Characterization and inactivation of the membrane-bound polyol dehydrogenase in *Gluconobacter oxydans* DSM 7145 reveals a role in meso-erythritol oxidation

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The growth of *Gluconobacter oxydans* DSM 7145 on meso-erythritol is characterized by two stages: in the first stage, meso-erythritol is oxidized almost stoichiometrically to L-erythrulose according to the Bertrand–Hudson rule. The second phase is distinguished from the first phase by a global metabolic change from membrane-bound meso-erythritol oxidation to L-erythrulose assimilation with concomitant accumulation of acetic acid. The membrane-associated erythritol-oxidizing enzyme was found to be encoded by a gene homologous to *sldA* known from other species of acetic acid bacteria. Disruption of this gene in the genome of *G. oxydans* DSM 7145 revealed that the membrane-bound polyol dehydrogenase not only oxidizes meso-erythritol but also has a broader substrate spectrum which includes C3–C6 polyols and D-gluconate and supports growth on these substrates. Cultivation of *G. oxydans* DSM 7145 on different substrates indicated that expression of the polyol dehydrogenase was not regulated, implying that the production of biomass of *G. oxydans* to be used as whole-cell biocatalysts in the biotechnological conversion of meso-erythritol to L-erythrulose, which is used as a tanning agent in the cosmetics industry, can be conveniently carried out with glucose as the growth substrate.

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INTRODUCTION

Gluconobacter oxydans is one of the most important organisms in industrial biotechnology because of its ability to incompletely oxidize various sugars, sugar alcohols and sugar acids (De Muynck *et al.*, 2007). The oxidation reactions are catalysed by membrane-bound dehydrogenases linked to the respiratory chain (Matsushita *et al.*, 1994). As the active sites of these dehydrogenases face the periplasm, substrates need not be transported into the cytoplasm and the corresponding oxidation products accumulate in the culture medium. Moreover, most substrates are oxidized regio- and stereoselectively, yielding enantiomerically pure products at high yields, thus enabling efficient whole-cell biocatalytic production processes that are superior to chemical substrate conversion

Abbreviations: DCIP, 2,6-dichlorophenol indophenol; Gm, gentamicin; Km, kanamycin; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone; RLM RACE, RNA ligase-mediated rapid amplification of cDNA ends.

A supplementary figure, showing an alignment of the amino acid sequences of the SldB and SldA proteins of *G. oxydans* ATCC 621H (Gox0855) and *G. oxydans* DSM 7145, is available with the online version of this paper.

due to the absence of complicated and expensive protective chemistry reactions (Schweiger *et al.*, 2007). Examples of the industrial application of *Gluconobacter* spp. are the production of L-sorbose, an intermediate of vitamin C synthesis (Adachi *et al.*, 2003) and the conversion of 1-amino-D-sorbitol to 6-amino-L-sorbose for the synthesis of the antidiabetic drug Miglitol (Schedel, 2000).

All of the biochemically studied membrane-bound dehydrogenases are either quinoproteins, containing pyrroloquinoline quinone (PQQ) as prosthetic group, or flavoproteins, containing a covalently bound FAD molecule (Davidson, 2004; Matsushita *et al.*, 1994). Additionally, some enzymes contain a haem *c* moiety, such as the quinohaemoprotein alcohol dehydrogenase (Kondo & Horinouchi, 1997). The reducing equivalents derived from the oxidation reactions are transferred via ubiquinone to a chinol oxidase of the bo₃ type (Matsushita *et al.*, 1987), with concomitant build-up of an electrochemical proton gradient across the cytoplasmic membrane. Therefore, the incomplete oxidation reactions provide energy for growth, while the cytoplasmic, NAD(P)-dependent dehydrogenases present in *Gluconobacter* spp. most likely provide precursors for biosynthetic pathways (Matsushita *et al.*, 1994).

The membrane-bound quinoprotein glycerol dehydrogenase of the *Gluconobacter suboxydans* strains IFO 3257 and IFO 3255, which contains PQQ as prosthetic group, has been shown to be a versatile enzyme, catalysing the oxidation of the sugar acid D-gluconate and the sugar alcohols glycerol, D-arabitol, D-mannitol and D-sorbitol (Matsushita *et al.*, 2003). It is composed of the large SldA subunit (79.6 kDa), which contains the active site, and the small SldB subunit (14 kDa), which is essential for activity development and seems to act as a molecular chaperone (Shinjoh *et al.*, 2002). The enzyme catalyses the regio- and stereoselective oxidation of its substrates according to the Bertrand–Hudson rule, which states that polyols with a *cis* arrangement of two secondary hydroxyl groups in D-configuration to the primary alcohol group (D-erythro configuration) are oxidized to the corresponding ketoses (Kulhanek, 1989). Also among these substrates is the tetritol *meso*-erythritol, which is oxidized to the corresponding ketose L-erythrulose (Matsushita *et al.*, 2003). L-Erythrulose is commonly used as tanning agent and constitutes a precursor for the production of L-erythrose, a derivative of which, dideoxyerythrose, has shown some anti-HIV activity (Tschamber *et al.*, 1996).

Despite the fact that *Gluconobacter oxydans* is used for the industrial conversion of *meso*-erythritol to L-erythrulose, the utilization and oxidation of erythritol by this species has not been studied in detail. In this study we have shown that the membrane-bound glycerol dehydrogenase catalyses *meso*-erythritol oxidation in *G. oxydans* DSM 7145, and that both *meso*-erythritol and L-erythrulose are utilized by *G. oxydans* DSM 7145. Additionally, the genetic organization of the genes *sldA* and *sldB* was investigated.

METHODS

Bacterial strains and growth conditions. All strains and plasmids and their relevant characteristics are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani medium (Sambrook *et al.*, 1989) at 37 °C and 150 r.p.m. *G. oxydans* DSM 7145 and its derivatives were routinely grown in a complex medium containing 5 g yeast extract l⁻¹, 3 g tryptone l⁻¹ and 50 mM D-mannitol (pH 6.0). When appropriate, D-mannitol was replaced by 50 mM D-glucose. Growth was recorded by measuring the OD₆₀₀ in a UV-Vis spectrophotometer (Amersham). Growth tests were performed in complex medium, using cultures pre-grown on the respective substrate as inoculum. The following substrates were tested at final concentrations of 50 mM: D-mannitol, D-glucose, glycerol and *meso*-erythritol. A mixture of D-glucose and D-mannitol was added at a final concentration of 25 mM each. Antibiotics were used at the following concentrations: kanamycin (Km; 50 µg ml⁻¹), cefoxitin (50 µg ml⁻¹) and gentamicin (Gm) 50 µg ml⁻¹ (*G. oxydans*) or 10 µg ml⁻¹ (*E. coli*).

Resting cell experiments. *G. oxydans* DSM 7145 was grown to stationary phase (OD₆₀₀ ~1.1) in 300 ml complex medium with 50 mM D-glucose. The cells were harvested by centrifugation at 10 000 r.p.m. and subsequently washed twice with 10 mM sodium phosphate buffer (pH 6.2). The pellets of 150 ml culture were resuspended in 100 ml 10 mM sodium phosphate buffer (pH 6.2) containing either 50 or 200 mM *meso*-erythritol, transferred to a 1 l flask and incubated at 30 °C, 150 r.p.m. for 24 h.

DNA manipulations. Routine molecular biology experiments were carried out with commonly used standard procedures. Genomic DNA from *G. oxydans* DSM 7145 for PCRs and Southern blot analysis was prepared using the MasterPure DNA purification kit (Epicentre). *Pfu* DNA polymerase (Fermentas) was used for amplification of fragments that were subsequently cloned. *Taq* DNA polymerase (Fermentas) was employed in test reactions. Primers used in this study are listed in Table 2 and were deduced from the whole genome sequence of *G. oxydans* ATCC 621H (Prust *et al.*, 2005). Restriction enzymes (Fermentas), DNA ligase and antarctic phosphatase (New England Biolabs) were all used according to the manufacturers' instructions.

Table 1. Strains used and constructed in this study

Abbreviations: Ap^R, ampicillin resistant; Gm^R, gentamicin resistant; Km^R, kanamycin resistant; Strep^R, streptomycin resistant.

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
XL-1 blue	<i>recA</i> ⁻ , <i>thi</i> , <i>hsdR1</i> , <i>supE44</i> , <i>relA1</i> , <i>lacF'</i> , <i>proAB</i> , <i>lacIq</i> , <i>lacZΔM15</i> , Tn10[Tet]	Bullock <i>et al.</i> (1987)
S17-1	Sm ^R , Tp ^R , <i>mod</i> ⁺ , <i>res</i> ⁻ , <i>thi</i> , <i>pro</i> , <i>recA</i> ⁻ , <i>hsdR17</i> , RP4-Tc::Mu-Km::Tn7	Simon <i>et al.</i> (1983)
Solopack	<i>lacZΔM15</i> , <i>endA</i> , <i>recA</i> ⁻ , Strep ^R , <i>cre</i>	Stratagene
<i>G. oxydans</i> strains		
DSM 7145	Type strain	Mason & Claus (1980)
<i>sldA</i> ::Gm	DSM 7145 derivative, Gm ^R inserted in <i>sldA</i> gene	This study
Plasmids		
pSC-A	Cloning vector, Ap ^R	Stratagene
pBBR-1 MCS2	Broad-host-range vector, Km ^R	Kovach <i>et al.</i> (1995)
pBBR-1 MSC5	Broad-host-range vector, Gm ^R	Kovach <i>et al.</i> (1995)
pK19mobsacB	Gene replacement vector, Km ^R , <i>sacB</i>	Schäfer <i>et al.</i> (1994)
pSC-A: <i>sldAB</i>	<i>sldBA</i> genes in pSC-A, Ap ^R	This study
pSC-A: <i>sldA</i> 'B':Genta	<i>sldBA</i> genes in pSC-A, <i>sldA</i> gene disrupted by Gm ^R gene, Km ^R , Gm ^R	This study
pJV5	pK19mobsacB derivative containing <i>sldBA</i> genes, <i>sldA</i> gene disrupted by Gm ^R , Km ^R , Gm ^R	This study
pJV8	pBBR-1 MCS2 derivative containing <i>sldB</i> promoter fused to <i>sldA</i> gene, Km ^R	This study

Table 2. Primers used in this study

Primer	Sequence (5'→3')	Source or reference
<i>sldB_for</i>	GACTTACCGTCCGGACTTTCAGTTCTG	This work
<i>sldB_rev</i>	CGTGGCACAGGCAACGGTGGCGAGAAG	This work
<i>sldA_for</i>	GTCTGATGCGCAGATCCCATCTTCTCG	This work
<i>sldA_rev</i>	CGCTTTCTCAGCCCTTGTGATCAGGCAGTG	This work
<i>sldA_w1</i>	GCTTTCGTCTATCACACCGGTAGC	This work
<i>sldA_w2</i>	GTAGAGGCCGTACCCACCTTGATC	This work
<i>sldA_w3</i>	GATACCGTAAGGCGTGCCGTC	This work
<i>sldA_w4</i>	ACGTAGACGAGGCCGACGCGATT	This work
<i>sldA_w5</i>	GACAATGCGCTGGGCGCTCGTC	This work
<i>sldA_w6</i>	GACCAGCTCGTAACCCGCAAG	This work
<i>sldB_v_for</i>	ATGCCGCGGTCAGTTACAACAACAC	This work
<i>sldA_v_rev</i>	GCCGGAAGAACCTGAAGGATGACGGGAG	This work
<i>sldA_p_for</i>	GCGTCCATGGACGGCAGCCTTA	This work
<i>sldB_prom_for</i>	CATTTGAATTTGTTGAAAGTGCAACC	This work
<i>sldB_prom_rev</i>	ATCTGCGCATTGGTGAAAGCCTCCAGAACTGAAAG	This work
<i>sldA_compl_for</i>	GCTTTCACCAATGCGCAGATCCCATCTTCTCGCCA	This work
<i>sldA_compl_rev</i>	GCTTCTCAGCCCTTGTGATCAGG	This work
<i>sldB_RC_out</i>	GCTCGAAGGAATTGCCGCTGCCAT	This work
<i>Genta_1_for</i>	CCATGGACGCACACCGTGGAACGGATGA	This work
<i>Genta_2_rev</i>	CCATGGACGCCCTGGGGCCGTTGAATC	This work
5' RACE_outer	GCTGATGGCGATGAATGAACACTG	Ambion
5' RACE_inner	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	Ambion

Nucleotide sequence analysis. Sequencing was performed using an ABI Prism 3730XL sequencer (Applied Biosystems). The nucleotide sequences were determined on both DNA strands. Sequence data were analysed using the STADEN software package (Bonfield *et al.*, 1995). Homology searches and alignment analysis were performed using BLAST (Altschul *et al.*, 1997) and CLUSTAL W (Larkin *et al.*, 2007), respectively. Potential signal peptides and transmembrane helices were predicted with the SignalP 3.0 software (Bendtsen *et al.*, 2004) and TMHMM software, respectively.

Southern blot analysis. Two micrograms of genomic DNA was digested with *KpnI* and electrophoresed on an 0.8% agarose gel, transferred to a positively charged Hybond XL membrane (Amersham Biosciences), and hybridized with a probe generated by amplification with primers *sldA_p_for/sldA_rev* (Table 2) and plasmid pJV5 (Table 1) as template. Labelling and detection were carried out using the Biotin DecaLabel DNA Labeling and Biotin Chromogenic Detection kit (both Fermentas) according to the manufacturer's protocols.

Transcription analysis. *G. oxydans* DSM 7145 total RNA was prepared from 40 ml cells grown to late exponential phase on complex medium with either D-glucose or D-glucose/D-mannitol using the RNeasy Midi kit (Qiagen) according to the manufacturer's protocol with the following modifications: cells were resuspended in 200 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and opened by mechanical disruption, and the frozen powder was then instantly resuspended in 4 ml of the RLT (RNeasy lysis) buffer provided. The prepared RNA was then treated with DNase (Roche) followed by phenol/chloroform extraction and subsequent precipitation with 0.3 M sodium acetate and 70% (v/v) ethanol. RNA integrity was verified using a Bioanalyser 2100 (Agilent).

RT-PCR. RT-PCRs were performed using the OneStep RT-PCR kit (Qiagen) according to the instructions of the manufacturer and using 800 ng total RNA as template. To verify co-transcription of the *sldBA*

genes, primers *sldB_for* and *sldA_w2* (Table 2) were used. Primer *sldB_for* binds at the 5' end of the *sldB* gene, while primer *sldA_w2* binds in the opposite direction starting at basepair 504 of the *sldA* gene. A polycistronic mRNA was expected to yield a product of 924 bp.

Mapping of the *sldBA* transcription start site. RNA ligase-mediated rapid amplification of cDNA ends (RLM RACE) was used to determine the transcription start site of the *sldBA* genes, using the protocol of the manufacturer (FirstChoice RLM-RACE kit, Ambion). Two independent replicates were carried out, including a control without tobacco acid pyrophosphatase treatment. The generated cDNA was subsequently used in a nested PCR with *Taq* DNA polymerase (Fermentas) and the primers listed in Table 2. After column purification (Qiagen), products were cloned using a TOPO TA cloning kit (Invitrogen). A total of four colonies were analysed, and the insert was sequenced in both directions.

Construction of a *G. oxydans* DSM 7145 *sldA* mutant. To construct a *G. oxydans* DSM 7145 *sldA* mutant by allelic replacement, the *sldBA* genes were amplified with forward primer *sldB_for* and reverse primer *sldA_rev* (Table 2) from genomic DNA of *G. oxydans* DSM 7145 and cloned into plasmid pSC-A (Stratagene). The central 1.5 kb *NcoI* fragment from the *sldBA* insert in pSC-A: *sldAB* was then replaced with a 1 kb Gm-resistance cassette which had previously been amplified from plasmid pBBR-1 MCS5 (Kovach *et al.*, 1995) using primers *Genta_1_for* and *Genta_2_rev* (Table 2). The correct insertion of the Gm-resistance cassette in the same transcriptional direction as the *sldB* gene was confirmed by restriction endonuclease digestion. The insert was finally cloned between the *HindIII* and *EcoRI* sites of plasmid pK19mobsacB (Schäfer *et al.*, 1994), resulting in plasmid pJV5. Plasmid pJV5 was transformed into *E. coli* S17-1 (Simon *et al.*, 1983) and mobilized into *G. oxydans* DSM 7145. Single-crossover mutants were selected by plating on complex medium with Km. Double-crossover mutants were identified based on their Km^r/Gm^r phenotype.

Construction of a complementation vector. To complement the *sldA* mutant, a 143 bp fragment containing the putative promoter region of the *sldBA* genes was amplified using primers *sldB_prom_for* and *sldB_prom_rev* (Table 2) and genomic DNA as template. Similarly, the *sldA* structural gene (2.2 kb) was amplified using primers *sldA_compl_for* and *sldA_compl_rev* (Table 2). Both fragments were fused in a long flanking homology PCR (Wach, 1996), and the obtained 2.3 kb fragment was subcloned into vector pSC-A (Stratagene). The insert was excised from pSC-A: *sldAB_prom* by *EcoRI* digestion and finally cloned between the *EcoRI* sites of pBBR-1 MCS2 (Kovach *et al.*, 1995), resulting in plasmid pJV8. The construct was transferred into *G. oxydans* DSM 7145 by biparental mating using *E. coli* S17-1.

Preparation of membrane fractions. *G. oxydans* DSM 7145 and *G. oxydans* DSM 7145 *sldA::Gm* were grown on 300 ml complex medium with D-glucose or D-glucose/D-mannitol to late exponential phase. Cultures were harvested by centrifugation (9000 r.p.m., 15 min), washed twice with 10 mM Tris/HCl buffer (pH 7.2) and resuspended in 10 ml of the same buffer per gram wet weight. Cells were lysed by twofold passage through a French press, and after centrifugation (11 000 r.p.m., 20 min) to remove intact cells, the supernatant (crude extract) was centrifuged at 100 000 g for 2 h. The resulting pellet was suspended in 10 mM Tris/HCl buffer (pH 7.2), centrifuged again (100 000 g, 60 min), resuspended in the same buffer and designated membrane fraction.

Protein determination. Protein concentrations were determined with a Bradford assay with the modification for determining protein concentrations of membrane fractions described by Pestov & Rydström (2007). BSA was used as standard.

Dehydrogenase assay. Dehydrogenase activities were assayed spectrophotometrically by following the reduction of 2,6-dichlorophenol indophenol (DCIP) at 578 nm using phenazine methosulfate (PMS) as a redox mediator. The reaction mixture contained enzyme solution, MacIlvaine buffer (pH 5.0), 25 mM substrate, 0.67 mM PMS and 0.1 mM DCIP. Reactions were started by addition of substrate and followed in a Varian Model 100 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μ mol substrate min^{-1} . The specific activity was calculated using a millimolar extinction coefficient for DCIP of $5.016 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 5.0 and 578 nm.

Substrate/product analyses. Samples from cultures for the determination of *meso*-erythritol and L-erythrulose were obtained by centrifugation at 13 000 r.p.m. for 10 min and subsequent freezing at -20°C . *meso*-Erythritol and L-erythrulose were quantified by HPLC using a CarboPac MA1 or PA1 column (Dionex). Sample volumes of 100 μ l were injected and the temperature was kept constant at 25°C . Each run was carried out isocratically using 480 mM (CarboPac MA1) or 250 mM (CarboPac PA1) NaOH elution buffer. *meso*-Erythritol and L-erythrulose peaks were detected electrochemically after approximately 1.8 and 2.5 min, respectively (CarboPac MA1 10.8 and 26.8 min, respectively). Acetate was assayed enzymically as described by Bergmeier (1974) with an acetate detection kit (R-Biopharm) according to the instructions of the manufacturer.

RESULTS

Growth of *G. oxydans* DSM 7145 on *meso*-erythritol-containing medium

As shown in Fig. 1, *G. oxydans* DSM 7145 grew at 30°C on complex medium supplemented with 50 mM *meso*-

erythritol at a growth rate of 0.51 h^{-1} and to a final optical density of 1.82. HPLC analysis of culture supernatants revealed that *meso*-erythritol was nearly quantitatively oxidized to L-erythrulose within the first 8 h of the experiment, which coincided with an increase in optical density from 0.05 to 0.8. However, the extracellular L-erythrulose concentration decreased by approximately 60 % in a second growth phase from t_8 to t_{24} , where a further increase in optical density was observed (0.8–1.82). To identify reaction products secreted in the second growth phase, culture supernatants were analysed by HPLC and GC-MS analysis. These analyses identified acetic acid as the main reaction product (data not shown), which was also confirmed enzymically. Acetic acid starts to accumulate as the supplied *meso*-erythritol is completely oxidized. While the extracellular L-erythrulose concentra-

Fig. 1. Growth and substrate/product analysis of *G. oxydans* DSM 7145 on complex medium supplemented with 50 mM *meso*-erythritol. A representative experiment is shown. (a) Growth of the culture was followed spectrophotometrically after starting the culture at OD₆₀₀ 0.07. (b) Substrate consumption and product formation during growth were quantified as described in Methods. Symbols: ■, *meso*-erythritol concentration (mM); □, L-erythrulose concentration (mM); ♦, acetic acid concentration (mM).

tion decreased by 30 mM, only 10 mM acetic acid was detectable in the supernatant (Fig. 1b).

Resting cells of *G. oxydans* DSM 7145 rapidly converted 50 mM *meso*-erythritol to L-erythrulose within 6 h with a yield of 93 % (Fig. 2). Interestingly, neither a decrease in extracellular L-erythrulose concentration nor an accumulation of acetic acid was observed in the resting cell experiments. Taken together, these results indicate that L-erythrulose consumption and acetic acid production can be attributed to growth of *G. oxydans* DSM 7145 on L-erythrulose.

Sequence analysis of *sldBA* from *G. oxydans* DSM 7145

As *meso*-erythritol oxidation was solely localized in membrane fractions of *G. oxydans* DSM 7145 (data not shown) it was hypothesized that an enzyme similar to the membrane-bound glycerol dehydrogenase of *G. suboxydans* (Matsushita *et al.*, 2003) catalyses oxidation of *meso*-erythritol in *G. oxydans* DSM 7145. Therefore, potential homologues of the *sldBA* genes, encoding the glycerol dehydrogenase, were amplified. Not only the product sizes obtained, ~380 bp for *sldB*, ~2200 bp for *sldA*, but also the sequences of the *sldBA* genes of *G. oxydans* DSM 1745 and ATCC 621H were highly similar.

Based on the deduced amino acid sequences a complete identity of the SldB and a 99 % identity of the SldA proteins of *G. oxydans* DSM 7145 and *G. oxydans* ATCC 621H was observed (Supplementary Fig. S1). The SldB protein contains three putative transmembrane helices, while no transmembrane helices were predicted for the SldA protein. Furthermore, a 24 amino acid signal sequence was identified at the N terminus of the SldA protein.

Based on the structure of the *Methylobacterium extorquens* methanol dehydrogenase (Ghosh *et al.*, 1995) two highly conserved residues could be identified in the SldA amino acid sequence. Among these residues is Trp-340, which lies

at the same position as Trp-243 of the methanol dehydrogenase, where this residue together with two sulfur atoms of a disulfide ring structure plays a key role in positioning the PQQ molecule. Interestingly, the Trp residue alone, and neither the two cysteine residues nor a histidine residue, which replaces the cysteine residues in the *E. coli* quinoprotein glucose dehydrogenase (Cozier & Anthony, 1995), was detectable in the SldA amino acid sequence. However, the SldA protein retains an Asp residue (Asp-404, Supplementary Fig. S1), conserved in all PQQ-dependent dehydrogenases, which corresponds to Asp-303 of the *M. extorquens* methanol dehydrogenase and is thought to be the catalytic base that initiates the reaction by abstraction of a proton from the substrate (Ghosh *et al.*, 1995).

Genetic organization of *sldA* and *sldB* in *G. oxydans* DSM 7145

Sequencing of the *sld* genes revealed that the stop codon of the *sldB* gene (TGA) overlaps with the start codon (ATG) of the *sldA* gene, suggesting that the *sldBA* genes are organized in an operon. To analyse whether the *sldBA* genes are co-transcribed, an RT-PCR with primers spanning the entire *sldB* gene and the 5' portion of the *sldA* gene was performed. As shown in Fig. 3, the expected ~920 bp fragment, originating from a polycistronic mRNA, was obtained. Additionally, the transcription start of the *sldBA* genes was mapped by 5' RLM RACE at the thymidine nucleotide 24 bp upstream of the *sldB* start codon, which further supports the notion that in *G. oxydans* DSM 7145 the *sldBA* genes are co-transcribed from a common promoter upstream of *sldB* (data not shown).

Disruption of the *sldA* gene in *G. oxydans* DSM 7145

To test the hypothesis that *meso*-erythritol oxidation in *G. oxydans* DSM 7145 is catalysed by the glycerol dehydrogenase, a mutant was constructed by chromosomal

Fig. 2. Conversion of 50 mM *meso*-erythritol to L-erythrulose by resting cells of *G. oxydans* DSM 7145. Symbols: ●, OD₆₀₀; ■, *meso*-erythritol concentration (mM); □, L-erythrulose concentration (mM).

Fig. 3. Genetic organization and operon analysis of the *sldBA* genes in *G. oxydans* DSM 7145. (a) Arrangement of the *sldBA* genes (grey arrows) in *G. oxydans* DSM 7145. (b) Operon analysis of the *sldBA* genes. cDNA synthesized from total RNA of cells grown either on D-glucose (lane 1) or on D-glucose/D-mannitol (lane 2) was used as template in PCR experiments with primers *sldB_for* and *sldA_w2* (Table 2). Negative controls without prior reverse-transcriptase treatment are shown in lanes 3 and 4, respectively. The product obtained from a PCR with genomic DNA is depicted in lane 6. Lanes 5 and 7 represent non-template controls of the PCRs. The predicted fragment size was 924 bp.

inactivation of the *sldA* gene. For inactivation, an internal 1.5 kb fragment of the *sldA* gene was replaced by a 1 kb Gm-resistance cassette. In this mutant, designated *G. oxydans* DSM 7145 *sldA*::Gm, more than 67 % of the *sldA* gene was deleted. The successful disruption of the *sldA* gene and the absence of the vector in the chromosome were verified by PCR and Southern blot analysis (data not shown).

Growth of *G. oxydans* DSM 7145 and *G. oxydans* DSM 7145 *sldA*::Gm on different carbon sources

Growth of the mutant was monitored on complex medium supplemented with *meso*-erythritol and other carbon sources known to be utilized by *G. oxydans* DSM 7145. Disruption of *sldA* severely impaired growth of the resulting strain *G. oxydans* DSM 7145 *sldA*::Gm on complex medium with glycerol, *meso*-erythritol and D-mannitol. No growth was observed on *meso*-erythritol and D-mannitol, while growth on glycerol was slowed down by a factor of three. After 24 h the mutant reached an optical density on glycerol resembling that of the wild-type strain DSM 7145.

Growth of *G. oxydans* DSM 7145 *sldA*::Gm on D-glucose was slightly impaired and the mutant also reached a final optical density that was lower than that of the wild-type. On a mixture of 25 mM D-glucose/25 mM D-mannitol the growth rate of *G. oxydans* DSM 7145 *sldA*::Gm resembled that of the wild-type grown on D-glucose (0.65 h^{-1}) and also the final optical density was comparable with the value reached by *G. oxydans* DSM 7145 grown on D-glucose ($\text{OD}_{600} \sim 0.7$). This seems noteworthy, because on D-glucose/D-mannitol the wild-type grew at a rate (0.46 h^{-1}) and to a final optical density ($\text{OD}_{600} \sim 2$) comparable with the values obtained for cultivation on D-mannitol.

These results suggest that the glycerol dehydrogenase indeed oxidizes *meso*-erythritol *in vivo*, and in addition seems to have a broader substrate spectrum that also includes at least glycerol and D-mannitol.

Biochemical characterization of *G. oxydans* DSM 7145 *sldA*::Gm

To biochemically examine the effect of *sldA* disruption, membrane fractions of *G. oxydans* DSM 7145 and *G. oxydans* DSM 7145 *sldA*::Gm grown on complex medium with D-glucose to late exponential phase were prepared. These were subsequently used to assay the dye-linked dehydrogenase activities with different substrates. As shown in Fig. 4, *sldA* disruption did not negatively affect membrane-bound D-glucose oxidation. In *G. oxydans* DSM 7145 *sldA*::Gm, D-glucose oxidation was even slightly higher than in the wild-type. Apart from D-glucose, significant D-gluconate oxidation [$0.18 \text{ U (mg protein)}^{-1}$, about 42 % of the wild-type level] was retained in the membrane fraction of *G. oxydans* DSM 7145 *sldA*::Gm. On the other hand, oxidation of the tested polyols glycerol, *meso*-erythritol, D-arabitol, D-mannitol and D-sorbitol was significantly reduced in the membrane fraction of *G. oxydans* DSM 7145 *sldA*::Gm as compared with the wild-type membranes. Thus, the glycerol dehydrogenase from *G. oxydans* DSM 7145 not only oxidizes *meso*-erythritol but has a broad substrate spectrum that includes the sugar acid D-gluconate and the C3–C6 polyols glycerol, D-arabitol, D-mannitol and D-sorbitol.

Glycerol dehydrogenase activities in membrane fractions of cells grown on complex medium with 25 mM D-glucose/25 mM D-mannitol

To exclude the possibility that other membrane-bound dehydrogenases catalysing the oxidation of *meso*-erythritol are induced during growth on carbon sources other than D-glucose, *G. oxydans* DSM 7145 and *G. oxydans* DSM 7145 *sldA*::Gm were cultivated on complex medium with 25 mM D-glucose/D-mannitol to late exponential phase. Based on the data depicted in Fig. 4, SldA seems to be the main polyol dehydrogenase in *G. oxydans* DSM 7145. Oxidation of glycerol, *meso*-erythritol, D-arabitol, D-mannitol, D-sorbitol and D-gluconate was also clearly reduced in the membrane fraction of *G. oxydans* DSM 7145 *sldA*::Gm grown in a mixture of D-glucose/D-mannitol.

Fig. 4. Membrane-bound dehydrogenase activities in *G. oxydans* DSM 7145. Membrane fractions were prepared from cells grown on complex medium supplemented with 50 mM D-glucose or 25 mM D-glucose/25 mM D-mannitol to late exponential phase. Black bars, membrane-bound enzyme activities in *G. oxydans* DSM 7145 grown on 50 mM D-glucose; grey bars, membrane-bound activities in *G. oxydans* DSM 7145 *sldA::Gm* grown on 50 mM D-glucose; white bars, membrane-bound activities in *G. oxydans* DSM 7145 grown on 25 mM D-glucose/25 mM D-mannitol; hatched bars, membrane-bound activities in *G. oxydans* DSM 7145 *sldA::Gm* grown on 25 mM D-glucose/25 mM D-mannitol. Assays were performed as described in Methods. The data shown are mean values from three replicate experiments.

Moreover, Fig. 4 shows that the glycerol dehydrogenase seems to be expressed constitutively, irrespective of the utilized carbon source, because similar PMS-DCIP reductase activities were detected in membrane fractions of cultures grown on D-glucose and D-glucose/D-mannitol. In contrast, differences in membrane-bound D-glucose oxidation were observed. In wild-type membrane fractions, glucose dehydrogenase decreased with the D-glucose concentration in the medium, while membrane fractions of *G. oxydans* DSM 7145 *sldA::Gm* revealed elevated D-glucose oxidation rates.

Complementation of *G. oxydans* DSM 7145 *sldA::Gm*

To complement *G. oxydans* DSM 7145 *sldA::Gm*, a plasmid, pJV8, containing the putative *sldB* promoter region fused to the *sldA* gene was constructed. This construct efficiently restored growth of *G. oxydans* DSM 7145 *sldA::Gm* on complex medium with D-mannitol, *meso*-erythritol and glycerol (data not shown). Differences were observed in the final optical densities. While *G. oxydans* DSM 7145 *sldA::Gm*(pJV8) grown on glycerol and *meso*-erythritol reached values comparable with those of the vector control, only half of the final optical density of the control was reached when *G. oxydans* DSM 7145 *sldA::Gm*(pJV8) was cultivated on D-mannitol. Membrane fractions of the complemented mutant again oxidized glycerol, *meso*-erythritol, D-arabitol, D-mannitol, D-sorbitol and D-gluconate. The observed ratio of the dehydrogenase activities of the vector control and the complemented mutant was 0.7 for most substrates, except for D-gluconate, where the ratio was 0.89.

DISCUSSION

In this study it was shown that *G. oxydans* DSM 7145 not only utilizes *meso*-erythritol but also L-erythrulose as growth substrate, and that both substrates are metabolized by different pathways. Oxidation of *meso*-erythritol is membrane-associated, mediated by the glycerol dehydrogenase (discussed below) and results in nearly quantitative accumulation of L-erythrulose in culture supernatants. Depletion of extracellular *meso*-erythritol leads to a global metabolic change from membrane-bound *meso*-erythritol oxidation to L-erythrulose assimilation accompanied by acetate accumulation. In the cytoplasm, L-erythrulose is most likely reduced to *meso*-erythritol by an NAD(P)⁺-dependent dehydrogenase, as similarly discussed with ribitol dehydrogenase from *G. suboxydans* IFO 12528 (Adachi *et al.*, 2001), and then channelled into the pentose-phosphate pathway. Acetate is probably produced from the glyceraldehyde 3-phosphate generated in the pentose-phosphate pathway via pyruvate and acetaldehyde, as the genome sequence of *G. oxydans* ATCC 621H reveals a missing acetate kinase pathway but the presence of a pyruvate decarboxylase and an NADP⁺-dependent acetaldehyde dehydrogenase (Prust *et al.*, 2005; Schweiger *et al.*, 2007). *Gluconobacter frateurii* CHM 43 has also been shown to grow on *meso*-erythritol and L-erythrulose (Moonmangmee *et al.*, 2002), but L-erythrulose consumption linked to extracellular accumulation of acetic acid has not been previously reported.

Excretion of acetic acid by *G. oxydans* DSM 7145 is most likely only a by-product of intracellular L-erythrulose metabolism, because 30 mM L-erythrulose was consumed but only 10 mM acetic acid was detectable in the culture

supernatants (Fig. 1b). As has been shown for *E. coli* aerobic acetate production, this is a manifestation of an imbalance between glucose uptake and the demands for both biosynthesis and energy production (El-Mansi & Holms, 1989). Thus, it can be assumed that L-erythrulose cannot be channelled into the cytoplasmic pathways fast enough and therefore part of the internalized L-erythrulose is converted to acetic acid.

A further physiological explanation of the observed biphasic metabolism of *meso*-erythritol could be that *G. oxydans* DSM 7145 first quickly oxidizes *meso*-erythritol to an unfavourable product, L-erythrulose, thereby preventing growth and utilization of erythritol by other bacteria. Afterwards, L-erythrulose is slowly utilized, similar to the hypothesis of L-sorbose utilization by *G. frateurii* CHM 43 (Soemphol *et al.*, 2007).

As *meso*-erythritol oxidation was solely localized in membrane fractions of *G. oxydans* DSM 7145, it seemed reasonable that an enzyme similar to the membrane-bound glycerol dehydrogenase of *G. suboxydans* (Matsushita *et al.*, 2003) catalyses *meso*-erythritol oxidation in *G. oxydans* DSM 7145. Sequencing of the identified homologues from strain DSM 7145 revealed not only that they are highly similar to the *sldBA* genes of *G. oxydans* ATCC 621H (*gox* 0854–0855) (Prust *et al.*, 2005) and *G. suboxydans* IFO 3255 (Miyazaki *et al.*, 2002) but also that the genes seem to be organized in an operon (Fig. 3). Only few promoters of acetic acid bacteria have been characterized to date, so we decided to elucidate the mode of transcription of the *sldBA* genes. Our experiments showed that these two genes are indeed transcribed polycistronically from a promoter upstream of *sldB*. Co-transcription may also take place in *G. suboxydans* IFO 3255, as was speculated by Miyazaki *et al.* (2002), and may also occur in other *Gluconobacter* strains with a similar genetic organization of the *sldBA* genes.

To test the hypothesis that *meso*-erythritol is a substrate of the membrane-bound glycerol dehydrogenase, the *sldA* gene was inactivated by insertion of a Gm-resistance gene. Growth experiments with *meso*-erythritol and other substrates known to be utilized by *G. oxydans* DSM 7145 indicated that *meso*-erythritol and other C3–C6 polyols but not D-glucose are substrates of the glycerol dehydrogenase. The broad substrate spectrum of the enzyme could be further confirmed by biochemical characterization of the mutant. While D-glucose oxidation was retained in membrane fractions of *G. oxydans* DSM 7145 *sldA*::Gm, oxidation of the C3–C6 polyols glycerol, *meso*-erythritol, D-arabitol, D-mannitol and D-sorbitol, and also of the sugar acid D-gluconate was significantly reduced. The substrate spectrum determined closely matches that of the glycerol dehydrogenases of *G. suboxydans* IFO 3255 and 3257, which have also been shown to catalyse the oxidation of some polyols and D-gluconate (Matsushita *et al.*, 2003; Shinjoh *et al.*, 2002).

The glycerol dehydrogenase seems to be the main enzyme involved in the oxidation of the substrates mentioned

above, as the corresponding oxidation activities were also significantly reduced in membrane fractions of *G. oxydans* DSM 7145 *sldA*::Gm grown on a different carbon source. The residual oxidation of polyols can be attributed to other dehydrogenases present in the membrane fraction of *G. oxydans* DSM 7145, which most likely oxidize the *SldA* substrates at a low rate.

Interestingly, expression of the polyol dehydrogenase seems not to be regulated, as PMS-DCIP reductase activities measured in membrane fractions of cells grown on D-glucose (non-*SldA* substrate) did not increase by addition of the substrate D-mannitol to the growth medium (Fig. 4). Similarly, De Ley & Dochy (1960) and DeLey & Stouthamer (1959) concluded from the examination of D-glucose metabolism in *Acetobacter* spp. that expression of the enzymes involved is not regulated. Genome-wide transcription analysis of *G. oxydans* ATCC 621H further confirmed that most membrane-bound dehydrogenases are expressed constitutively, irrespective of the utilized carbon source (our unpublished results). Therefore, we conclude that for industrial production of L-erythrulose, *G. oxydans* DSM 7145 can also be cultured on glucose-containing medium to obtain biomass, which then can be used in resting cell preparations to oxidize *meso*-erythritol to L-erythrulose (Fig. 2).

In contrast to the glycerol dehydrogenase, differences were observed in membrane-bound D-glucose oxidation. In the wild-type background, membrane-bound D-glucose oxidation activity was lower in cultures grown on D-glucose/D-mannitol than in cultures grown on D-glucose. Additionally, elevated dye-linked glucose dehydrogenase activities were detectable in *G. oxydans* DSM 7145 *sldA*::Gm (Fig. 4). At present, the reasons for these differences are not clearly understood, as no differences in expression of the *sldA* gene and the *mgdh* gene, encoding the membrane-bound D-glucose dehydrogenase, were detectable (our unpublished results). Gupta *et al.* (1997) attributed elevated gluconate- and 2-keto-D-gluconate-oxidizing activities after transposon-induced inactivation of the membrane-bound glucose dehydrogenase in *G. oxydans* ATCC 9937 to a decontrol or activation of the genes encoding the respective enzymes. Such polar effects on the expression of genes distal to the insertion site have been described before (Kleckner *et al.*, 1977) but can be ruled out in this case because neither in *G. oxydans* ATCC 621H nor in *G. oxydans* DSM 7145 are the *sldBA* and the *mgdh* genes organized in operons.

The ongoing work with *G. oxydans* DSM 7145 showed that this strain seems to be genetically similar to *G. oxydans* ATCC 621H. Nonetheless, physiological differences between the two strains were noted. With *G. oxydans* ATCC 621H, only a minor decrease in extracellular L-erythrulose concentration was observed after the supplied *meso*-erythritol was depleted. Moreover, in contrast to *G. oxydans* ATCC 621H, the *sacB* selection strategy (Gay *et al.*, 1985) could be applied in *G. oxydans* DSM 7145 to identify

double-crossover mutants. Based on these observations, sequencing of other *G. oxydans* genomes seems interesting in order to elucidate whether physiological differences can be traced back to genomic differences or whether other regulatory mechanisms are involved.

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