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A new shuttle vector for gene expression in biopolymer-producing *Ralstonia eutropha*☆

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ABSTRACT

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) is a fascinating microorganism with a great scientific importance and an immense commercial potential. A new genetic transformation system for the organism would greatly facilitate the biological study and molecular engineering of this organism. We report here a versatile gene expression method for the genetic engineering of *R. eutropha*. This method, based on a simplified electroporation protocol, uses a recombinant plasmid, pBS29-P2, containing a *Pseudomonas syringae* promoter (P2) and two antibiotic-resistance markers (i.e., genes coding for kanamycin (Km)- and tetracycline (Tc)-resistance). Using this method, we successfully achieved transformation of wild-type *R. eutropha* and its poly(hydroxyalkanoate)-negative mutant, *R. eutropha* PHB^{-}4, with various pBS29-P2-based recombinants. A transformation frequency as high as 4×10^3 Km-resistance colonies/μg DNA was obtained per electroporation experiment. We further demonstrated the successful expression of a heterologous gene coding for green-fluorescent-protein by fluorescence measurement. In addition, our results indicated the expression of a truncated but active *Streptomyces coelicolor* α-galactosidase in *R. eutropha*.

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1. Introduction

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) is a versatile microorganism of high interest in research and industrial communities for many reasons. Since its discovery in the 1960s as having the ability to accumulate a large quantity of intracellularly sequestered poly(3-hydroxybutyric acid) (PHB), which was subsequently shown to be an important biodegradable polymeric substance, it has become one of the most studied organisms in the field of poly(hydroxyalkanoate) (PHA) research because of the immense potential of PHAs to replace polymers and plastics derived from nonrenewable feedstocks (Reinecke and Steinbüchel, 2008). The field of PHA research has benefited from the identification of the mutant *R. eutropha* strain (i.e., PHB^{-}4), which is incapable of synthesizing PHB (Schlegel et al., 1970) and has proven invaluable in the genetic complementation studies involving PHA biosynthesis. *R. eutropha* is also intensively studied for use in the fields of bioenergy (Goldet et al., 2008; and ref. in Pohlmann et al., 2006) and bioremediation (Paul et al., 2005; Harms and Wick, 2006) because of its ability to oxidize H_2 gas and to degrade various halide-containing aromatic compounds that are potential pollutants. The importance of this organism is attested to by the undertaking and completion of two separate genomic sequencing initiatives (Pohlmann et al., 2006; DOE Joint Genome Institute, 2005).

Because of its great importance, genetic engineering efforts abound to study the biology of *R. eutropha* and to augment the metabolic capabilities of *R. eutropha* for specific applications. Early undertakings relied on conjugative transfer of genetic elements to yield transformed cells. Commonly used conjugative transfer agents are the mobilizable plasmid, pBBR1MCS (Kovach et al., 1994), and its numerous derivatives (see, for example, Dennis et al., 1998). Subsequently, the more straightforward electroporation method was adopted to supplant the rather involving conjugative transfer approach. For example, Park et al. (1995a,b) developed an electroporation protocol using pKT230 vector to transform *R. eutropha*. However, this and other similar vectors have not been characterized with respect to the presence or functionality of a promoter sequence, which is critical for the proper expression of a cloned gene. In the present study, we report the development of a new expression shuttle vector containing a functional promoter for use in the transformation of *R. eutropha* and the important PHB^{-}4 mutant by a straightforward electroporation protocol. We also demonstrate the successful expression of heterologous genes using this method. The added advantage of multiple antibiotic selection markers of the vector is also discussed.

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2. Material and methods

2.1. Bacteria, growth conditions, and plasmids

Transformationally competent *Escherichia coli* DH5α and α-Select cells used in routine DNA subcloning and plasmid maintenance were obtained from Invitrogen (Carlsbad, CA) and Bioline (Randolph, MA), respectively. *Ralstonia eutropha* wild-type and the PHB^{-}4 mutant were kind gifts from Prof. A. Steinbüchel (University of Münster, Münster, Germany). A truncated gene encoding the N-terminal catalytic domain of a *Streptomyces coelicolor* α-galactosidase (herein referred to as dAG or d-α-*gal*) was obtained as cloned in the form of a plasmid pET28-dAG (Kondoh et al., 2005) from Prof. H. Kobayashi (Koibuchi College of Agriculture and Nutrition, Koibuchi, Japan). The source of the green-fluorescent-protein gene (*gfp*) is the plasmid pBS12, which was constructed by one of the authors (Swingle et al., 2008). The construction of plasmids pBS29-P2-gfp and pBS29-P2-dAG, which are used in the present study to demonstrate the promoter activity of P2 sequence in the *Ralstonia* host background, had been described (Solaiman and Swingle, 2010). Recombinant plasmid pKK-II 89-6 was generated by cloning the class III poly(hydroxyalkanoates) synthase genes, *phaE* and *phaC*, of *Allochromatium vinosum* ATCC 35206 into a broad-host-range plasmid, pBHR1, as described (Aneja et al., 2009). Bacterial strains were maintained on Luria medium (LB; 1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl). Growth of *E. coli* was performed at 37 °C and *Ralstonia* at 30 °C unless otherwise specified. Agar media were prepared by including 1.2–1.5% w/v of agar to the corresponding liquid broths before autoclaving. Kanamycin (Km, 35 μg/ml for *E. coli* and 200 μg/ml for *R. eutropha*), tetracycline (Tc, 12 μg/ml except indicated otherwise) and carbenicillin (Cb, 50 μg/ml) were added to the growth media as needed.

2.1.1. Molecular biological procedures

Transformation of *Ralstonia* wild-type and PHB^{-}4 strains was accomplished by an electroporation protocol described in Aneja et al. (2009). Plasmids in *E. coli* transformants were isolated by using a GenElute Miniplasmid Kit (Sigma, St. Louis, MO, USA) for subsequent restriction analysis. A slightly modified alkaline lysis method (Birnboim and Doly, 1979), in which the initial cell suspension was subjected to lysozyme treatment (2 mg/ml at 37 °C for 15 min) and the final step of ethanol precipitation was carried out overnight in a −20 °C freezer, was used to screen the plasmids of the *Ralstonia* transformants.

2.1.2. Assays for green-fluorescent-protein expression and α-galactosidase activity

Both assay procedures were as described previously (Solaiman and Swingle, 2010). Briefly, *Ralstonia* transformants were cultured for 2 days at 30 °C and 200 rpm rotary shaking in 3.5 ml of LB medium supplemented with kanamycin. The cultures were centrifuged to obtain the clear supernatant for fluorescence measurement. Fluorescence intensity was measured at $\lambda_{emission}$ of 527 nm and $\lambda_{excitation}$ of 481 nm. Two clones from each strain or transformant were separately cultured and used in the experiment in order to assess the reproducibility of the results. Three fluorescence readings were recorded and averaged for each sample. The standard errors for the averages of these fluorescence measurements were less than 1% for each sample. For α-galactosidase activity assay, *Ralstonia* transformants were cultured for 2 days (at 30 °C and 200 rpm rotary shaking) in LB + Km medium (50 ml) in 125-ml Erlenmeyer flasks. Cells were harvested by centrifugation (Sorvall RC-5B centrifuge, SS-34 rotor, 6500 rpm, 15 min, 4 °C) and washed once with 5 ml of HEPES buffer (0.1 M, pH 7). The cell pellet was resuspended in 1 ml of HEPES buffer in a 15-ml COREX centrifuge tube, and the resultant cell suspension was subjected to sonication (Model W-385 Sonicator with the Output Control set at 5; Heat Systems-Ultrasonics, Inc., Farmingdale, NY; fitted with a standard tapered microtip) to disrupt the cells. Each sample was sonicated for 5 times at 10 s each time, with cooling in ice-water between sonication. We also tested the effects of lysozyme and lysostaphin to aid in cell lysis. Lysozyme (1 mg/ml; Sigma) and lysostaphin (0.5 mg/ml; Sigma) were added to the 1 ml-cell suspension, and the mixture was incubated at 37 °C for 1 h, and sonicated as described. The cell extract was obtained by centrifugation (Sorvall RC-5 centrifuge, SS-34 rotor, 9000 rpm, 30 min, 4 °C) of the sonicated cell supension. Alpha-galactosidase activity of the cell extract was assayed essentially as described (Kondoh et al., 2005). Briefly, 0.1 ml of cell extract was added to 0.9 ml of 1 mM *p*-nitrophenyl-α-D-galacto-pyranoside (*p*-NP-α-gal) in 0.1 M HEPES (pH 7) in a test tube. After a 20-min incubation at 40 °C, 1 ml of 0.2 M Na_2CO_3 solution was added to stop the reaction. The nitrophenol moiety released by the α-galactosidase was monitored by absorbance measurement at 400 nm.

3. Results and discussion

3.1. Electroporative transformation of R. eutropha and mutant PHB^{-}4

We had previously reported the construction of a shuttle expression vector, pBS29-P2 (Fig. 1A), and its use in expressing heterologous genes in *Pseudomonas* species (Solaiman and Swingle, 2010). Briefly, a chromosomal DNA sequence (P2; genomic coordinate 6143993–6144829 in GenBank AE016853 entry) of *Pseudomonas syringae* pv.

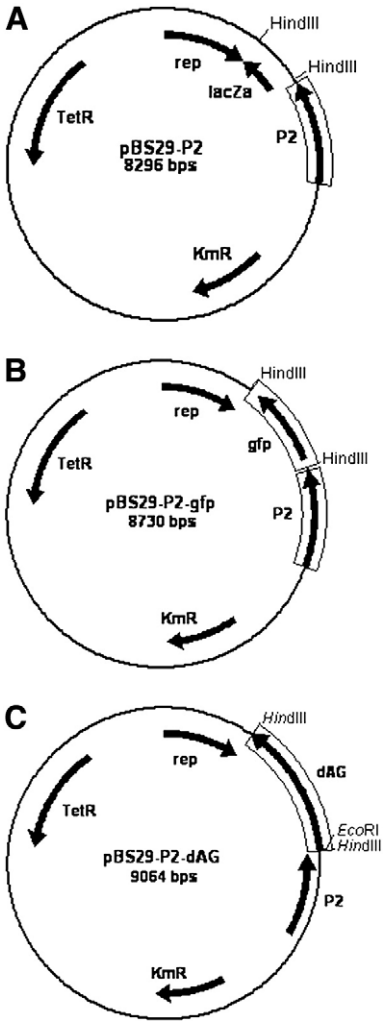

Fig. 1. Map of plasmids. *P2*, promoter P2; *TetR* and *KmR*, tetracycline- and kanamycin-resistance markers, respectively; *lacZα*, alpha-complementation fragment of *lacZ* gene; *rep*, gene coding for a replication protein; *gfp*, green fluorescent protein; *dAG*, a truncated but active α-galactosidase gene of *Streptomyces coelicolor*.