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(54) Titre : INTEGRATION MULTICOPIE ET EXPRESSION DE GENES HETEROLOGUES DANS UN METHYLBACTERIUM

(54) Title: MULTICOPY-INTEGRATION OF HETEROLOGOUS GENES AND EXPRESSION IN METHYLOBACTERIUM

(57) Abrégé/Abstract:
The integration of genes into methylobacterium, such as M. extorquens ATCC 55366 is disclosed, using a transposon system, preferably the mini-Tn7 transposon system, and under the control of a promoter, such as the strong methanol dehydrogenase promoter (P_{mxaF}). Multicopy integration of genes of interest is also disclosed. The unique and specific attachment site for the Tn7 attachment (attTn7) is identified for M. extorquens.

ABSTRACT

The integration of genes into methylobacterium, such as *M. extorquens* ATCC 55366 is disclosed, using a transposon system, preferably the mini-Tn7 transposon system, and under the control of a promoter, such as the strong methanol dehydrogenase promoter (P_{mxaF}). Multicopy integration of genes of interest is also disclosed. The unique and specific attachment site for the Tn7 attachment (*att*Tn7) is identified for *M. extorquens*.

Multicopy-Integration of Heterologous Genes and Expression in Methylobacterium

FIELD OF THE INVENTION

The invention relates to the transformation of host prokaryotic cells with one or more copies of genes, such that the genes are expressed in the host cells.

BACKGROUND OF THE INVENTION

[01] *Methylobacterium extorquens* is a pink-pigmented facultative methylotroph (PPFM) capable of growth on simple and inexpensive single-carbon compounds, such as methanol, as the sole carbon and energy source in a completely synthetic mineral salt medium (Bourque et al., 1992). These simple requirements combined with the fully automated nutrient non-limiting high cell density fed-batch bioprocesses developed for *M. extorquens* ATCC 55366 (Bourque t al, 1995; Bélanger et al., 2004; Béland et al., 2004), render large-scale *M. extorquens* fermentations very cost-effective. This feature, along with the availability of genetic tools (Marx and Lidstrom, 2001; Figueira et al., 2003; Choi et al., 2004) and abundant genome sequence information and stochiometric models for evaluating its metabolic capabilities (Van Dien and Lidstrom, 2002), makes *M. extorquens* very interesting economically as a host for the production of recombinant proteins. Overexpression in *M. extorquens* of recombinant green fluorescent protein, esterase from *Lactobacillus casei*, catechol 2,3-dioxygenase from *Pseudomonas putida*, enterocin P from *Enterococcus faecium*, and haloalkane dehalogenase from *Xanthobacter autotrophicus* have been described in the literature (Fitzgerald and Lidstrom, 2003; Bélanger et al., 2004; Choi et al., 2004; Gutiérrez et al., 2005).

[02] *Methylobacterium* strains are ubiquitous in nature, inhabiting soils (Sy et al., 2001), sediments and fresh water environments (Rickard et al., 2002). *Methylobacterium* strains have also been detected and isolated from the surface of leaves from almost all plants tested (Romanovskaya 2001; Omer et al., 2004; Koopman and Kutschera, 2005; Gallego et al., 2005). Furthermore, there are a growing number of reports describing favourable interactions between PPFMs and plants. *M. extorquens* has been described as an endophytic microorganism, found in the stem and leaves of citrus plants (Lacava et al., 2004), as a bud endophyte of Scots pine (Pirttila, 2000), and in the rhizosphere of flowering plants (Idris et al., 2004).

[03] Recently, our laboratory has successfully cloned and expressed *in M. extorquens* the cry1Aa gene. The toxin protein encoded by *cry1Aa* is highly active against the spruce budworm, a powerful forest defoliating pest. These observations in view of the ubiquitous nature of *M. extorquens* in the environment and the ease by which the strain can be genetically transformed to over-express recombinant proteins, suggests that this microorganism could be utilized as an attractive delivery system of recombinant biocontrol agents against crop and forest insect pests. However, the utilization of expression systems in the environment or under high cell density fermentation bioprocesses can be problematic. The reason for this being plasmid segrational instability in the absence of selective pressure. The loss of plasmid and consequently the loss of expression of the desired recombinant product occurs gradually but surely under growth conditions where antibiotics can not be used for practical or safety issues. We have shown in a previous study (Bélanger et al., 2004) that approximately 50% of recombinant expression is lost following 15 generations of growth in the absence of selective pressure in *M. extorquens*. Integration of expression cassettes in bacterial chromosomes would not necessitate the use of antibiotic selection for the stable expression of recombinant products. It has been recently shown that the Tn7 system integrates, in a stable and site-specific manner, recombinant DNA fragments into a site of the chromosome called *att*Tn7. This *att*Tn7 is located in the intergenic region downstream of the *glm*S gene in

many Gram negative bacteria such as *E. coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Pseudomanas putida* and *Yersinia pestis* (Craig, 1989; Lichtenstein and Brenner, 1982; Choi et al., 2005).

SUMMARY OF THE INVENTION

[04] A first object of the invention is to provide Methylobacterium cells, and preferably *M. extorquens* cells, capable of expressing genes of interest, preferably without need for antibiotic selective pressures, and preferably without inactivation of host cell genes. This is preferably achieved using the mini-Tn7 transposon system, at least one gene of interest, and a suitable promoter.

[05] A further object of the invention is to provide Methylobacterium cells, and preferably *M. extorquens* cells, capable of expressing multiple copies of genes of interest.

[06] A further object of the invention is to identify the mini Tn7 transposon system integration site of *M. extorquens*.

[07] A first aspect of the invention provides for a vector for the integration of at least one gene into the genetic material of a *Methylobacterium* host cell such that the gene may be expressed by the host cell, the vector comprising a promoter operably linked to said at least one gene, and a transposon system.

[08] A second aspect of the invention provides for a *Methylobacterium* host cell that has been transformed with a vector comprising at least one gene of interest, a promoter operably linked to said at least one gene, and a transposon system.

[09] A further aspect of the invention provides for a method for producing a polypeptide encoded by at least one gene, comprising the step of culturing

Methylobacterium host cells that have been transformed with a vector comprising at least one gene of interest, a promoter operably linked to said at least one gene, and a transposon system.

[010] A further aspect of the invention provides for the use of a *Methylobacterium* host cell that has been transformed with a vector comprising at least one gene of interest, a promoter operably linked to said at least one gene, and a transposon system, as a biopesticide or for tracking the movement and growth of the host cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[011] Figure 1 is a schematic drawing illustrating construction of mini-Tn delivery plasmids.

[012] Figure 2 is a series of southern hybridization blot profiles of BGL, EST and GFP, wherein M; marker, *bgl*; B-galactosidase, *est*I; esterase, *gfp*; green fluourescent protein, and wherein chromosomal DNA was isolated from each recombinant, and digested with *sal*I, then hybridized.

[013] Figure 3 is a schematic illustration showing the mini-Tn7 integration site (*att*Tn7) in *M. extorquens*.

[014] Figure 4 is a schematic illustration showing the PCR identification of mini-Tn7 integration in *M. extorquens*. Verification of transposition events by colony PCR using the primer pairs shown by convergent arrows yields PCR fragments whose sizes are indicated in bp. Lane 1; wild type, lane 2 and 5; *gfp* integrants, lane 3 and 6; *est* integrants, lane 4 and 7; *bgl* integrants, M; marker.

[015] Figure 5 is a graph comparing the yield of target protein from recombinant *M. extorquens* for one copy of gene integrated in the chromosome under the control of the P_{mxaF}.

[016] Figure 6 is is a series of drawings illustrating the integrative expression of multi-copy GFP in *M. extorquens*.

[017] Figure 7 is a graph comparing the specific yield of GFP from recombinant *M. extorquens* for various GFP gene copy numbers integrated in the chromosome and for GFP gene encoded in the plasmid DNA.

DETAILED DESCRIPTION OF THE INVENTION

[018] The invention provides transposon-based single and multicopy expression of recombinant genes in Methylobacterium cells, including *M. extorquens* ATCC 55366. Using a suitable promoter such as a *lac* promoter, a *T5* promoter, a λ promoter, or a methanol dehydrogenase promoter (P_{mxaF}))preferably a methanol dehydrogenase promoter) and a suitable transposon system such as mini-Tn7, high level expression of chromosomally integrated genes in Methylobacterium such as *M. extorquens* ATCC may be obtained. The methodology also permits multicopy integration of genes of interest. Multicopy transformants (1-5 copies) were obtained with significantly increased GFP yield in correlation with the corresponding copy numbers.

[019] Further, the unique and specific attachment site for the Tn7 attachment (*att*Tn7) was identified for *M. extorquens.* Insertion of heterologous genes in this location did not cause any insertional inactivation of host genes.

[020] More specifically according to the invention, we have established procedures for the construction of genetically engineered Methylobacterium, including *M. extorquens*,

harbouring chromosomally integrated expression constructs of heterologous DNA sequences encoding such proteins as β-galactosidase, esterase and green fluorescent using the mini-Tn7 integration system. The recombinant *M. extorquens* described herein, contains the methanol dehydrogenase promoter (P_{mxaF}) which drives the efficient production of heterologous proteins in the absence of selective pressure for the maintenance of target genes. However, other promoters such as *lac* promoter, a *T5* promoter, a λ promoter have been tested with this system and found to be effective. Further, it is expected that other Methylobacterium strains, including methanotrophic strains, having genetic properties highly similar to those of *M. extorquens*, could be similarly transformed with a gene if interest and the mini-Tn7 transposon system. All of the integrated genes tested, *bgl*, *est*I and *gfp* were very stably maintained during fermentation processes in a simple chemically defined mineral salts medium, known as CHOI medium. The stable inheritance of the heterologouus genes in *M. extorquens* without selective pressure is of particular interest for "green" bioprocesses, where the use of antibiotics is not desirable. Furthermore, this integration system allows for multi-copy integration of genes of interest in *M. extorquens* resulting in the over-production of recombinant proteins.

[021] Unlike the Tn5-based integration system which randomly integrates DNA fragments into the chromosome potentially causing insertional inactivation of host genes, the Tn7-based system results in stable expression of integrated gene(s). The Tn7 inserts at a specific intergenic site called *att*Tn7, a non-coding region of *M. extorquens* chromosome.

[022] The highest level of GFP expression produced by the five copies of GFP integrated transformants (GFP5) was approximately 20-fold greater than that produced by the single copy integration transformant (GFP1), and about 50 % that produced by transformants harboring the expression cassette on a multicopy plasmid (10 to 30 copies of plasmid per cell). This invention also renders the stable over production of

recombinant proteins in *M. extorquens* in the absence of antibiotics possible. Furthermore, using the novel integration system, it is contemplated to simultaneously integrate and express different genes of interest in *M. extorquens*. The present invention also demonstrates that the mini-Tn7 mediated integration system is a valuable tool for the overproduction of multiple enzymes in *M. extorquens* and other methylobacterium, and possesses interesting environmental and commercial applications. For example, a gene encoding a labeling protein could be integrated into host methylobacterium cells in order to track the labeled host cells in plant or soil samples. Further, a gene encoding a toxic protein may be integrated into host methylobacterium cells so that the host cells may be applied to plants as a biopesticide.

[023] Construction of an integrative expression vector for *M. extorquens*. A strong homologous promoter (P_{mxaF}), which was derived from the *mxa*F operon of *M. extorquens* (Marx and Lidstrom, 2001) was applied in the construction of the integrative expression vector. In previous studies, we have used this promoter combined with the T7 RBS to express heterologous proteins in *M. extorquens* (Choi et al., 2004). The promoter and RBS cassette was cloned into a mini-Tn7 transposon system to construct the expression plasmid, pBRI70 (Fig. 1A). Chromosomal integration of the mini-Tn7-P_{mxaF}-RBS-genes of interest derived from pBRI70 was achieved in *M. extorquens* by co-electroporation with a helper plasmid pUX-BF13 providing the Tn7 transposition function *in trans* (Bao et al., 1991).

Identification of Tn7 integration site in *M. extorquens*.

[024] It has been recently shown that the Tn7 system integrates, in a stable manner, recombinant DNA fragments into a specific site of the chromosome called *att*Tn7. This *att*Tn7 is located in the intergenic region downstream of the *glm*S gene in many Gram negative bacteria such as *E. coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Pseudomanas putida* and *Yersinia pestis* (Craig, 1989; Lichtenstein and Brenner, 1982; Choi et al., 2005). Southern hybridization analysis of three recombinants confirmed that

Tn7 integration occurred in the chromosomal DNA of *M. extorquens* (Fig. 2). Nucleotide sequence analyses of the of the cloned genes tested (*bgl, est*I and *gfp*) revealed the identify of the Tn7 integration site(s) in *M. extorquens*. Interestingly, all three genes were integrated at the same site of the chromosome. The Tn7 insertion site was located in a 61 bp intergenic region between *glm*S, which encodes the essential glucosamine-6-phosphate synthetase, and *dha*T, which encodes 1,3-propanediol dehydrogenase in the chromosome of *M. extorquens*. Sequence analysis of cloned DNA fragments showed that Tn7 system was inserted between nucleotides 24 and 25 downstream of the *glm*S stop codon (Fig. 3), and this site seem to lie in one of the inverted repeats of the putative *glm*S transcriptional terminator, as had been previously suggested (Choi et al., 2005). To confirm the integration of target genes in the chromosome of *M. extorquens*, colony PCR was carried out by using two sets of Tn7 based primers and two sets of strain specific primers as described in material and methods. The PCR products resulting from different colonies were similar in size as expected, showing that the mini-Tn7 transposon had inserted in one orientation into one specific area of the *M. extorquens* chromosome downstream of *glm*S (Fig. 4).

[025] Taken together, these results indicate that *M. extorquens* has a unique Tn7 attachment site (*att*Tn7), and the insertion does not cause any insertional inactivation of host genes. This *att*Tn7 is a useful site for the integration of recombinant genes in *M. extorquens* with the ultimate purpose of heterologous protein production.

[026] Integrative expression of heterologous proteins in *M. extorquens*. The mini-Tn7 based recombinant plasmids were integrated into the *att*Tn7 locus of *M. extorquens* by electroporation. Electroporation of the *M. extorquens* strain with these constructs in conjunction with the helper plasmid yielded about ~10^3 transformants on selective plates containing 50 μg/ml of kanamycin. The mini-Tn7 integrated expression cassettes containing either *bgl, est*I or *gfp* under the control of P_{mxaF} promoter were successfully integrated and the respective genes were expressed in *M. extorquens*. The positive clones

producing active recombinant proteins were screened on CHOI plates containing chromogenic substrates, X-gal for β-galactosidase, X-caprylate for esterase. Recombinant GFP was detected by fluorescence microscopy or by spectrofluorophotometry as mentioned in the materials and methods.

[027] High-cell-density fermentations were performed with strains BGL, EST, and GFP1. A previously developed fermentation protocol for *M. extorquens* was conducted (Bélanger et al., 2004). This strategy was proven to be very effective in achieving high biomass yields of *M. extorquens* ATCC 55366, using methanol as a carbon source and energy source (Bourque et al., 1995). In this study, the nitrogen source was not limiting, in order to reduce cellular poly-β-hydroxy butyric acid (PHB) production and accumulation. PHB production, a result of substrate limitation, was monitored by microscopy and by chemical means throughout the duration of the fed-batch fermentation. The PHB granules accumulated only at the end of the fermentation, approximately after 50-60 h run time, and never exceeded 20 % of the biomass (data not shown). The growth of recombinant *M. extorquens* carrying either the *bgl*, *est*I or *gfp* genes showed that the maximum recombinant protein yield was reached at late exponential phase (0.9, 1.1 and 1.9 mg per g dry biomass, respectively), and subsequently decreased slightly as the culture reached early stationary phase (Fig. 5).

[028] Multi-copy integration and expression of GFP. In yeasts, multi-copy gene integration methods have been applied for the purpose of increasing recombinant protein expression levels. However, this approach has not been commonly used in prokaryotes. Typically, during high cell density pilot scale production of recombinant proteins, segregational instability resulting in partial or complete loss of plasmids is a common occurrence. 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 achieve robust and productive recombinant cell processes. Since integration of gene(s) into the chromosomes

eliminates the segregational instability and copy number variation associated with plasmid-based systems, we constructed one-, two-, three-, and five-copy integrations of the *gfp* expression cassettes in the chromosome of *M. extorquens*, and protein expression levels were evaluated. Expression cassettes, GFP1, GFP2, GFP3, and GFP5, were made, each copy with the cassette containing the open reading frame of *gfp* under the control of the P_{mxaF} promoter and a RBS as shown Fig.1. The expression cassette was cloned into the integration vector pBRI70, generating four separate integration vectors containing one, two, three and five copies of the *gfp* gene. A wild type culture (non-transformed 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 activity was verified under the fluorescence microscopy (Fig. 6). Growth of recombinant cultures containing chromosomally integrated multi-copies of *gfp* genes (GFP1, GFP2, GFP3 and GFP5) resulted in the production of 1.9, 2.9, 5.5, and 35.1 mg GFP/g dry biomass, respectively (Fig 7). In this experiment, the amount of biomass generated from these multi-copy integrants at the end of fermentation (~48h) was essentially identical to the wild type strain (~40 g dry mass per litre, data not shown), which indicates that gene dosage does not negatively affect the fermentation capability of *M. extorquens*.

[029] The results of the specific yields showed that the GFP production was enhanced as additional copies of the *gfp* gene were integrated in the chromosome. The specific yield was proportional to the number of integrated genes. However, when 5 copies of *gfp* were integrated, proportionality was lost. The specific yield of the five copy construct showed approximately a 20 fold higher yield (35.1 mg/g) than the levels produced by single copy integrants, and this reached almost 50 % of the production yield obtained by the plasmid-based production system (Fig. 7). To evaluate the stability of multi-copy *gfp* integrated clones, GFP yields were determined once every 30 generations for a total of 120 generations in the absence of antibiotic selection. GFP production yields remained constant (data not shown).

[030] Taken all together, we believe that the multi-copy integration system is be a useful and efficient tool for the purpose of stable recombinant protein production in the absence of selective pressure in *M. extorquens*.

Materials and Methods

[031] Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1.

[032] *E. coli* was cultured in Luria Bertani broth (LB) at 37°C. Strain of *M. extorquens* ATCC 55366 was grown in CHOI medium, as previously described (Bourque at al., 1995, Bélanger et al., 2004, the disclosures of which are incorporated herein by reference) and 1 % (v/v) methanol was used as sole carbon source. Both media were solidified by 1.8% agar (Difco) when appropriate. Antibiotics were used for *E. coli* and *M. extorquens* at the following concentrations (in µg/ml): ampicillin, 100; kanamycin (Km), 40; tetracyclin (Tc), 35. The mini-Tn7 recombinant plasmids and the helper plasmid pUX-BF13, were purified from *E. coli*.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
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 copies integrant of the gfp cassette derived from pBRI-gfp2	This study
GFP3	Three copies integrant of the gfp cassette derived from pBRI-gfp3	This study
GFP5	Five copies integrant of the gfp cassette derived from pBRI-gfp5	This study
E. coli strains		
Top10	Strain for cloning and propagating plasmid DNA	Invitrogen Inc.
S-17/λ *pir*	Host strain for pUX-BF13	Bao et al., 1991
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen Inc.
pCR-bgl	pCR2.1-TOPO plasmid containing lactase expression cassette	This study
pCR-est	pCR2.1-TOPO plasmid containing esterase expression cassette	This study
pCR-gfp1	pCR2.1-TOPO plasmid containing one copy of gfp expression cassette	This study
pCR-gfp2	pCR2.1-TOPO plasmid containing two copies of gfp expression cassette	This study
pUC19	Multi-purpose cloning vector	Invitrogen Inc.

Strain or plasmid	Description	Reference or source
pCM-bgl	pCM110 plasmid containing lactase expression cassette	This study
pCM-est	pCM110 plasmid containing esterase expression cassette	This study
pCM-gfp	pCM110 plasmid containing gfp expression cassette	This study
pBK-miniTn7-ΩSm2	pUC19-based delivery plasmid for a miniTn7-Km transposon; Km^r, Sm^r	Koch et al., 2001
pBRI70	pUC19-based delivery plasmid for a miniTn7-Km transposon; Km^r	This study
pBRI-bgl	pBRI70 plasmid containing lactase expression cassette	This study
pBRI-est	pBRI70 plasmid containing esterase expression cassette	This study
pBRI-gfp1	pBRI70 plasmid containing one copy of gfp expression cassette	This study
pBRI-gfp2	pBRI70 plasmid containing two copies of gfp expression cassette	This study
pBRI-gfp3	pBRI70 plasmid containing three copies of gfp expression cassette	This study
pBRI-gfp5	pBRI70 plasmid containing five copies of gfp expression cassette	This study
pUX-BF13	R6K replicon based helper plasmid	Bao et al., 1991
pCESTa	Esterase gene source	Choi et al., 2004
pBGLIII	Lactase gene source	Hung et al., 2001
pQBI63	GFP gene source	Qbiogene Inc.

[033] DNA isolation and manipulations. Plasmids from *E. coli* were prepared with the Qiagen mini plasmid purification kit according to the manufacturer's instructions (Qiagen Inc., Mississauga, ON, Canada). Recombinant plasmids were constructed and agarose gel electrophoresis was performed according to the method of Sambrook and Russell (2000). DNA fragments were isolated from agarose gels by using QIAquick gel extraction system (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 used as recommended by the manufacturer. Electroporation was performed with a Gene-Pulser II electroporation apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada).

[034] Construction of Tn7 vectors. The mini-Tn7 base vector pBRI70 for *M. extorquens* was constructed as follows: the P_{mxaF}-ribosomal binding site (RBS) was amplified from pCESTc (Choi et al., 2004) using primers MDH-F-*Pst*I (5'-GGCTGCAGGTTGACGACAACGGTGCGATG-3') and MDH-R-*Mlu*I (5'-CCGACGCGTATGTATATCTCCTTCTTAAAG-3'). The PCR fragment containing P_{mxaF}-RBS was cloned into pBK-miniTn7-KmΩSm1 (Koch et al., 2001) which was partially digested with PstI/MluI to delete *Sm*R/*Sp*R cassette, to generate pBRI70 (Fig. 1A).

[035] The 2.1 kb fragment carrying the lactase gene (*bgl*) was amplified from pEGIG4 (Hung et al., 2001) using primers bgl-F-*Mlu*I (5'-CACGCGTATG GAACATAGAGCGTTCAAGTG-3') and bgl-R-*Not*I (5'-GCGGCCGCTTACAGCTTGACGACGAGTACGCCG-3'). For the amplification of esterase gene (1.8 kb, *est*I), pCESTa (Choi et al., 2004) was used as a template with primers est-F-*Mlu*I (5'-GACGCGTATGGATCAATCTAAAACAAATC-3') and est-R-*Kpn*I (5'-CGGTACCTTATTTATTTGTAATACCGTCTGC-3').

[036] The 0.8 kb fragment carrying the *gfp* gene was amplified with pCM110-gfp using primers gfp-F-*Mlu*I (5'-GACGCGTATGGCTAGCAAAGGAGAAGAAC-3') and gfp-R-*Afl*II (5'-CCTTAAGTCAGTTGTACAGTTCATCCATGC-3'). All of PCR products were then cloned into pCR2.1-TOPO vector generating pCR-bgl, pCR-est, and pCR-gfp, respectively. The expression cassette was then cloned into the integration vector pBRI70 to form pBRI-bgl, pBRI-est, and pBRI-gfp, respectively (Fig. 1B, C, D). Similarly, three recombinant plasmids pBRI-gfp2, pBRI-gfp3, and pBRI-gfp5, containing two, three and five copies of the *gfp* expression cassette, were constructed with different restriction enzyme sites available in the MCS of pBRI70, respectively. (Fig. 1E-G).

[037] Chromosomal integration of constructs by electroporation. 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 Tn7 cassette into the genomic target site (Bao et al., 1991). 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 thereafter, 1 ml of CHOI medium was added to the cuvette. The cell suspension was transferred to 15 ml tube and incubated at 30°C for 5 h, 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 300-500 transformants per plate were obtained.

[038] Southern blot analysis. Chromosomal DNA was purified from mini-Tn7-Km-target gene transformed *M. extorquens* by using AquaPure Genomic DNA kit (Bio-Rad) as recommended by the manufacturer. DNA samples (~2 µg) were digested with *Sal*I separated electrophoretically on a 0.7% agarose gel, and transferred to a Hybond N membrane (Amersham Biotech. Inc.) according to the instructions of the supplier. The PCR fragment of each target genes (*bgl*, *est*I and *gfp*) were labeled separately with digoxigenin-11-dUTP (DIG) (Roche Applied Science) and used as a probe. After

hybridization at 42°C for 12 h and being washed twice in 2× SSC with 0.2% sodium dodecyl sulfate (SDS) at room temperature, 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 substrate solution (NBT/BCIP) for 5 min.

[039] Determination of Tn7 insertion site in *M. extorquens*. For the verification of Tn7 insertion site, we cloned the DNA flanking the Tn7 insertion site in recombinant *M. extorquens*. To subclone the Tn7 insertion site from recombinant *M. extorquens*, *Sal*I digested chromosomal DNA was cloned into the unique *Sal*I site of the pUC19 vector and transformed into *E. coli* TOP10. Since the P_{mxaF} promoter is not recognized by *E. coli,* a kanamycin resistant clone was selected for sequencing. The sequencing was done by primer walking on purified plasmid DNA. The first primers recognized the vector sequences and both strands were sequenced. The nucleotide sequences of both strands were determined by AmpliTaq 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 sequences were conducted on *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).

[040] The integration of target genes were also confirmed by colony PCR using primers (P_{Tn7L}R; 5'-ATTAGCTTACGACGCTACACCC-3', P_{Tn7R}F; 5'-CACAGCATAACTGGACTGATTTC-3', P_{dhaT}F; 5'-CATCGCGATTGTCGATTCGG-3', and P_{glmS}R; 5'-CTGAAGGAAATCAGCTACATC-3') as shown in Fig. 4.

[041] Gene expression and protein assays. Detection of GFP was carried out by fluorescence microscopy, and quantified by spectrofluorophotometry (Shimadzu RF-5001PC). The measurements were carried out with whole cells resuspended in phosphate-

buffered saline (PBS). The excitation wavelength was 397 nm and the emission wavelength was 510 nm. The esterase activity was determined by a spectrophotometric method using para-nitrophenyl caprylate (pNP-caprylate) as substrate. The rate of hydrolysis of pNP-caprylate at 37°C was measured in 50 mM sodium phosphate buffer (pH 7.0) according to Kademi et al. (1999). The β-galactosidase activity was measured with o-nitrophenol-β-D-galactoside (ONPG) as a substrate and calculated based on pure enzyme from *E. coli* (Sambrook and Russell, 2001). Protein concentration was estimated by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

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

[043] Fed-batch bioreactor experimenst were conducted at pH 7.0 and 30°C. Ammonia solution (30%) was used as both pH control and nitrogen source, and was added as needed during fermentation. A 1% inoculum grown in 1 L culture grown shake flasks was used to inoculate a 20 L fermentor containing 9 L of medium CHOI medium.

[044] On-line measurements of the methanol concentration in the culture medium was performed using a silicone membrane probe (Bioengineering Inc.) coupled with a

semiconductor gas sensor (Bourque et al., 1995). The methanol concentration was controlled by using an the adaptive control algorithm described previously (Bélanger et al., 2004). Methanol was added using a variable-speed peristaltic pump and the methanol concentration was controlled at 1.4 g/l. Off-gas measurements were performed for O_2 (Servomex Paramagnetic analyzer) and CO_2 (Servomex Infrared analyzer) concentrations.

[045] It is understood that the examples described above in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

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WHAT IS CLAIMED IS:

1. A vector for the integration of at least one gene into the genetic material of a *Methylobacterium* host cell such that the gene is expressible by the host cell, the vector comprising a promoter operably linked to said at least one gene, and a mini-Tn7 transposon system, wherein the promoter is a methanol dehydrogenase promoter, a lac promoter, a T5 promoter, or a λ promoter.

2. The vector of claim 1 wherein the host cell is a *Methylobacterium extorquens* cell.

3. The vector of any one of claims 1-2 wherein the promoter is a methanol dehydrogenase promoter.

4. The vector of any one of claims 1-3 comprising one, two, three, four or five copies of the at least one gene.

5. The vector of claim 2 wherein the *Methylobacterium extorquens* host cell is of the species designated as ATCC 55366.

6. The vector of any one of claims 1-5 wherein the at least one gene encodes a toxic protein.

7. The vector of claim 6 wherein the toxic protein is cry1Aa.

8. The vector of any one of claims 1-7 wherein the at least one gene encodes a marker protein.

9. The vector of claim 8 wherein the marker protein is selected from the group consisting of β-galactosidase, esterase, and green fluorescence.

10. A *Methylobacterium* host cell transformed with a vector for the integration of at least one gene into the genetic material of the host cell such that the gene is expressible by the host cell, the vector comprising a promoter operably linked to said at least one gene, and a mini-Tn7 transposon system.

11. The host cell of claim 10 that is a *Methylobacterium extorquens* cell.

12. The host cell of claim 10 or 11 wherein integration of the vector does not cause any insertional inactivation of host genes.

13. The host cell of any one of claims 10-12 wherein the vector is integrated at the attTn7 site.

14. A method for producing a polypeptide encoded by said at least one gene, comprising the step of culturing host cells as claimed in claim 12.

15. A method for using the host cell as claimed in claim 12 as a biopesticide, wherein the at least one gene encodes a pesticide protein, the method comprising the step of applying one or more of the host cells to growing plants.

16. A method for using the host cell as claimed in claim 12 for tracking the movement and growth of the host cell, wherein the at least one gene encodes a marker protein, the method comprising the step of tracking the marker protein.

17. The method of claim 15 wherein the promoter is a methanol dehydrogenase promoter.

Application number/numéro de demande: 02553485

Figures: Fig. 1, 2, 4, 6, 7,

Pages:

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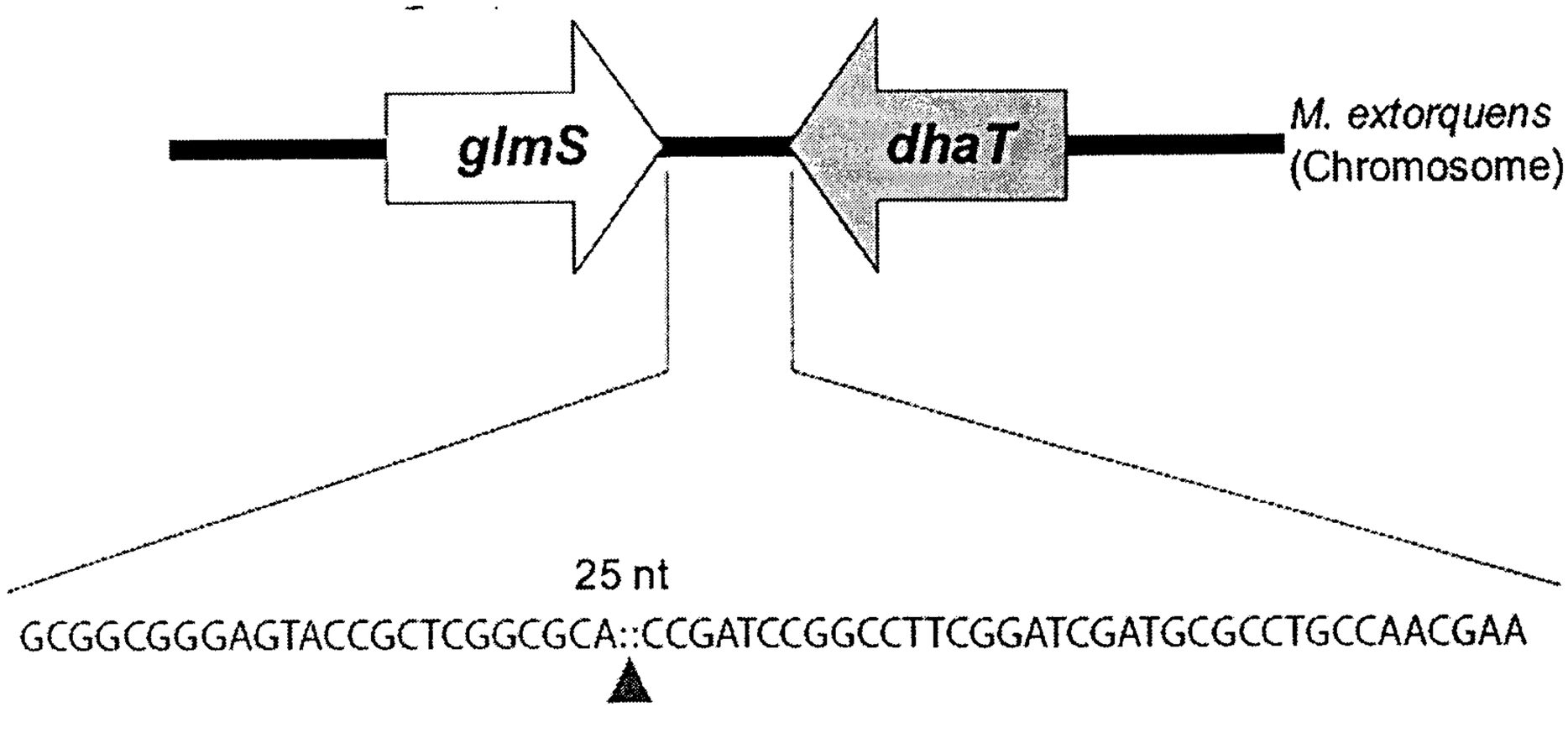
glmS
dhaT
M. extorquens
(Chromosome)
25 nt
GCGGCGGGAGTACCGCTCGGCGCA::CCGATCCGGCCTTCGGATCGATGCGCCTGCCAACGAA

Fig. 3

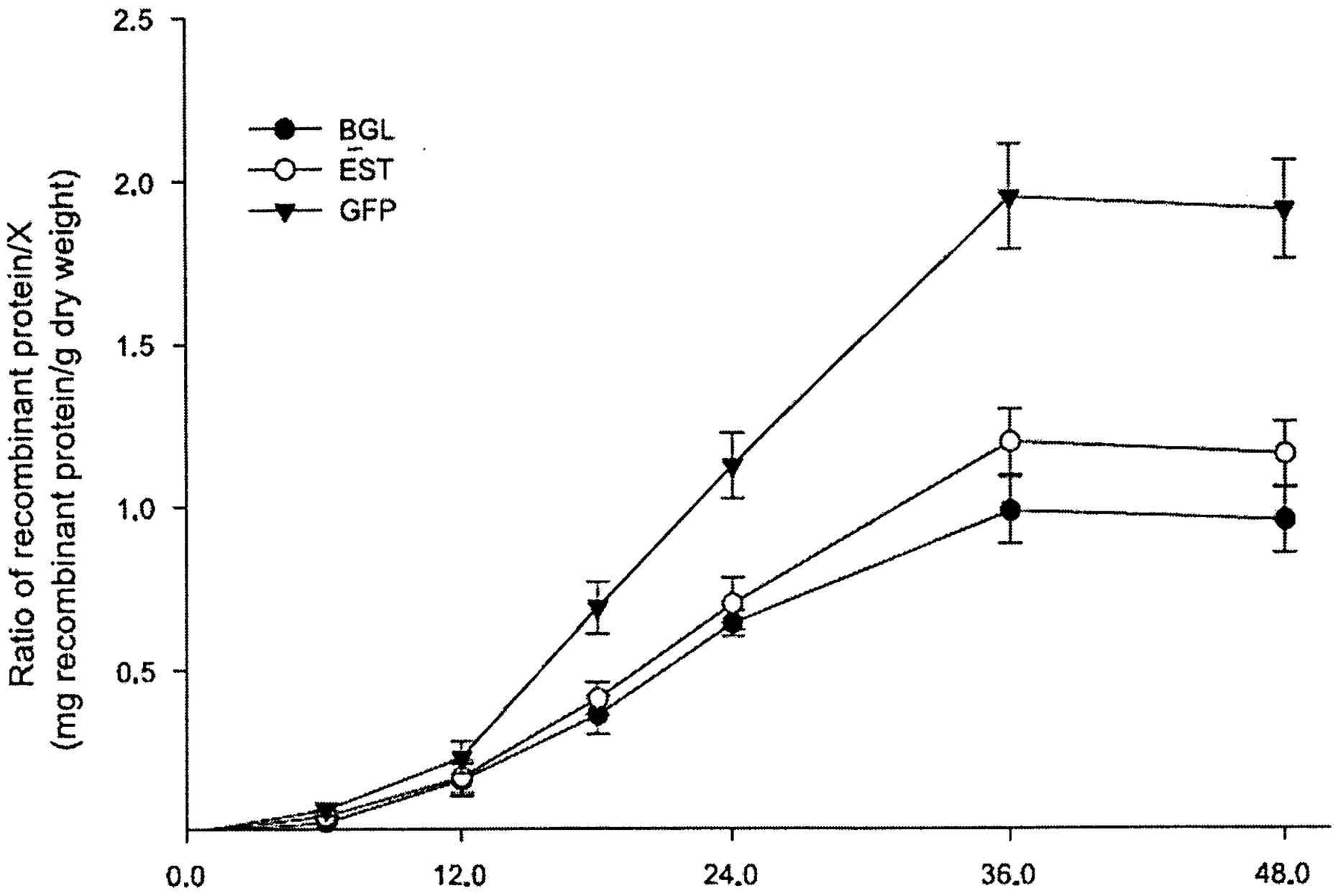
BGL
EST
GFP
Ratio of recombinant protein/X
(mg recombinant protein/g dry weight)
2.5
2.0
1.5
1.0
0.5
0.0
12.0
24.0
36.0
48.0
Fermentation time (h)

Fig. 5