



Production of a periplasmic trehalase in *Gluconobacter oxydans* and growth on trehalose



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ABSTRACT

Gluconobacter strains are specialized in the incomplete oxidation of monosaccharides. In contrast, growth and product formation from disaccharides is either very low or impossible. A pathway that allows growth on trehalose was rationally designed to broaden the substrate range of *Gluconobacter oxydans*. Expression vectors containing different signal sequences and the gene encoding alkaline phosphatase, *phoA*, from *Escherichia coli* were constructed. The signal peptide that exhibited the strongest periplasmic PhoA activity was used to generate a *G. oxydans* strain able to utilize the model disaccharide trehalose as a carbon and energy source by expressing the periplasmic trehalase TreA from *E. coli*. The strain had a doubling time of 3.7 h and reached a final optical density of 1.7 when trehalose was used as a growth substrate. In comparison, the wild-type harboring the empty vector and the strain expressing *treA* without a signal sequence grew slowly to a final OD of only 0.15. The trehalose concentration in *treA* expressing cultures decreased continuously during the exponential growth phase indicating that the substrate was hydrolyzed to glucose by TreA. In contrast to the wild-type growing on glucose, the *treA* expression strain mainly formed acetate and 5-ketogluconate as end products rather than gluconate.

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1. Introduction

Gluconobacter oxydans is a Gram-negative rod-shaped aerobic *alpha* proteobacterium, belonging to the *Acetobacteraceae* (De Ley et al., 1984). The organism has the ability to grow at high sugar concentrations and low pH values, which reflects the adaptation to its native habitat of fruits and flowers (Sievers and Swings, 2005; Gupta et al., 2001; Deppenmeier and Ehrenreich, 2009). The organism is able to catalyze the regioselective and incomplete oxidation of many alcohols and monosaccharides at high oxidation rates. The reactions are catalyzed by pyrroloquinoline quinone (PQQ) or flavin-dependent dehydrogenases that are located in the cytoplasmic membrane with their active sites oriented toward the periplasm. Substrates can diffuse into the periplasm via porins in the outer membrane where they are incompletely oxidized and the products are released into the medium. The electrons derived from the oxidative reactions are channeled into the respiratory chain and finally transferred to O₂ to form H₂O. These features give *Gluconobacter* strains great biotechnological potential and industrial importance. For example, *Gluconobacter* strains are used for

the production L-sorbose in the course of vitamin c synthesis (Reichstein and Grüssner, 1934) and for the synthesis of 1-amino-L-sorbose as a precursor for antidiabetic drugs (Schedel, 2000). Industrial application also includes the production of gluconic acid (e.g. Bionade© fermentation), ketogluconates and dihydroxyacetone (a tanning agent). Furthermore, enzymes and whole cells are used in sensor systems for the detection of alcohols, sugars and polyols (Macauley et al., 2001; Reshetilov et al., 1996). Another biotechnological application of *Gluconobacter* strains is the production of flavoring ingredients used as food additives that are synthesized from aliphatic and aromatic alcohols (Rabenhorst et al., 2001).

Gluconobacter strains specialize in the oxidation of monosaccharides, whereas growth and product formation from disaccharides is either very low (e.g. sucrose) or impossible (e.g. lactose). Therefore, as the first step in design of a *G. oxydans* strain that can hydrolyze disaccharides or oligosaccharides, we investigated the possibility of conferring the ability to use trehalose as substrate through metabolic engineering. To our knowledge transporter proteins for the uptake of disaccharides are not known and are likely absent in *Gluconobacter* sp. (Prust et al., 2005). Production of periplasmic hydrolases in *G. oxydans* would enable the cleavage of disaccharide and oligosaccharides to monosaccharides that could function as substrates for membrane-bound

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Table 1
Strains, plasmids and primers.

Strain, plasmid, primer	Description or sequence	Source or restriction site (underlined)
<i>Strain</i>		
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80dIacZ Δ M15, Δ (<i>lacZYA-argF</i>) U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk ⁻ , mk ⁺), <i>phoA</i> , <i>supE44</i> , λ -, <i>thi-1</i> , <i>gyrA96</i> , <i>rel A1</i>	Hanahan (1983), DSM 6897
<i>G. oxydans</i> 621H Δ hsdR	Δ hsdR (Δ gox2567) derivative of <i>G. oxydans</i> 621 H	S. Bringer-Meyer, Forschungszentrum Jülich GmbH
<i>Plasmid</i>		
pBBR1MCS-2	OriT, mob, Kan ^R , OriRep, MCS	Kovach et al. (1994)
pBBR1p264-SP <i>pelB</i> -Streplong	pBBR1MCS-2 derivative containing the 5'-UTR of <i>gox0264</i> , an elongated streptag and the signal sequence of <i>pelB</i>	Zeiser et al. (2014)
pBBR1p264-SP <i>pelB</i> - <i>phoA</i>	pBBR1p264-SP <i>pelB</i> -Streplong derivative expressing <i>phoA</i> (<i>b0383</i>) from <i>E. coli</i>	This study
pBBR1p264-SPgox0605- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative with the signal sequence of <i>gox0605</i>	This study
pBBR1p264-SPgox0748- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative with the signal sequence of <i>gox0748</i>	This study
pBBR1p264-SPgox0854- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative with the signal sequence of <i>gox0854</i>	This study
pBBR1p264-SPgox0952- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative with the signal sequence of <i>gox0952</i>	This study
pBBR1p264-SPgox2077- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative with the signal sequence of <i>gox2077</i>	This study
pBBR1p264-SPgox2219- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative with the signal sequence of <i>gox2219</i>	This study
pBBR1p264-NoSP- <i>phoA</i>	pBBR1p264-SP <i>pelB</i> - <i>phoA</i> derivative expressing <i>phoA</i> without any signal sequence	This study
pBBR1p264-SP <i>pelB</i> - <i>treA</i>	pBBR1p264-SP <i>pelB</i> -Streplong derivative expressing <i>treA</i> from <i>E. coli</i> (<i>b1197</i>)	This study
pBBR1p264-NoSP- <i>treA</i>	pBBR1p264-SP <i>pelB</i> - <i>treA</i> derivative expressing <i>treA</i> without any signal sequence	This study
<i>Primer</i>		
SPgox0605.fw	CTCTCGAGGAGAGACTGCATGAGA	XhoI
SPgox0605.rev	CTGTACGTAGGCATTGGCGAGCGTC	SnaBI
SPgox0748.fw	AACCTCGAGGAGGTTTCAATGTCCAG	XhoI
SPgox0748.rev	GGTTACGTACGCTGTGCCACACCGG	SnaBI
SPgox0854.fw	CTTACTCGAGAGGGGAACCGTCTGATGCGC	XhoI
SPgox0854.rev	ACTCTACGTAGGCATTGGCAGCCAGCGGTG	SnaBI
SPgox0952.fw	ATCCTCGAGGAGGCTAAGCATGAGC	XhoI
SPgox0952.rev	CAGTACGTAGGCAGAGCGGACGACACA	SnaBI
SPgox2077.fw	TCCACTCGAGGGGACCGTCCATGAAATC	XhoI
SPgox2077.rev	TGTTACGTATGCAAATGCTGCGCATAC	SnaBI
SPgox2219.fw	GGTCTCGAGGAGGAATACACTTATGAAAACG	XhoI
SPgox2219.rev	GTTTACGTATGCATGAGCCTGACC	SnaBI
SP <i>pelB</i> .fw	ATCGCTCGAGGTTTAACCTTAAAGAAGGAG	XhoI
SP <i>pelB</i> .rev	ATCGTACGTAGGCCATCGCCGGCTGGGC	SnaBI
<i>phoA</i> .fw	AATTTACGTACCTGTTCTGAAAACCGGG	SnaBI
<i>phoA</i> .rev	TTAAGGCGCGCCTTTCAGCCCCAGAGCGGCT	AscI
<i>phoA</i> -NoSP.fw	TCCGACTCGAGGTTCTGAAAACCGGGCT	XhoI
<i>phoA</i> -NoSP.rev	ATTAGGCGCGCCTTTCAGCCCCAGAG	AscI
<i>treA</i> .fw	GGGTGACTAGTAAGAAACACCGGTAACAC	SnaBI
<i>treA</i> .rev	AGTAGGCGCGCCAGGTGTGGTTGTGCCT	AscI
<i>treA</i> -NoSP.fw	GGTGCTCGAGGAAGAAACACCGGTAACAC	XhoI

dehydrogenases and could serve as precursors for biosynthesis. For strain improvement, basic expression vectors with constitutive promoter of different strengths were already constructed from the broad-host-range plasmid pBBR1MCS-2 (Kovach et al., 1995) and the upstream regions of ribosomal protein encoding genes *gox0264* and *gox0452* (Kallnik et al., 2010). These expression vectors are suitable for the overproduction of cytoplasmic and membrane-bound proteins (Meier et al., 2012). However, the ability to export proteins into the periplasmic space is still lacking. To obtain a suitable and biotechnologically valuable expression system for the specific production of proteins in the periplasm of *G. oxydans*, the translocation efficiency of several different signal peptides was investigated. The signal peptide that exhibited the strongest periplasmic activity of PhoA was used to generate a *G. oxydans* strain able to utilize the model disaccharide trehalose as a carbon and energy source. The production and translocation of the *Escherichia coli* trehalose hydrolase, TreA, into the periplasm of *G. oxydans* was investigated.

2. Materials and methods

2.1. Materials

Reagents and chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany) and Sigma-Aldrich (Munich, Germany). T4

ligase, restriction endonucleases, Taq DNA polymerase and PCR reagents were obtained from Fermentas (St. Leon-Rot, Germany). Phusion DNA polymerase was purchased from New England Biolabs (Frankfurt am Main, Germany). All oligonucleotides were synthesized by Eurofins (Ebersberg, Germany).

2.2. Strains and culture conditions

E. coli DH5 α was grown in lysogeny broth (Miller, 1972). *G. oxydans* Δ hsdR (Table 1) was used for all experiments and was grown in yeast mannitol (YM) medium consisting of 2% D-mannitol and 0.6% yeast extract. For growth experiments with trehalose, 50 mM trehalose replaced mannitol. Kanamycin was added to 50 μ g/ml for plasmid maintenance.

2.3. Construction of signal peptide and reporter containing expression systems

All routine molecular techniques were performed as described in Sambrook et al. (1989). Genomic DNA from *G. oxydans* and *E. coli* was isolated by CTAB extraction (Ausubel, 1994) and used as a template for the PCR. Transformation of *G. oxydans* was done via electroporation as described by Kallnik et al. (2010). The alkaline phosphatase encoding gene *phoA* was amplified from *E. coli*

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