selective plates (CHOI agar with 35 μg of kanamycin per ml). The plates were incubated at 30°C for 48 h until Km^r colonies appeared. Typically, about 300 to 500 transformants per plate were obtained.

Southern blot analysis. Chromosomal DNA was purified from mini-Tn*7*-Km target gene-transformed *M. extorquens* cells using an AquaPure genomic DNA kit (Bio-Rad) as recommended by the manufacturer. DNA samples (~2 μg) were digested with SalI and separated electrophoretically on a 0.7% agarose gel, and then they were transferred to a Hybond N membrane (Amersham Biotech. Inc.) according to the instructions of the supplier. The PCR fragment of each target gene (*bgl*, *estI*, or *gfp*) was labeled separately with digoxigenin-11-dUTP (DIG) (Roche Applied Science) and used as a probe. After hybridization at 42°C for 12 h and two washes in 2× SSC with 0.2% sodium dodecyl sulfate at room temperature (× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the DIG-labeled fragments were detected by reaction with anti-DIG antibodies coupled to alkaline phosphatase according to a protocol supplied by the manufacturer (Roche Applied Science). Nylon membranes were stained with a substrate solution (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate) for 5 min as recommended by the manufacturer.

Determination of Tn*7* insertion site in *M. extorquens*. For verification of the Tn*7* insertion site, we cloned the DNA flanking the Tn*7* insertion site in recombinant *M. extorquens*. To subclone the Tn*7* insertion site from recombinant *M. extorquens*, SalI-digested chromosomal DNA was cloned into the unique SalI site of the pUC19 vector and transformed into *E. coli* TOP10. Since the P_{mxaF} promoter is not recognized by *E. coli*, screening of the appropriate clone could not be done by determining target protein expression. Clone selection was therefore performed by detecting clones exhibiting kanamycin resistance. Sequencing was done by primer walking with purified plasmid DNA. The first primers recognized the vector sequences, and both strands were sequenced. The nucleotide sequences of both strands were determined by Ampli*Taq* FS DNA polymerase fluorescent dye terminator reactions as recommended by the supplier (Perkin-Elmer). Sequencing products were detected by using an Applied Biosystems 373 stretch automated sequencer (Applied Biosystems). Nucleotide sequence analyses and comparisons were conducted using the *M. extorquens* genome databases provided by Integrated Genomics and PEDANT (Protein Extraction, Description and ANalysis Tool; http://www.integratedgenomics.com and http://pedant.gsf.de).

The integration of target genes was also confirmed by colony PCR using general Tn*7* primers (P_{Tn7L}R [5′-ATTAGCTTACGACGCTACACCC-3′] and P_{Tn7R}F [5′-CACAGCATAACTGGACTGATTTC-3′]) and *M. extorquens*-specific primers (P_{dhaT}F [5′-CATCGCGATTGTCGATTCGG-3′] and P_{glmS}R [5′-CTGAAGGAAATCAGCTACATC-3′]), as shown in Fig. 2B.

Gene expression and protein assays. GFP was detected by fluorescence microscopy and was quantified by spectrofluorophotometry (Shimadzu RF-5001PC). The measurement procedures were carried out with whole cells resuspended in phosphate-buffered saline. The excitation wavelength was 397 nm, and the emission wavelength was 510 nm. The concentration of GFP expressed by *M. extorquens* was calculated based on a linear relationship between known concentrations of purified GFP solutions (Qbiogene) and fluorescence units, as previously described (5, 15). The esterase activity was determined by a spectrophotometric method using *para*-nitrophenyl caprylate as the substrate. The rate of hydrolysis of *para*-nitrophenyl caprylate at 37°C was measured in 50 mM sodium phosphate buffer (pH 7.0) as described by Kademi et al. (20). The β-galactosidase activity was measured with *o*-nitrophenol-β-D-galactoside as the substrate and was calculated based on pure enzyme from *E. coli* (34). Protein concentrations were estimated by the method of Bradford (8) using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

Fed-batch fermentation. Recombinant *M. extorquens* fed-batch cultures were grown using a 20-liter continuously stirred baffled fermentor (Chemap, Volketswil, Switzerland) equipped with pH and pO_2 probes (Ingold), a foam sensor, and a mechanical foam breaker. For agitation, the bioreactor was equipped with three Rushton impellers. The dissolved oxygen level was controlled at 15% saturation by first increasing the agitation speed from 500 rpm to 1,000 rpm and then increasing the airflow supply from 7 to 8 liters/min with pure oxygen. This O_2 enrichment procedure was initiated after 30 h of fermentation at a initial feed rate of 0.2 liter/min of pure oxygen, which was later increased to 3 liters/min. At the same time, the airflow was reduced to keep the overall inlet gas rate at 8 liters/min. The pressure in the fermentor was also increased to 0.8 $\times 10^5$ Pa after around 25 h of fermentation to increase the oxygen mass transfer.

Fed-batch bioreactor experiments were conducted at pH 7.0 and 30°C. An ammonia solution (30%) was used as both a pH control and a nitrogen source and was added as needed during fermentation. A 1% inoculum grown in 1-liter culture shake flasks was used to inoculate a 20-liter fermentor containing 9 liters of CHOI medium.

On-line measurements of the methanol concentration in the culture medium were obtained using a silicone membrane probe (Bioengineering Inc.) coupled to a semiconductor gas sensor (7). The methanol concentration was controlled by using an adaptive control algorithm described previously (5). Methanol was added using a variable-speed peristaltic pump, and the methanol concentration was controlled at 1.4 g/liter. Off-gas measurements were obtained to determine O_2 (Servomex paramagnetic analyzer) and CO_2 (Servomex infrared analyzer) concentrations.

RESULTS AND DISCUSSION

Construction of an integrative expression vector for *M. extorquens*. A strong homologous promoter (P_{mxaF}), which was derived from the *mxaF* operon of *M. extorquens* (1, 29), was used for construction of the integrative expression vector. In previous studies, we used this promoter combined with the T*7* RBS to express heterologous proteins in *M. extorquens* (11). The promoter and RBS cassette were cloned into a mini-Tn*7* transposon system to construct the expression plasmid,

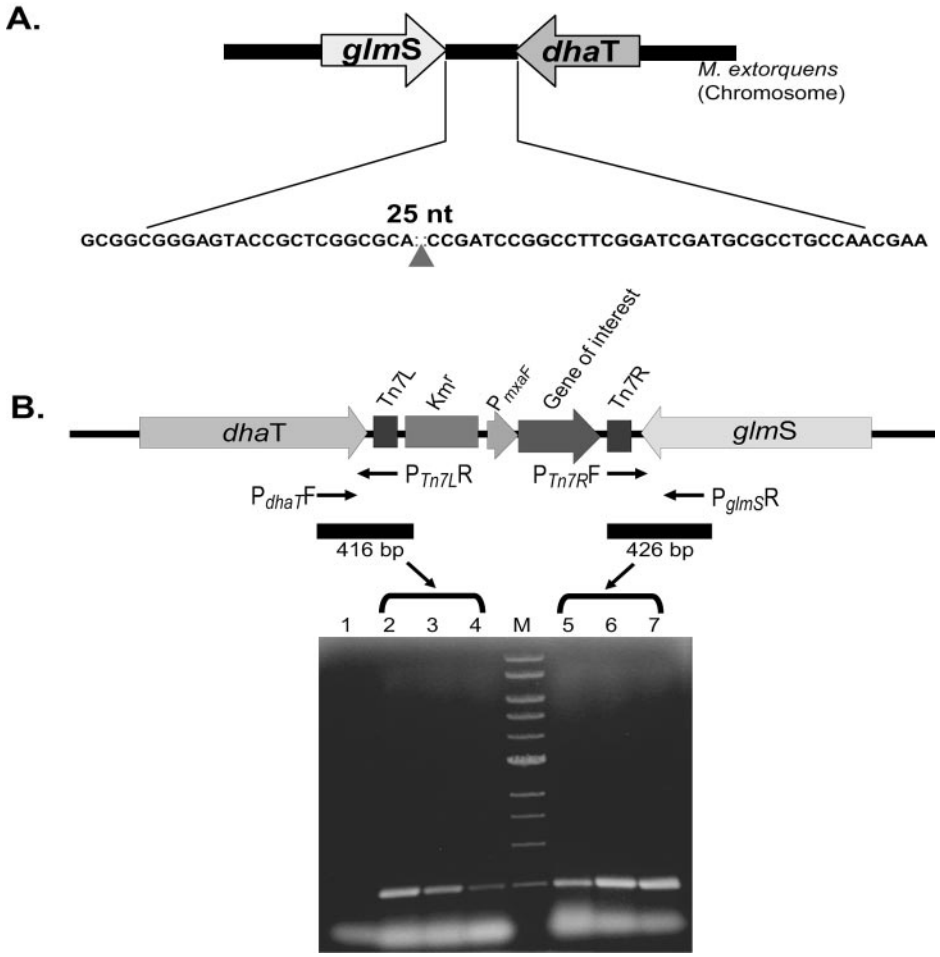

FIG. 2. (A) Identification of the mini-Tn*7* integration site (*att*Tn*7*) in *M. extorquens*. The insertion site is indicated by a triangle between nucleotides 24 and 25 downstream of the *glmS* stop codon in the chromosome of *M. extorquens*. nt, nucleotides. (B) Identification of mini-Tn*7* integration in *M. extorquens* by colony PCR. Verification of transposition events by colony PCR using the primer pairs indicated by convergent arrows yielded PCR fragments (solid bars) whose sizes are indicated. Lane M, 1-kb DNA ladder; lane 1, wild type; lanes 2 and 5, *gfp* integrants; lanes 3 and 6, *est* integrants; lanes 4 and 7, *bgl* integrants.

pBRI70 (Fig. 1A). Chromosomal integration of the mini-Tn*7*–P_{mxaF} RBS genes of interest derived from pBRI70 was achieved in *M. extorquens* by coelectroporation with helper plasmid pUX-BF13, which provided the Tn*7* transposition function in *trans* (3). The mini-Tn*7*-based recombinant plasmids were integrated into the *att*Tn*7* locus of *M. extorquens* by electroporation. Electroporation of the *M. extorquens* strain with these constructs in conjunction with the helper plasmid yielded about 1,000 transformants per μg DNA on selective plates containing 50 μg/ml of kanamycin.

Identification of Tn*7* integration site in *M. extorquens*. It was shown recently that the Tn*7* system integrates in a stable manner recombinant DNA fragments into a specific site of the chromosome called the *att*Tn*7* site. The *att*Tn*7* site is located in the intergenic region downstream of the *glmS* gene in many gram-negative bacteria, including *E. coli*, *K. pneumoniae*, *S. marcescens*, *P. putida*, and *Y. pestis* (10, 12, 25, 26). Southern hybridization analysis of three recombinants confirmed that Tn*7* integration occurred in the chromosomal DNA of *M. extorquens* (data not shown). Nucleotide sequence analyses of the cloned genes tested (*bgl*, *estI*, and *gfp*) revealed the identity of the Tn*7* integration site in *M. extorquens*. Interestingly, all three genes were integrated into the same site of the chromosome. The Tn*7* insertion site was located in a 61-bp intergenic region between *glmS*, which encodes the essential enzyme glucosamine-6-phosphate synthetase, and *dhaT*, which encodes 1,3-propanediol dehydrogenase in the chromosome of *M. extorquens*. Sequence analysis of cloned DNA fragments showed that the Tn*7* system was inserted between nucleotides 24 and 25 downstream of the *glmS* stop codon (Fig. 2A), and this site seems to lie in one of the inverted repeats of the putative *glmS* transcriptional terminator, as suggested previously (10). To confirm integration of the target genes into the chromosome of *M. extorquens*, colony PCR was carried out by using two sets of Tn*7*-based primers and two sets of strain-specific primers, as described in Materials and Methods. The sizes of the PCR products derived from different colonies were similar, as expected, and the data revealed that the mini-Tn*7* transposon is always inserted in only one orientation and is always inserted at the same site of the *M. extorquens* chromosome downstream of *glmS* (Fig. 2B).

Taken together, these results indicate that *M. extorquens* has

a unique Tn*7* attachment site (*att*Tn*7* site), and the insertion should not cause any insertional inactivation of host genes. This *att*Tn*7* site is a useful site for integration of recombinant genes in *M. extorquens* with the ultimate purpose of heterologous protein production.

Integrative expression of heterologous proteins in *M. extorquens*. The mini-Tn*7* integrated expression cassettes containing either *bgl*, *estI*, or *gfp* under control of the P_{mxaF} promoter were successfully integrated, and the genes were expressed in *M. extorquens*. The positive clones producing active recombinant proteins were screened on CHOI medium plates containing the chromogenic substrates 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for β-galactosidase and X-caprylate (5-bromo-4-chloro-3-indolylcaprylate) for esterase. Recombinant GFP was detected by fluorescence microscopy or by spectrofluorophotometry, as mentioned in Materials and Methods.

High-cell-density fermentations were performed with strains BGL, EST, and GFP1. A previously developed fermentation protocol for *M. extorquens* was used (5). This strategy was shown to be very effective for obtaining high biomass yields of *M. extorquens* ATCC 55366, using methanol as a carbon and energy source (7). In this study nitrogen limitation was avoided in order to reduce cellular poly-β-hydroxybutyric acid production and accumulation. PHB accumulation was monitored by microscopy and by chemical means throughout the fed-batch fermentation. The PHB granules accumulated only at the end of the fermentation, after approximately 50 to 60 h, and never accounted for more than 20% of the biomass (data not shown). The growth of recombinant *M. extorquens* carrying either the *bgl*, *estI*, or *gfp* gene showed that the maximum recombinant protein yield was obtained in the late exponential phase (0.9, 1.1, and 1.9 mg per g [dry weight], respectively), and the yield subsequently decreased slightly as the culture reached the early stationary phase (Fig. 3).

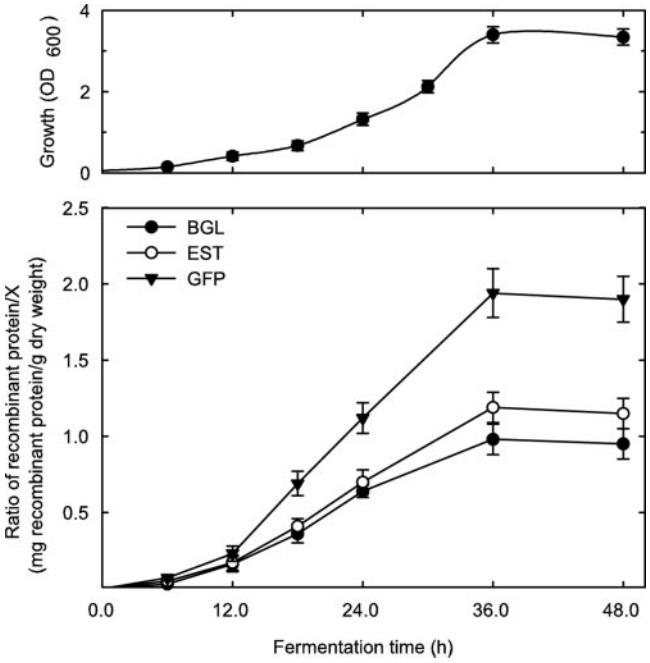

FIG. 3. Specific yields of target proteins produced by recombinant *M. extorquens* for one-copy gene integration into the chromosome under control of P_{mxaF}. BGL, β-galactosidase; EST, esterase. Almost identical growth profiles were obtained for each *M. extorquens* recombinant. All measurements were obtained two or more times, and the deviations are indicated by error bars. OD_{600}, optical density at 600 nm.

Multicopy integration and expression of GFP. In yeasts, multicopy gene integration methods have been used to increase recombinant protein expression levels. However, this approach has not been used commonly in prokaryotes. Typically, during high-cell-density pilot-scale production of recombinant proteins, segregational instability, resulting in partial or complete loss of a plasmid, is common. Furthermore, utilization of antibiotics for selection in bioprocesses can be a regulatory issue, as well as a major problem for downstream processing. However, cloned genes must be stably maintained in the culture in order to obtain robust and productive recombinant cell processes. Since integration of a gene(s) into the chromosome eliminates the segregational instability and copy number variation associated with plasmid-based systems, we integrated one, two, three, and five copies of the *gfp* expression cassette into the chromosome of *M. extorquens*, and the levels of protein expression were evaluated. Expression cassettes GFP1, GFP2, GFP3, and GFP5 were constructed, and each copy had the cassette containing the open reading frame of *gfp* under control of the P_{mxaF} promoter and an RBS, as shown Fig. 1. The expression cassette was cloned into the integration vector pBRI70, which generated four separate integration vectors containing one, two, three, and five copies of the *gfp* gene. A wild-type culture (nontransformed competent cells) of *M. extorquens* was electroporated with these vectors, and colonies were selected on a CHOI medium plate containing kanamycin. One colony from each copy number construct (GFP1, GFP2, GFP3, and GFP5) was selected, and GFP fluorescence was verified by fluorescence microscopy (Fig. 4). Growth of recombinant cultures containing chromosomally integrated multiple copies of the *gfp* gene, GFP1, GFP2, GFP3, and GFP5, resulted in production of 1.9, 2.9, 5.5, and 35.1 mg GFP/g [dry weight], respectively (Fig. 4). In this experiment, the amount of biomass generated from these multicopy integrants at the end of fermentation (~48 h) was essentially identical to the amount observed for the wild-type strain (~40 g [dry weight] per liter) (data not shown), which indicates that gene dosage does not negatively affect the fermentation ability of *M. extorquens*.

The results for the specific yields showed that GFP production was enhanced as additional copies of the *gfp* gene were integrated into the chromosome. The specific yield was proportional to the number of integrated genes. However, when five copies of *gfp* were integrated, proportionality was lost. The specific yield of the five-copy construct was approximately 20-fold higher (35.1 mg/g) than the levels produced by single-copy integrants (Fig. 4), and this accounted for almost 50% of the production yield obtained for the plasmid-based production system. To evaluate the stability of multicopy *gfp* integrated clones, GFP yields were determined once every 30 generations for a total of 120 generations in the absence of antibiotic pressure. The GFP production yields remained constant (data not shown).

In summary, we believe that the multicopy integration sys-

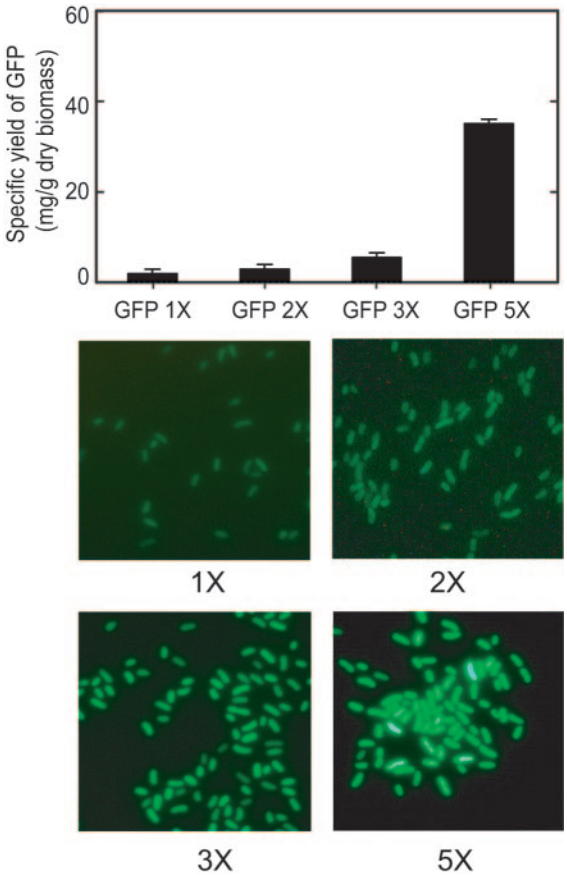

FIG. 4. Specific yield and fluorescence profiles for strains containing one (1×), two (2×), three (3×), and five (5×) copies of the GFP expression cassette integrated into the chromosome. The deviations in the specific yield determinations are based on three or more independent experiments with different positive colonies on the same plate.

tem used in the present study is a useful and efficient tool for stable recombinant protein production in the absence of selective pressure in *M. extorquens*.

Conclusions. In the present study, we established procedures for construction of genetically engineered *M. extorquens* harboring chromosomally integrated expression constructs of heterologous DNA sequences encoding β-galactosidase, esterase, and green fluorescent protein using the mini-Tn*7* integration system. The recombinant *M. extorquens* described here contains the methanol dehydrogenase promoter (P_{mxaF}), which drives efficient production of heterologous proteins in the absence of selective pressure for maintenance of target genes. Indeed, all of the integrated genes tested, *bgl*, *estI*, and *gfp*, were very stably maintained during fermentation in a simple chemically defined mineral salts medium. The stable inheritance of the heterologous genes in *M. extorquens* without selective pressure is of particular interest for "green" bioprocesses, in which the use of antibiotics is not desirable. Furthermore, this integration system allows multicopy integration of genes of interest in *M. extorquens*, resulting in overproduction of recombinant proteins.

Unlike the Tn*5*-based integration system, which randomly integrates DNA fragments into the chromosome, potentially causing insertional inactivation of host genes, the Tn*7*-based system results in stable expression of an integrated gene(s). Tn*7* inserts at a specific intergenic site called the *att*Tn*7* site, a noncoding region of the *M. extorquens* chromosome.

The highest level of GFP expression exhibited by the GFP5 integrated transformants was approximately 20-fold greater than the level exhibited by the single-copy integration transformant (GFP1) and about one-half the level exhibited by transformants harboring the expression cassette on a multicopy plasmid (10 to 30 copies of plasmid per cell). This technology makes stable overproduction of recombinant proteins in *M. extorquens* in the absence of antibiotics possible. Furthermore, utilization of this integration system offers two interesting bioprocess possibilities. First, the modulated expression ability may be used to express potentially difficult proteins or proteins toxic to the host, and second, it might be possible to simultaneously integrate and express different genes of interest in *M. extorquens*. The present study demonstrates that the mini-Tn*7*-mediated integration system is a valuable tool for overproduction of multiple proteins in *M. extorquens* and could have interesting environmental and commercial applications.

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