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Construction of expression vectors for protein production in *Gluconobacter oxydans*

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ABSTRACT

The characteristic ability of *Gluconobacter oxydans* to incompletely oxidize numerous sugars, sugar acids, polyols, and alcohols has been exploited in several biotechnological processes, for example vitamin C production. The genome sequence of *G. oxydans* 621H is known but molecular tools are needed for the characterization of putative proteins and for the improvement of industrial strains by heterologous and homologous gene expression. To this end, promoter regions for the genes encoding *G. oxydans* ribosomal proteins L35 and L13 were introduced into the broad-host-range plasmid pBBR1MCS-2 to construct two new expression vectors for gene expression in *Gluconobacter* spp. These vectors were named pBBR1p264 and pBBR1p452, respectively, and have many advantages over current vectors for *Gluconobacter* spp. The *uid*A gene encoding β-D-glucuronidase was inserted downstream of the promoter regions and these promoter–reporter fusions were used to assess relative promoter strength. The constructs displayed distinct promoter strengths and strong (pBBR1p264), moderate (pBBR1p452) and weak (pBBR1MCS-2 carrying the intrinsic lac promoter) promoters were identified.

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1. Introduction

Gluconobacter oxydans is a member of the Gram-negative Acetobacteraceae that perform rapid incomplete oxidation of many sugars, sugar acids polyols and alcohols (De Ley et al., 1984). This feature of *Gluconobacter* spp. is currently used in several biotechnological processes, for example the production of vitamin C (Adachi et al., 2003) and the production of the antidiabetic drug miglitol (Schedel, 2000). The genome sequence of *G. oxydans* 621H is known and it was found to contain over 70 uncharacterized oxidoreductases (Prust et al., 2005). Some of these proteins have been functionally overproduced heterologously in *Escherichia coli* and characterized (Cheng et al., 2005; Saichana et al., 2007; Schweiger et al., 2008, 2010). However, protein production in the natural host is preferred, especially for membrane-bound dehydrogenases, which are of major importance for industrial bioconversions as the products are excreted into the medium to almost quantitative yields. *Gluconobacter* spp. and the related *Acetobacter* have attracted increasing attention from industry because of their large oxidative potential and relative ease of product recovery from the medium. Improvements of industrially important strains have led to increased productions of, for example, acetic acid (Beppu, 1993) and 5-keto-D-gluconate (Merfort et al., 2006a,b). Vectors derived from broad-host-range plasmids (Condon et al., 1991; Merfort et al., 2006a,b; Schleyer et al., 2008) or cryptic plasmids (Creavan et al., 1994; Fukaya et al., 1985; Okumura et al., 1985; Saito et al., 1997; Shinjoh and Hoshino, 1995; Tonouchi et al., 1994, 2003; Trček et al., 2000; Zhang et al., 2010) have been reported in *G. oxydans* and *Acetobacter*. However, many of these vectors have limited use and/or effectiveness. Therefore, effective vectors are needed for functional analysis of genes/proteins and for further strain improvements. To take advantage of the biotechnological potential of the *G. oxydans* genome, two expression vectors were constructed from the broad-host-range plasmid pBBR1MCS-2. Putative constitutive promoters for the genes *gox0264* and *gox0452* encoding ribosomal proteins L35 and L13, respectively, were introduced into this vector and the relative promoter strength of each promoter was investigated.

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Sigma–Aldrich (Munich, Germany) or Carl Roth GmbH (Karlsruhe, Germany). Restriction endonucleases, T4 ligase, *Taq* DNA polymerase and PCR reagents were purchased from Fermentas (St. Leon-Rot, Germany). Phusion DNA polymerase was purchased from New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were synthesized by Eurofins (Ebersberg, Germany).

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Table 1
Strains, plasmids and primers for vector construction and cloning.

Strain or plasmid or primer	Description or primer sequence	Source or added site[a]
Strain		
E. coli DH5α	F⁻, *sup*E44, Δ*lac*U169, *ϕ*80, *lacZ*ΔM15, *end*A1, *hsd*R17 (r_K-m_K+), *thi*-1, λ⁻, *rec*A1, *gyr*A96, *rel*A1	Hanahan (1983)
G. oxydans Δ*hsd*R	Δ*hsd*R derivative of *G. oxydans* 621H (deletion of *gox2567*)	S. Bringer-Meyer, Forschungszentrum Jülich GmbH
Plasmid		
pASK-IBA5plus	*Amp*^{R}	IBA GmbH
pASK-*gox2181*	pASK-IBA5plus derivative expressing *gox2181*	This study
pBBR1MCS-2	*mod*, *rep*, *Kn*^{R}, *lacZ*	Kovach et al. (1995)
pBBR1p264	pBBR1MCS-2 derivative containing the 5′-UTR of *gox0264*	This study
pBBR1p452	pBBR1MCS-2 derivative containing the 5′-UTR of *gox0452*	This study
pBBR1MCS-*uid*A	pBBR1MCS-2 expressing *uid*A	This study
pBBR1p264-*uid*A	pBBR1p264 derivative expressing *uid*A	This study
pBBR1p264-*gox2181*	pBBR1p264 derivative expressing *gox2181*	This study
pBBR1p452-*uid*A	pBBR1p452 derivative expressing *uid*A	This study
pBBR1p452-*gox2181*	pBBR1p452 derivative expressing *gox2181*	This study
Primer		
upGox264_f	ATGGTAGAGCTCGTTGCGCCTGAATGAGAGG	*Sac*I
upGox264_r	GACGTATTTAAATTCGGTCTCCCTCGCCGTAA	*Dra*I
upGox452_f	ATAGAGCTCGGCTTCGTGGTGAACGCC	*Sac*I
upGox452_r	CTGTGCTTTAAATAGTGACATTCCAGCTTGGG	*Dra*I
UidAE1f	CCGGGAATTCGAGGAGTCCCTTATGTTACG	*Eco*RI
UidAE1r	AATTCTCGAGTCATTGTTTGCCTCCCTGCT	*Xho*I
Gox2181_f	ATGGTAGGTCTCAGCGCCTACATGGAAAAACTCCGCCTCGA	*Bsa*I
Gox2181_r	ATGGTAGGTCTCATATCACCAGACGGTGAAGCCCGCATC	*Bsa*I
pASK_f	TGGGAATTCGAGTTATTTTACCACTCCCT	*Eco*RI
pASK_r	CGCAGTGCGGTAAACG	
pBBR1_f	ACTCACTATAGGGCGAATTG	
pBBR1_r	CCCAGGCTTTACACTTTATG	
RT*gap*_f	TCCGACTTCAACCATGACAA	
RT*gap*_r	GTTGTCGTACCACGAGCAGA	
RT*uid*A_f	TGCTGTCGGCTTTAACCTCT	
RT*uid*A_r	CTGTAAGTGCGCTTGCTGAG	

[a] Restriction endonuclease sites underlined.

2.2. Bacterial strains and culture conditions

G. oxydans Δ*hsd*R (Table 1) was grown in yeast mannitol (YM) medium consisting of 0.6% yeast extract and 2% D-mannitol. Electrocompetent cells were prepared from *G. oxydans* Δ*hsd*R grown in electroporation medium (8.0% D-mannitol, 0.05% glycerol, 1.5% yeast extract, 0.25% $MgSO_4 \cdot 7H_2O$, 0.15% $CaCl_2$, pH 6). *Gluconobacter* were grown at 30 °C and 200 rpm. *E. coli* DH5α was grown in lysogeny broth (LB). Addition of 50 μg/ml kanamycin or 25 μg/ml kanamycin was used for plasmid maintenance in *Gluconobacter* and *E. coli*, respectively.

2.3. Standard molecular biology techniques

Routine molecular biological techniques were done according to Sambrook et al. (1989). Transformation of *G. oxydans* was done by electroporation essentially as described by Mostafa et al. (2002). Briefly, cells were harvested by centrifugation (4500 × *g*, 15 min at 4 °C) between an $OD_{600\,nm}$ 0.8 and 1.0, washed three times with ½ volume 1 mM HEPES and resuspended in 250 μl HEPES. Glycerol was added to approximately 20% and electrocompetent cells were flash frozen in 65 μl aliquots and stored at −70 °C. Electroporation was performed by a modified method of Mostafa et al. (2002) with a Gene Pulser II (Bio-Rad, München, Germany) set at 2.0 kV, 25 μF and the pulse controller set at 200 Ω with a 1 mm electrode distance.

2.4. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was done on a 12.5% (w/v) slab gel as described by Laemmli (1970) with a 5% (w/v) polyacrylamide stacking gel. Samples were diluted in sample loading buffer (2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 50% [v/v] glycerol, 20% [v/v] collecting buffer pH 6.8, 0.001% [w/v] bromophenol blue) and boiled for 10 min prior to application. Molecular mass was calculated by comparison to a molecular mass standard (Fermentas, St. Leon-Rot, Germany). Proteins were visualized by silver stain (Blum et al., 1987).

2.5. Vector and promoter fusion construction

The 5′-UTR (5′-untranslated region) of *gox0264* and *gox0452* were amplified by PCR with oligonucleotides containing extended 5′ *Sac*I/*Dra*I or *Sac*I/*Sma*I restriction endonuclease sites (Table 1). The amplified products were ligated into similarly cut pBBR1MCS-2 to produce the expression vectors pBBR1p264 and pBBR1p452 (Fig. 1). The *uid*A gene, including the native ribosomal binding site, was amplified from *E. coli* DH5α with extended 5′ *Eco*RI/*Xho*I endonuclease sites and cloned into similarly digested pBBR1p264 and pBBR1p452 to produce promoter–reporter fusion constructs pBBR1p264-*uid*A and pBBR1p452-*uid*A. A control vector pBBR1MCS-*uid*A was created similarly from pBBR1MCS-2 by cloning *uid*A behind the lac promoter. The promoter fusions were transformed into *E. coli* DH5α and transformants were screened for proper insertion with PCR using sequencing primers (Table 1). Positive transformants were sequenced by the StarSEQ GmbH (Mainz, Germany). The promoter–reporter fusions were transformed into *G. oxydans* Δ*hsd*R by electroporation.

2.6. Cloning of protein production constructs

The *gox2181* gene from *G. oxydans* was cloned into pASK-IBA5 (IBA GmbH, Göttingen, Germany) as described in Schweiger et al. (2010). The gene was amplified from pASK-IBA5 with the addition of a 5′ *Eco*RI restriction endonuclease site. The amplicon, containing a 5′ Strep-tag II and a plasmid encoded ribosomal binding site, was cut with endonucleases *Eco*RI/*Hind*III and ligated into similarly cut pBBR1p264 and pBBR1p452 to produce pBBR1p264-*gox2181*

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