NRC·CNRC Biotechnology Research Institute
CNRC·NRC Institut de recherche en biotechnologie

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Powerful *Methylobacterium*-based technology platform for the production of recombinant proteins and other bioproducts

SUMMARY

Modern biotechnology uses living cells as cell factories for the production of bioproducts. This novel expression platform uses the non-pathogenic bacterium *Methylobacterium extorquens* as a natural source for the production of industrially important compounds and as an alternative to current bacterial expression systems for the production of recombinant proteins. The identification and development of efficient genetic tools and protocols render *M. extorquens* a powerful and efficient biocatalyst for the expression of heterologous value-added peptides and proteins for environmental, biopharmaceutical and industrial applications. Furthermore, *M. extorquens* is a promising microorganism for the commercial production of natural products, including polyhydroxybutyrate (PHB) and the very valuable co-polymer P(HB/HV).

APPLICATIONS

- A versatile and efficient genetic tool box alternative for cloning and expression of heterologous genes and for metabolic engineering in *Methylobacterium* bacteria.
- High-yield production of recombinant peptide/proteins (enzymes, bioactive peptides, bio-insecticides) or other bioproducts, including biopolymers.
- Expression of chromosomally-integrated genes in the absence of antibiotic selective pressure.
- Multicopy expression of integrated heterologous genes in *Methylobacterium* cells allowing over-production of recombinant proteins or simultaneous expression of different genes of interest in the absence of antibiotic selective pressure.
- Expression of potentially difficult proteins or proteins toxic to the host using a genetic switch tightly regulated by the low-cost and non-toxic inducer cumate.
- Inoculation of soil and plants with genetically modified *Methylobacterium* cells for field applications, e.g. the control of pathogens with biopesticide(s) or the tracking (growth, persistence, and spreading) of microorganisms that are introduced into the environment.

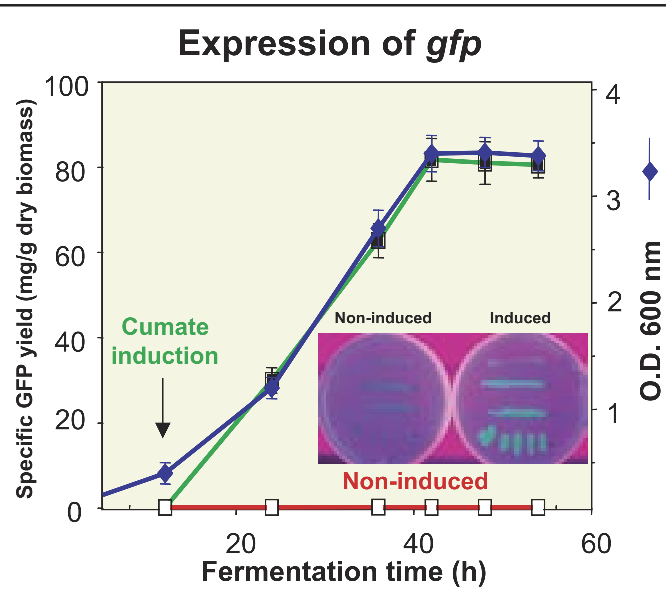
CONCEPT

Methylotrophic bacteria are a diverse group of microorganisms that have the ability to utilize single-carbon compounds (e.g. methanol) as the sole source of carbon and energy. Methanol is a "non-food" and inexpensive renewable substrate that can be derived from various biomasses via a simple process known as gasification. *Methylobacterium* strains are commonly found in soil, as well as in the internal tissues and on the surface of stems and leaves of a wide variety of plants. The bacterium *M. extorquens* is one of the best-characterized methylotrophs, both genetically and physiologically. Hence, a technology platform that takes advantage of the wealth and depth of understanding of this microorganism was developed for the production of recombinant proteins and industrially pertinent natural products. This platform relies on a new isolate of *M. extorquens* (ATCC 55366), obtained from a hydrocarbon-contaminated sandy soil, and the development of specific genetic tools and protocols.

An important step towards the development of this bacterial expression system was achieved with the identification of stable cloning/expression vectors and a strong and efficient promoter, P*mxaF*, from the methanol dehydrogenase gene of *M. extorquens*. Furthermore, a protocol was developed for stable integration of expression cassettes into the *M. extorquens* chromosome. This protocol, based on the application of the Tn7-based broad-range bacterial cloning and expression system for the integration of recombinant genes, was shown to allow stable segregation and expression of heterologous genes without generation of secondary mutations in the host. Single-copy integration and expression of several recombinant proteins under the control of the strong P*mxaF* promoter was successfully achieved. Also, the ability to integrate and express multiple copies of the same gene was shown to enhance the specific yields of recombinant proteins.

The existing promoters for genetic engineering in *M. extorquens* are insufficiently repressible and, hence, unsuitable in applications where regulated expression is paramount. In order to have the ability to control gene expression from the P*mxaF* promoter, the regulatory elements of *Pseudomonas putida* F1 were applied to *M. extorquens*. Tightly regulated expression of several heterologous proteins validated the inducibility afforded by cumate (*p*-isopropylbenzoate) in the *M. extorquens* host strain.

The availability of high cell density fermentation protocols is also critical for the large-scale production of biomolecules. A bioprocess operated in a fed-batch mode in bioreactors was first developed and optimized for endogenous PHB production by *M. extorquens* ATCC 55366. This strategy was shown to be very effective for obtaining high biomass yields and intense PHB production, using methanol as a carbon and energy source as well as a completely defined, minimal culture medium. Bioreactor scale-up was achieved up to 150 liters. A similar approach was successfully applied to high yield production of recombinant proteins.



Cumate regulated expression of the green fluorescent protein (gfp) in *M. extorquens*.

FEATURES AND BENEFITS

Stable expression in the absence of antibiotic selective pressure

A Tn7 transposon-based system for chromosomal integration of recombinant genes was adapted for use in *M. extorquens*. The integration event is site specific and targets a non-coding region of the chromosome, therefore permitting construction of strains that differ only in the nature of the added DNA (isogenic). This strategy overcomes instability problems associated with plasmid-based systems and affords stable recombinant protein production in the absence of selective pressure. This feature is of particular interest for industrial fermentation bioprocesses in which the use of antibiotics is not desirable in view of economic, downstream processing, and regulatory safety concerns.

Production of toxic or unstable proteins

Regulatory elements of *P. putida* F1 were adapted to modulate the expression of heterologous genes in *M. extorquens*. In this system, recombinant protein expression from the strong *PmxaF* is under the control of cumate, a nontoxic and inexpensive inducer. Cumate was shown to tightly regulate the expression of several recombinant proteins in *M. extorquens*. Such ability is of great interest for the expression of toxic or capricious proteins requiring low but controlled expression levels or in metabolic flux and pathway engineering applications. Alternatively, the proficiency of the Tn7 transposon-based system to integrate and express multiple copies of a gene of interest also confers the ability to modulate the expression of potentially difficult proteins or proteins toxic to the host.

High-yield and cost-effective scalable production

A fully automated and nutrient non-limiting high cell density fed-batch bioprocess was developed for fermentations employing *M. extorquens* ATCC 55366. This robust and scalable process takes advantage of the simple growth requirements of *M. extorquens* using an optimized minimal culture medium with methanol as the sole carbon and energy source. The use of methanol as sole substrate for fermentation processes has many advantages. Methanol is miscible with water and is easily separated from fermentation products and biomass. It can also be considered a renewable non-food substrate, as it could be derived from woody materials or from natural gas obtained after anaerobic digestion of organic substances. Consequently, the *M. extorquens* technology platform allows reproducible, cost-effective, and high yield production of heterologous proteins and endogenously-produced biomolecules, such as PHB.

Potential for *in situ* environmental applications

Members of the genus *Methylobacterium* have been described as being ubiquitous endophytic microorganisms found in the stem, leaves, and rhizosphere of some plants and participating in a myriad of favorable interactions with nature. This ability, combined with the ease with which *M. extorquens* can be genetically transformed to over-express recombinant proteins, suggests that this microorganism could be utilized as an attractive delivery system to improve crop performance (e.g., drought tolerance, growth promotion, and disease resistance). For instance, the *cry1Aa* gene, which encodes for a highly active toxic protein against the spruce budworm, a forest defoliating pest, was successfully cloned and expressed in *M. extorquens*. Alternatively, a gene encoding for a labeling protein could be integrated into *M. extorquens* for tracking its movement and growth in plant or soil samples.

Convenient and flexible molecular tool box for heterologous protein expression

The *M. extorquens* technology platform offers an extensive set of complementary genetic tools. These tools, which comprise a set of efficient cloning vectors and promoters, a chromosomal integration strategy, a tightly-regulated inducible gene expression system, and a high-cell-density fed-batch fermentation process, could form the basis of a flexible and convenient microbial research tool box for gene cloning and expression. *M. extorquens* obviates *E.coli* drawbacks such as inclusion body formation, high level of endotoxins, reduced yields with high G-C content genes, and glucose consumption. As such, it offers an alternative for the production of heterologous peptides/proteins to support R&D in various biological fields, including proteomics.

Highly versatile cell factory in the bioproducts area

A technology platform based on the methylotrophic bacterium *M. extorquens* strain ATCC 55366 was developed for biosynthesis of recombinant proteins/peptides and industrially important chemicals. The potential of this platform for commercial applications was demonstrated by the overexpression of a variety of heterologous proteins at yields ranging from milligrams to grams per litre, including the green fluorescent protein (*gfp*), the *Bifidobacterium infantis beta*-galactosidase (*bgl*), an esterase (*est*) from *Lactobacillus casei*, enterocin P from *Enterococcus faecium*, and *cry1Aa* from *Bacillus thuringiensis*. The potential of *M. extorquens* as a cell factory is further enhanced by its inherent ability to produce natural products of great importance, including coenzyme Q10, vitamin B12, and PHB and the co-polymer P(HB/HV), both promising candidates for the manufacture of biodegradable thermoplastics.

PROTECTION STATUS

Cumate regulated heterologous recombinant protein expression in *Methylobacterium extorquens* (NRC no. 11725); Multicopy-integration of heterologous genes and expression in *Methylobacterium* (NRC no. 11724); Methylotrophic bacterium for the production of recombinant proteins and other products (NRC no. 11404, 11327); *Methylobacterium extorquens* microorganism useful for the preparation of poly-beta-hydroxybutyric acid polymers (NRC no. 10230).

CONTACTS

Daniel Desmarteaux
Tel.: (514) 496-5300
Business Development Officer
E-mail: daniel.desmarteaux@cnrc-nrc.gc.ca

Yves Quenneville
Tel.: (514) 496-8507
Business Development Officer
E-mail: yves.quenneville@cnrc-nrc.gc.ca

Dr. Carlos B. Míguez
Tel.: (514) 496-6280
Microbial & Enzymatic Technology Group
E-mail: carlos.miguez@cnrc-nrc.gc.ca

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