FNR-Type Regulator GoxR of the Obligatorily Aerobic Acetic Acid Bacterium *Gluconobacter oxydans* Affects Expression of Genes Involved in Respiration and Redox Metabolism

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ABSTRACT Gene expression in the obligately aerobic acetic acid bacterium *Gluconobacter oxydans* responds to oxygen limitation, but the regulators involved are unknown. In this study, we analyzed a transcriptional regulator named GoxR (GOX0974), which is the only member of the fumarate-nitrate reduction regulator (FNR) family in this species. Evidence that GoxR contains an iron-sulfur cluster was obtained, suggesting that GoxR functions as an oxygen sensor similar to FNR. The direct target genes of GoxR were determined by combining several approaches, including a transcriptome comparison of a Δ goxR mutant with the wild-type strain and detection of *in vivo* GoxR binding sites by chromatin affinity purification and sequencing (ChAP-Seq). Prominent targets were the *cioAB* genes encoding a cytochrome *bd* oxidase with low O₂ affinity, which were repressed by GoxR, and the *pnt* operon, which was activated by GoxR. The *pnt* operon encodes a transhydrogenase (*pntA1A2B*), an NADH-dependent oxidoreductase (GOX0313), and another oxidoreductase (GOX0314). Evidence was obtained for GoxR being active despite a high dissolved oxygen concentration in the medium. We suggest a model in which the very high respiration rates of *G. oxydans* due to periplasmic oxidations cause an oxygen-limited cytoplasm and insufficient reoxidation of NAD(P)H in the respiratory chain, leading to inhibited cytoplasmic carbohydrate degradation. GoxR-triggered induction of the *pnt* operon enhances fast interconversion of NADPH and NADH by the transhydrogenase and NADH reoxidation by the GOX0313 oxidoreductase via reduction of acetaldehyde formed by pyruvate decarboxylase to ethanol. In fact, small amounts of ethanol were formed by *G. oxydans* under oxygen-restricted conditions in a GoxR-dependent manner.

IMPORTANCE *Gluconobacter oxydans* serves as a cell factory for oxidative biotransformations based on membrane-bound dehydrogenases and as a model organism for elucidating the metabolism of acetic acid bacteria. Surprisingly, to our knowledge none of the more than 100 transcriptional regulators encoded in the genome of *G. oxydans* has been studied experimentally until now. In this work, we analyzed the function of a regulator named GoxR, which belongs to the FNR family. Members of this family serve as oxygen sensors by means of an oxygen-sensitive [4Fe-4S] cluster and typically regulate genes important for growth under anoxic conditions by anaerobic respiration or fermentation. Because *G. oxydans* has an obligatory aerobic respiratory mode of energy metabolism, it was tempting to elucidate the target genes regulated by GoxR. Our results show that GoxR affects the expression of genes that support the interconversion of NADPH and NADH and the NADH reoxidation by reduction of acetaldehyde to ethanol.

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Gluconobacter oxydans is a Gram-negative, rod-shaped alphaproteobacterium belonging to the family of acetic acid bacteria (1). It has been used since the 1930s in biotechnology, mainly in production of vitamin C and 6-amino-L-sorbose, a key intermediate for the synthesis of the antidiabetes drug miglitol (2–4). The basis for its industrial application is the variety of membrane-bound dehydrogenases, which incompletely oxidize sugars, sugar alcohols, and a variety of other compounds regioselectively and stereoselectively in the periplasm, such as D-sorbitol to L-sorbose in vitamin C synthesis (5–7). The membrane-bound dehydrogenases use ubiquinone as an electron acceptor, which is reoxidized either by the proton-pumping cytochrome *bo*₃-type oxidase (8) or by a cytochrome *bd*-type oxidase (9). The latter enzyme was characterized as a cyanide-insensitive oxidase (CIO) and the corresponding genes named *cioA* and *cioB* instead of *cydA* and *cydB* (10, 11). Remarkably, the oxygen affinity of CIO was found to be 7-fold lower than that of cytochrome *bo*₃ (21 μ M versus 3 μ M), which is in contrast to the situation in *Escherichia coli*, where cytochrome *bd* is a high-affinity terminal oxidase (11, 12).

When *G. oxydans* is cultivated with substrates such as glucose or mannitol, about 90% of these carbon sources are converted by membrane-bound dehydrogenases to partially oxidized products, which remain unmetabolized in the medium. Only a small fraction of the substrate or its oxidation products enters the cell and is metabolized in either the pentose phosphate pathway (PPP) or the Entner-Doudoroff pathway (EDP). The PPP, which runs partially cyclically, is the major catabolic pathway for glucose in the cytoplasm, as shown by ¹³C metabolic flux analysis and studies of mutants lacking key enzymes of the PPP or the EDP (13–15). Neither glycolysis nor the tricarboxylic acid (TCA) cycle is functional, since *G. oxydans* lacks the genes for phosphofructokinase, succinyl-CoA synthetase, and succinate dehydrogenase (16, 17). The absence of a functional TCA cycle explains the inability of *G. oxydans* to oxidize acetate, which is a key difference in comparison with other acetic acid bacteria, such as *Acetobacter* and *Gluconacetobacter* species. Pyruvate formed by the PPP or the EDP is predominantly converted to acetate via the activities of pyruvate decarboxylase and an NADP⁺-dependent acetaldehyde dehydrogenase (18). Metabolic engineering of *G. oxydans*, including inactivation of glucose oxidation to gluconate and of pyruvate decarboxylation, combined with introduction of the missing TCA cycle genes, led to strains that showed increased biomass yield on glucose and formed pyruvate instead of acetate (17, 19).

According to the genome sequence and current literature, *G. oxydans* 621H is not capable of anaerobic growth by respiratory or fermentative pathways and therefore is described as a strictly aerobic bacterium (16, 20). However, it responds to changes in the oxygen concentration, because a shift from oxygen excess to oxygen limitation caused changes in the expression of about 500 genes, many of which are involved in respiration and oxidative phosphorylation (21). Although the expression changes of many genes of the oxygen starvation stimulon might have been triggered by the slowed, nonexponential growth occurring after the shift to oxygen limitation, oxygen- or redox-sensing transcriptional regulators might also be involved. In bacteria, several types of oxygen sensors have been described, e.g., those based on iron-sulfur clusters, exemplified by fumarate-nitrate reduction regulator (FNR)-type transcriptional regulators, and those based on heme, exemplified by the FixL sensor kinase (for reviews, see references 22 to 30).

In this study, we characterized a transcriptional regulator of the FNR family in *G. oxydans*, which we named *Gluconobacter redox* regulator, GoxR (GOX0974). Based on the determined target genes of GoxR, we propose a model for the response of *G. oxydans* to oxygen limitation.

RESULTS

In silico analysis of GoxR suggests an FNR-like function. Inspection of the genome of *G. oxydans* (16, 20) for transcriptional regulators that might be involved in oxygen-dependent gene expression revealed a gene (GOX0974, termed *goxR*) encoding a protein (26.5 kDa, with 240 amino acid residues) that belongs to the cAMP receptor protein (Crp)/FNR family of transcriptional regulators. GoxR is the only member of this family present in *G. oxydans*. The Crp/FNR family includes various subfamilies represented by Crp, FNR, or the FixK regulator involved in nitrogen fixation (25). A search using the NCBI BLASTP tool (31) revealed proteins with an E value of $<2e^{-63}$ and $>50\%$ sequence identity to GoxR in species of the genera *Gluconobacter*, *Saccharibacter*, *Kozakia*, *Asaia*, *Acidomonas*, *Gluconacetobacter*, *Komagataeibacter*, and *Acetobacter*, all of which belong to the family *Acetobacteraceae*. A comparison of GoxR with the *E. coli* proteome revealed FNR as the closest homolog, with 30% amino acid sequence identity, whereas Crp showed only 22% sequence identity with GoxR. The strongest evidence for GoxR being a member of the FNR subfamily was provided by the presence of five cysteine residues in the N-terminal region, C₁₄(X)₂C₁₇(X)₇C₂₅(X)₅C₃₁(X)₈₂C₁₁₃ (see Fig. S1 in the supplemental material). The presence of four cysteine residues is characteristic of FNR-like proteins, because they are required for the formation of the [4Fe-4S] cluster that is key for FNR function, and these residues are not present in members of the Crp subfamily. In *E. coli* FNR, the residues C_{20r}, C_{23r}, C_{29r}, and C₁₂₂ are known to coordinate the [4Fe-4S] cluster, whereas C₁₆ has been reported to serve as a stabilizing residue (32).

Comparison of global gene expression in the Δ goxR mutant with that in the parental strain by RNA-Seq. To explore the function of GoxR, a mutant with an in-frame deletion of *goxR* was constructed in the parental strain *G. oxydans* Δ hsdR. The *hsdR* gene (GOX2567) is part of a restriction-modification system located on the 163-kb plasmid pGOX1 and was deleted in order to improve transformation efficiency. Growth of *G. oxydans* Δ goxR in mannitol medium was comparable to that of the parental strain both in shake flasks and in bioreactors under oxygen- and pH-controlled conditions (15% dissolved oxygen [DO] [pH 6]) (data not shown). A transcriptome comparison of the Δ goxR mutant and the parental strain was performed by RNA sequencing (RNA-Seq) to identify genes with altered expression in the absence of GoxR. For this purpose, the two strains were cultivated in a bioreactor at 15% DO and pH 6 using mannitol medium. Because GoxR was assumed to be active under oxygen-restricted conditions, the oxygen supply was switched from 15% DO to 0% DO by gassing with a 2% O₂/98% N₂ mixture when the strains had reached an optical density at 600 nm (OD₆₀₀) of 2.5. The cells were harvested 20 min after the switch and used for RNA isolation.

In three independent biological replicates, two genes showed ≥ 2 -fold increased mRNA levels in the Δ goxR mutant and 12 genes demonstrated ≥ 2 -fold decreased mRNA levels, with *P* values of ≤ 0.05 (Table 1). The genes with increased expression in the Δ goxR mutant were *cioA* and *cioB*, encoding the two subunits of the *bd*-type terminal oxidase CIO (see above). Within the group of genes with decreased expression in the Δ goxR mutant, the ones showing the most strongly reduced mRNA levels were those of the *pnt* operon, i.e., *pntA1*-*pntA2*-*pntB*-GOX0313-GOX0314 (Fig. 1). The first three genes of this operon encode subunits $\alpha 1$, $\alpha 2$, and β of a membrane-bound transhydrogenase that catalyzes the reversible reduction of NADP⁺ with NADH coupled with the import of a proton from the periplasm to the cytoplasm (33). GOX0313 encodes a medium-chain alcohol dehydrogenase with a broad substrate spectrum that oxidizes various primary alcohols but has a preference for NADH-dependent reduction of aldehydes to alcohols and of α -diketones to (S)-hydroxyketones (34). GOX0314 was annotated as an alcohol dehydrogenase-like oxidoreductase, but it has not yet been experimentally characterized. A group of six genes involved in flagellum synthesis, organized in several operons, showed ~ 3 -fold reduced mRNA levels in the Δ goxR mutant, suggesting an influence of GoxR on motility. Another gene with a 2.5-fold reduced mRNA level in the Δ goxR mutant was GOX0090, which was originally annotated as a putative sugar kinase. However, the GOX0090 protein shows 30% sequence identity with the YjeF protein of *Escherichia coli*, which has been shown to

TABLE 1 Genes showing altered expression in the deletion mutant *G. oxydans* Δ *goxR*, compared to the parent *G. oxydans* strain, 20 min after shifting from oxygen excess (15% DO) to oxygen limitation (0% DO)^a

Locus tag and functional category ^b	Gene	Annotation	Fold change (Δ <i>goxR</i> vs parent strain)	P
Respiration and metabolism				
GOX0278	<i>cioA</i>	Terminal oxidase CIO, subunit I	6.60	0.001
GOX0279	<i>cioB</i>	Terminal oxidase CIO, subunit II	4.66	0.009
GOX0090		Putative ADP-dependent NAD(P)HX epimerase/dehydratase	0.40	0.05
GOX0310	<i>pntA1</i>	Transhydrogenase subunit α 1	0.11	<0.001
GOX0311	<i>pntA2</i>	Transhydrogenase subunit α 2	0.11	0.003
GOX0312	<i>pntB</i>	Transhydrogenase subunit β	0.19	0.010
GOX0313		Alcohol:NAD ⁺ oxidoreductase	0.15	<0.001
GOX0314		Probable alcohol:NAD(P) ⁺ oxidoreductase	0.36	0.06
Regulation				
GOX0974	<i>goxR</i>	GoxR; FNR-like transcriptional regulator	0.02	<0.001
Motility				
GOX0424	<i>fliF</i>	Flagellar MS ring protein	0.44	0.022
GOX0425	<i>flgG</i>	Flagellar basal body modification protein FlgG	0.38	0.018
GOX0426	<i>fliK</i>	Putative flagellar hook length control protein FliK	0.27	0.02
GOX0787	<i>flaB</i>	Flagellin B	0.34	0.028
GOX0788	<i>flaF</i>	Flagellin assembly protein	0.35	0.005
GOX1528	<i>flgB</i>	Flagellar basal body rod protein FlgB	0.36	0.017

^aThe strains were cultivated in a bioreactor in mannitol medium (pH 6) with 15% DO to an OD₆₀₀ of 2.5. The gassing was then changed to 2% O₂/98% N₂, resulting in a measured DO level of 0%. Twenty minutes after the switch, the cells were harvested and used for RNA isolation. Gene expression was determined by RNA-Seq analysis as described in Materials and Methods. Genes with mRNA ratios of ≥ 2.0 (lower ones allowed in the case of operons) or ≤ 0.5 (higher ones allowed in the case of operons) and P values of ≤ 0.05 are listed. The data shown represent mean values from three biological replicates.

^bThe genes were grouped into functional categories, within which they were ordered according to their locus tags.

function as an enzymatic repair system for hydrated NAD(P)H (35), and a similar function can be assumed for the *G. oxydans* homolog.

Reporter gene assays for potential GoxR target genes. In order to confirm the RNA-Seq results, reporter gene assays were performed with three potential GoxR target genes, *cioA*, *pntA1*, and GOX0090. The DNA regions covering 314 to 340 bp upstream of the predicted start codons of the three genes were cloned into the promoter probe vector pLacZ_pPPP3 carrying a *lacZ* reporter gene. The resulting plasmids pLacZ_*cioA*, pLacZ_*pntA1*, and pLacZ_0090, as well as the promoterless *lacZ* control plasmid (pLacZ), were transferred into the *G. oxydans* parent and Δ *goxR* strains by electroporation. The strains were cultivated in a bioreactor in the same way as the strains used for the RNA-Seq analysis and were used for β -galactosidase assays 0, 20, and 60 min after the shift from 15% DO to 0% DO (by gassing with a 2% O₂/98% N₂ mixture).

As shown in Fig. 2, the background LacZ activity in the two control strains carrying pLacZ_control varied between 61 and 114 Miller units. The parental strain carrying pLacZ_*cioA* showed LacZ activities of around 30 Miller units, which were even lower than those seen with pLacZ_control at all time points tested. This suggests that *cioA* expression is very low under the chosen conditions and that in the plasmid

FIG 1 Chromosomal organization of the *pnt* operon, a major target of the FNR-type regulator GoxR in *G. oxydans*. Dark gray arrows mark genes regulated by GoxR, and light gray arrows show neighboring genes not regulated by GoxR.

FIG 2 Analysis of the promoter activities of *cioA*, *pntA1*, and GOX0090 in dependence on oxygen and GoxR using transcriptional *lacZ* fusions. The β -galactosidase activities of the parent strain (white) and the Δ *goxR* mutant (gray) carrying the reporter plasmids pLacZ_control, pLacZ_*cioA*, pLacZ_*pntA1*, or pLacZ_GOX0090 were measured. The strains were cultivated in a bioreactor at 15% DO to an OD₆₀₀ of 2.5 before the gassing was changed to 2% O₂/98% N₂. Samples were taken before the switch to oxygen limitation and 20 min and 60 min after the switch. Mean values and standard deviations of three biological replicates are shown.

pLacZ_control there is some residual *lacZ* expression from a plasmid-based promoter. In the Δ *goxR* mutant carrying pLacZ-*cioA*, the LacZ activity was 31-fold higher than that in the parental strain already at high oxygen levels and was 45-fold and 59-fold higher 20 min and 60 min, respectively, after the shift to oxygen limitation. These results suggest that GoxR inhibits *cioA* expression. The parental strain carrying pLacZ_*pntA1* showed very high LacZ activities (4,486 Miller units) already at 15% DO, which further increased about 1.5-fold after the shift to oxygen limitation. In the Δ *goxR* mutant with pLacZ_*pntA1*, the LacZ activity (959 Miller units) was 4.7-fold lower than that in the parental strain at 15% DO but also increased about 2-fold after the shift to oxygen limitation. These results suggest that GoxR stimulates expression of the *pnt* operon and that an additional regulator or regulatory mechanism is responsible for increased expression after the shift to oxygen limitation. The LacZ activity for pLacZ_0090 in the parental strain was 15-fold lower than that for *pntA1* and increased 2.6-fold after the switch to oxygen limitation. In the Δ *goxR* mutant with pLacZ_0090, the LacZ activities were at background levels, suggesting that GoxR stimulates GOX0090 expression.

The results from the reporter gene studies are in agreement with the RNA-Seq data. The observation that the LacZ activities of the Δ *goxR* mutant and the parental strain differed already before the switch to oxygen limitation for all three tested genes suggests that GoxR is active in the parental strain already at 15% DO.

Aldehyde reductase activity of the soluble cell fraction. As another approach to confirm the influence of GoxR on the expression of the *pnt* operon, we measured NADH-dependent reduction of propionaldehyde and hydrocinnamaldehyde in the soluble cell fraction of the parental strain and the Δ *goxR* mutant. These substances were previously identified as favorable substrates for the purified GOX0313 oxidoreductase (34). In the parental strain, the activities for both substrates increased 1.5- to 2-fold after the shift from 15% DO to 0% DO (Fig. 3). In the Δ *goxR* mutant, both activities were much lower (7- to 15-fold) than in the parental strain and did not change much after the shift from 15% DO to 0% DO (Fig. 3). These results are in agreement with the

FIG 3 NADH-dependent aldehyde reductase activity in the soluble fraction of cell extracts of the *G. oxydans* parental strain (Ref) and the $\Delta goxR$ mutant ($\Delta goxR$). Propionaldehyde and hydrocinnamaldehyde were previously shown to be favorable substrates of the alcohol:NAD⁺ oxidoreductase GOX0313 of *G. oxydans* (34), whose expression is repressed by GoxR. The strains were cultivated in bioreactors either at 15% DO (sat.) or under oxygen limitation (lim.), achieved by gassing with 2% O₂/98% N₂ (0% DO), as described in Materials and Methods. Mean values and standard deviations of three technical replicates are shown.

assumption that both reductase activities are predominantly catalyzed by GOX0313, whose expression was found to be 7-fold decreased in the $\Delta goxR$ mutant (Table 1). Furthermore, in agreement with the reporter gene studies, the enzyme activity differences between the $\Delta goxR$ mutant and the parental strain were already present under oxygen-saturating conditions, supporting the assumption that GoxR is active at a DO level of 15%.

Ethanol formation by *G. oxydans* under oxygen-restricted conditions. We previously speculated that GOX0313 activity might play a role in the adjustment of the redox balance of NAD⁺ and NADH under oxygen-limited conditions because expression of the GOX0313 gene, together with the other genes of the *pnt* operon, was upregulated under oxygen limitation (21). Of the various substrates identified for GOX0313 (34), acetaldehyde was reduced with the highest specific activity and also is a likely *in vivo* substrate, as cytoplasmic carbon catabolism involves decarboxylation of pyruvate to acetaldehyde (18, 19). Under conditions of a high NADH/NAD⁺ ratio caused by oxygen limitation or insufficient NADH dehydrogenase activity, acetaldehyde may serve as the electron acceptor for NADH reoxidation by an alcohol dehydrogenase, forming ethanol as product. Therefore, we examined ethanol production by cell suspensions of the parental strain and of the $\Delta goxR$ mutant incubated under oxygen-restricted conditions in a buffer containing glucose. As shown in Fig. 4, the parental strain indeed produced some ethanol in a time-dependent manner. The $\Delta goxR$ mutant also formed ethanol but at a much lower rate than the parental strain, correlating with lower expression of the GOX0313 oxidoreductase.

Identification of a putative DNA binding motif of GoxR. Because the amino acid sequence of the DNA recognition helix of the helix-turn-helix motif of GoxR (ETVSR) is almost identical to that of *E. coli* FNR (ETISR) (Fig. S1), we assumed that the DNA binding site of GoxR is similar to that of FNR. The consensus sequence reported for FNR binding sites in *E. coli* is TTGAT-N₄-ATCAA (25, 36). Therefore, we analyzed the promoter regions (500 bp upstream of the start codon plus a 50-bp coding region) of selected genes or operons with altered expression in the $\Delta goxR$ mutant for the presence of sequence motifs with similarity to the *E. coli* FNR consensus motif using the FIMO program (37) of the MEME bioinformatics suite (38).

In the case of *cioA*, a potential GoxR binding site (**TTGAT**tttt**GTCAA**; capital letters denote the binding half-sites, bold capitals indicate nucleotides identical to the *E. coli* FNR consensus sequence, and lowercase letters denote the variable nucleotides in between the two binding half-sites) was centered at position −56.5 upstream of the transcription start site (TSS) (128 bp upstream of the annotated ATG start codon), which was identified by 5′ rapid amplification of cDNA ends (5′-RACE) (Fig. S2) and by

FIG 4 Ethanol production under oxygen-deprived conditions by the *G. oxydans* parent strain (open circles) and the $\Delta goxR$ mutant (closed circles). The two strains were cultivated in mannitol medium in shake flasks with vigorous shaking to the stationary phase of growth. The cells were washed, resuspended to an OD_{600} of 0.5 in morpholineethanesulfonic acid (MES) buffer (pH 6.0), and incubated under oxygen-deprived conditions in the presence of 0.4% (wt/vol) glucose. Mean values and standard deviations from at least four parallel experiments are shown.

reanalysis of RNA-Seq mapping data (39). Another, less conserved, potential GoxR binding site (TTTAGagggTTCAA) was centered at position -2.5 with respect to the TSS, which would be compatible with *cioAB* repression by GoxR (Fig. S3). In a previous RNA-Seq analysis, a potential TSS was identified within the coding region of *cioA* and another potential TSS was located in the 3' region of the upstream gene GOX0277 (39). The relevance of these potential TSSs is unclear.

In the case of the *pnt* operon, a sequence perfectly matching the FNR consensus sequence (TTGATatccATCAA) was centered at position -41.5 with respect to the TSS (Fig. S3), which was located 80 bp upstream of the annotated start codon by RNA-Seq (39) and was confirmed in this work by 5'-RACE (Fig. S2). For the GOX0090 gene, a putative GoxR binding site (TTGATcctcGTCAT) was also located at position -41.5 with respect to the TSS (Fig. S3) identified by RNA-Seq 132 bp upstream of the start codon (39). However, this TSS was detected not by the automatic pipeline but also by rechecking of the respective mapping. Position -41.5 matches class II FNR-activated promoters (40) and supports an activating function of GoxR for the *pnt* operon and GOX0090.

Genome-wide identification of *in vivo* GoxR binding sites by ChAP-Seq analysis. In order to demonstrate that the GoxR sequence motifs identified by bioinformatic means are indeed bound by GoxR *in vivo* and to identify additional GoxR binding sites, we performed a chromatin affinity purification and sequencing (ChAP-Seq) experiment using a *G. oxydans* strain in which the chromosomal *goxR* gene was modified by a 3'-terminal extension, resulting in a GoxR variant with a C-terminal Strep-tag preceded by a short linker (SAWSHPQFEK). The strain was cultivated in a 5-liter baffled Erlenmeyer flask with 1 liter of medium at 130 rpm, and cells were harvested at an OD_{600} of about 2.8 and used for isolation of GoxR-bound DNA (see Materials and Methods). Forty-three GoxR binding sites were identified in the genome of *G. oxydans*, 34 of which were located upstream of open reading frames (ORFs) (Table S1). A search for the presence of the *E. coli* FNR consensus motif (TTGAT-N₄-ATCC) in a 100-bp DNA region comprising 50 bp each upstream and downstream of the peak maximum revealed 9 ChAP-Seq peaks that contained two binding motifs and 8 ChAP-Seq peaks with a single binding motif (Table S1). For 17 ChAP-Seq peaks, no binding motif was identified.

With the exception of six genes involved in flagellum synthesis, *in vivo* GoxR binding was found upstream of all genes and operons that were shown to have ≥ 2 -fold altered mRNA levels in the RNA-Seq analysis, i.e., the *pnt* operon, the *cioAB* operon, and GOX0090. In Fig. 5, the ChAP-Seq plots of the corresponding peaks, the identified binding site motifs, and the mRNA ratios ($\Delta goxR$ versus parental strain) are shown. In

ChAP-Seq peak	Peak intensity	GoxR binding motif sequence	P value	Distance to TSS	Regulated genes	Annotation	mRNA ratio	P value
7.6	TTTA Gaggg TCAA TTGAT tttt ATCAA	2.20E-03 4.32E-05	-2.5 -56.5	<i>cioA</i> <i>cioB</i>	Terminal oxidase CioA Terminal oxidase CioB	6.60 4.66	0.001 0.009
7.2	TTGA Gatat ATCAA	4.32E-05	-41.5	GOX2142	Hypothetical protein	0.46	0.058
7.1	TTGAT gcag ATCAA	3.98E-07	+37.5	<i>trxA</i>	Thioredoxin	3.06	0.103
4.3	TTGAT atcc ATCAA	1.00E-06	-41.5	<i>pntA1</i> <i>pntA2</i> <i>pntB</i> GOX0313 GOX0314	Transhydrogenase, α 1 Transhydrogenase, α 2 Transhydrogenase, β Alcohol:NAD ⁺ oxidored. Put. alcohol:NAD(P) ⁺ oxidoreductase	0.11 0.11 0.19 0.15 0.36	<0.001 0.003 0.010 <0.001 0.060
3.4	ATTCT cctt ATCAA TTGAT aagg AGAA T	3.53E-03 3.53E-03	+94.5 No TSS	<i>hemH</i> GOX0875	Ferrochelatase attachment protein	2.21 1.20	0.222 0.746
3.3	TGGT Tcggg GTCTA	6.29E-03	+13.5	GOX1500 GOX1501	Put. antitoxin with YefM-like domain Put. toxin with a PIN domain acting as nuclease	2.28 2.04	0.178 0.222
2.8	TTGAT cctc GTCA T ATGAC gagg ATCAA	1.89E-04 1.89E-04	-41.5 +144	GOX0090 GOX0091	ADP-dependent (S)-NADPH-hydrate dehydratase/NADPH-hydrate epimerase hypothetical protein	0.40 0.74	0.050 0.460

FIG 5 Overview of GoxR binding sites in the genome of *G. oxydans*, identified by ChAP-Seq analysis, that are correlated with increased or decreased expression of the target genes. A peak intensity of ≥ 2.8 was used as the cutoff value. From left to right, a plot of the relevant ChAP-Seq peak, the peak intensity, the GoxR binding motif sequence, the *P* value of this binding motif determined with the FIMO program using the consensus sequence (TTGATnnnnATCAA), the distance of the center of this motif to the TSS of the downstream gene, the genes regulated by this GoxR binding site, and the annotation of the corresponding genes are shown. Furthermore, the mRNA ratios (Δ *goxR* mutant versus parental strain) of the corresponding genes determined by RNA-Seq and the *P* values for the mRNA ratios are depicted.

all three cases, the binding motifs identified by bioinformatic means were present in the peak regions. In addition to the GoxR targets described above, ChAP-Seq identified GoxR binding sites upstream of five genes with ≥ 2 -fold altered mRNA levels in the Δ *goxR* mutant that were not included in Table 1 because the mRNA ratios did not meet the criterion of *P* values of ≤ 0.05 (Fig. 5; also see Table S1). Four of these genes showed increased expression in the Δ *goxR* mutant, i.e., *trxA* encoding thioredoxin (mRNA ratio of 3.06 [*P* = 0.10]), *hemH* encoding a ferrochelatase (mRNA ratio of 2.21 [*P* = 0.22]), and the GOX1500-GOX1501 operon encoding a putative toxin-antitoxin system (mRNA ratios of 2.28 and 2.04, respectively [*P* = 0.18 and *P* = 0.22, respectively]). The GoxR binding sites were centered at positions +37.5, +94.5, and +13.5 with respect to the TSSs of *trxA*, *hemH*, and GOX1500, respectively, supporting a repressor function of GoxR for these genes. In the case of GOX2142 encoding a hypothetical protein, expression was decreased in the Δ *goxR* mutant (mRNA ratio of 0.46 [*P* = 0.06]) and the GoxR binding motif was located at position -41.5 with respect to the TSS (39), suggesting that expression of GOX2142 is activated by GoxR.

Several genes that were found by ChAP-Seq to contain a GoxR binding site in the promoter region did not show altered expression in the Δ *goxR* mutant, such as GOX1988 (pyridoxine/pyridoxamine 5'-phosphate oxidase), GOX1070 (transcription termination factor Rho), GOX0691 (hemolysin D), GOX0692 (TetR family transcriptional

FIG 6 β -Galactosidase activity of an *E. coli* Δfnr strain transformed with the indicated pTrc99-based GoxR expression plasmids or the vector alone (minus GoxR) and the reporter plasmid pLacZ_*cioA* carrying the *lacZ* gene under the control of the *cioA* promoter or pLacZ_control carrying a promoterless *lacZ* gene (pl lacZ). The strains were cultivated under anaerobic conditions as described in Materials and Methods. Mean values and standard deviations of three independent biological replicates are shown.

regulator), GOX0875 (bacterial archaeo-eukaryotic release factor family 12), GOX0091 (hypothetical protein), and GOX0978 (riboflavin biosynthesis protein RibD) (Table S1). Therefore, the role of GoxR for expression of these genes remains unclear.

Evidence for the presence of an iron-sulfur cluster in GoxR. For biochemical characterization of GoxR, the expression plasmid pMal-c-*goxR*, which encodes a 69.6-kDa fusion protein composed of the *E. coli* maltose-binding protein (MBP) without its signal peptide followed by a tobacco etch virus (TEV) cleavage site and the GoxR sequence, was constructed. The fusion with MBP allowed overproduction of GoxR as a soluble protein in *E. coli* BL21(DE3), whereas overproduction of another GoxR variant, with an N-terminal decahistidine tag followed by a TEV cleavage site, using plasmid pET-TEV-*goxR* led to the formation of inclusion bodies (data not shown). In initial cultivation and purification experiments that were performed under aerobic conditions, induction with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.5 to 0.8 followed by cultivation overnight at 16°C were selected as suitable conditions for overproduction. For the purification of MBP-GoxR with amylose resin, the conditions suggested by the manufacturer (New England Biolabs) were applied. The anaerobic purification of MBP-GoxR was performed in an anaerobic chamber as described in Materials and Methods. The cell extract already had a dark green to brownish color, which was recovered in the elution fractions 3 to 6, suggesting the presence of an iron-sulfur cluster in the purified protein (Fig. S4). The fractions were analyzed by SDS-PAGE and found to contain a protein of the expected mass of ~69 kDa (data not shown). A UV-visible spectrum of elution fraction 3 of the anaerobically purified GoxR revealed, besides the 280-nm peak, a small peak at 420 nm, which suggested the presence of an iron-sulfur cluster (Fig. S4). After aerobic incubation of the same sample for 7 h, the brownish to dark green color and also the 420-nm peak of the spectrum disappeared, suggesting the destruction of an oxygen-labile iron-sulfur cluster (Fig. S4).

Identification of cysteine residues required for GoxR activity. In order to identify the cysteine residues that are involved in the formation of the iron-sulfur cluster, GoxR variants were constructed in which each of the five N-terminal cysteine residues, C14, C17, C25, C31, and C113, was exchanged individually to alanine using pTrc99a-*goxR* as the parental expression plasmid. The activity of the five GoxR variants was tested in an *E. coli* Δfnr strain that contained the *goxR* expression plasmids and the reporter plasmid pLacZ_*cioA*. As controls, pTrc99a and pLacZ were used. *E. coli* was used for this experiment because currently no compatible plasmid vectors are available for *G. oxydans*. As reported above, *cioA* expression is assumed to be repressed by GoxR, and inactive GoxR variants should lead to increased expression of *lacZ* under the control of the *cioA* promoter. After anaerobic cultivation, the LacZ activity of the various strains was measured; the values determined in the absence of GoxR were set as 100% (Fig. 6). In the presence of wild-type GoxR, LacZ activity was reduced by 70%, suggesting

that GoxR synthesized in *E. coli* is functional and is able to repress the *cioA* promoter. In contrast to wild-type GoxR, the variants GoxR-C17A, GoxR-C25A, and GoxR-C113A showed the same LacZ activity as the control without GoxR, suggesting that residues C17, C25, and C113 are essential for the synthesis of a repression-competent GoxR protein and therefore are presumably involved in formation of the Fe-S cluster. The variant GoxR-C14A showed intermediate LacZ activity, corresponding to about 50% of the value found in the absence of GoxR, suggesting that also C14 is important for GoxR-dependent repression and is presumably involved in Fe-S cluster formation. The variant GoxR-C31A showed the same LacZ activity as the strain with wild-type GoxR, suggesting that C31 is not required for GoxR activity and is presumably not involved in formation of the Fe-S cluster. This result is in accordance with the fact that C31 is not conserved in *Acetobacteraceae* GoxR homologs (Fig. S1). According to these data, C14, C17, C25, and C113 are likely to be involved in formation of the Fe-S cluster of GoxR. As the GoxR variants with C→A exchanges might not fold properly and are degraded, we attempted to determine the GoxR protein levels using an antiserum raised against *E. coli* FNR. However, the antiserum did not react with GoxR (data not shown).

DISCUSSION

Triggered by the finding that a shift from oxygen excess to oxygen limitation causes expression changes of about 500 genes in *G. oxydans* (21), we searched for putative oxygen-sensing transcriptional regulators and identified the GoxR protein (GOX0974) as a candidate. GoxR belongs to the FNR family of transcriptional regulators, which is part of the CRP superfamily of homodimeric transcription factors. Members of this superfamily are composed of an N-terminal sensory domain and a C-terminal DNA binding domain (25). FNR was first identified in *E. coli* and was shown to regulate the expression of genes involved in fumarate and nitrate respiration in dependence on oxygen (41, 42). FNR has become a paradigm for oxygen-sensing transcriptional regulators (22, 23, 26). The regulatory mechanism of FNR involves the assembly and degradation of an oxygen-sensitive [4Fe-4S] cluster in the N-terminal domain coordinated by the cysteine residues C20, C23, C29, and C122 (*E. coli* numbering). Under anoxic conditions, FNR contains a [4Fe-4S] cluster, which enables dimerization, site-specific DNA binding, and transcriptional regulation (43–46). Upon oxygen exposure, the [4Fe-4S] cluster is converted to a [2Fe-2S] cluster, which results in inactivation of FNR by monomerization and dissociation from DNA (47, 48). The [2Fe-2S] cluster of the monomeric FNR is completely lost after prolonged oxygen exposure (49). Recently, the crystal structure of the FNR protein of *Aliivibrio fischeri* was solved, which allowed a more precise description of the events occurring when the [4Fe-4S] cluster is converted to the [2Fe-2S] form (50).

The classification of *G. oxydans* GoxR as an FNR-type regulator was initially based on sequence similarity, particularly on the presence of the cysteine residues C14, C17, C25, C31, and C113 in the N-terminal domain and the presence of a DNA recognition helix in the C-terminal domain with an amino acid sequence very similar to that of *E. coli* FNR (see Fig. S1 in the supplemental material). Experimental support for the presence of an oxygen-labile iron-sulfur cluster in GoxR was obtained with the presence of a characteristic peak at 420 nm in the UV-visible spectrum and the brown to dark green color of an anaerobically purified MBP-GoxR fusion protein. The peak disappeared upon prolonged exposure to oxygen. Reporter gene assays in an *E. coli* Δ *fnr* strain with GoxR variants carrying C14A, C17A, C25A, C31A, and C113A amino acid substitutions suggested that C14, C17, C25, and C113 are involved in forming an iron-sulfur cluster. A notable difference between GoxR and *E. coli* FNR is the distance between the second and third cysteine residues involved in cluster formation, which spans five amino acids in the case of FNR but seven in the case of GoxR (Fig. S1). This difference can be assumed to affect properties of the iron-sulfur cluster, such as its oxygen sensitivity. FNR homologs with a seven-amino-acid distance between the second and third cysteine residues were reported previously (e.g., for *Bradyrhizobium japonicum* FixK1 [51]

and *Rhizobium leguminosarum* FnrN [52]), but to our knowledge the biochemical properties of these proteins have not yet been studied.

FNR-type regulators are characteristic of facultative anaerobic bacteria, in which they control the transition between aerobic and anaerobic energy metabolism. The presence of an FNR homolog in *G. oxydans* is surprising because, according to current knowledge, this species is not capable of anaerobic growth by respiratory or fermentative pathways and therefore is considered a strict aerobe. Consequently, the regulon of GoxR might differ from that of FNR-type regulators in facultative anaerobes. The GoxR regulon was determined by identifying the genes with altered expression in a ΔgoxR mutant by RNA-Seq, reporter assays, and enzyme activity measurements and by identifying the *in vivo* binding sites of GoxR by ChAP-Seq. Seven genes organized in three transcriptional units were identified as major GoxR targets. Two of them (*cioA* and *cioB*), encoding the *bd*-type ubiquinol oxidase CIO, showed increased expression, and two GoxR binding sites similar to the *E. coli* FNR consensus binding site TTGAT-N₄-ATCAA were found in the promoter region of *cioA*, centered at positions -2.5 and -56.5 with respect to the TSS of *cioA*. Their occupation by GoxR was confirmed by the ChAP-Seq experiment, indicating that transcription of *cioAB* is directly repressed by GoxR. Repression of *cioAB* by GoxR was also confirmed by reporter gene assays, in which LacZ activity was 30- to 58-fold higher in the ΔgoxR mutant than in the parental strain. In an *E. coli* Δfnr background, expression of *lacZ* under control of the *cioA* promoter was 3-fold higher in the absence of GoxR than in its presence, further supporting a repressor function of GoxR for *cioAB* expression. As described above, the non-proton-pumping terminal oxidase CIO has a lower oxygen affinity than the proton-pumping cytochrome *bo*₃ oxidase. Therefore, repression of the *cioAB* genes under oxygen deficiency seems reasonable, as it favors usage of oxygen by the energetically more efficient cytochrome *bo*₃ oxidase. It is interesting to note that the FNR homolog CydR from *Azotobacter vinelandii*, which like *G. oxydans* is an obligately aerobic bacterium, also represses the expression of the *cydA* and *cydB* genes encoding a cytochrome *bd*-type oxidase (53). Like CIO of *G. oxydans*, this oxidase from *A. vinelandii* has a low apparent K_m for oxygen of $4.5\ \mu\text{M}$ (54), and its synthesis increases with the oxygen supply to enable respiratory protection of the oxygen-sensitive nitrogenase (53). *G. oxydans* does not possess nitrogenase but is confronted with conditions requiring high terminal oxidase activities due to the rapid oxidation of substrates in the periplasm by membrane-bound dehydrogenases (16).

Among the genes showing reduced mRNA levels in the ΔgoxR mutant, the five genes of the *pnt* operon (*pntA1*, *pntA2*, *pntB*, GOX0313, and GOX0314) were the most striking. The results of the RNA-Seq analysis were supported by reporter gene assays, as well as by reduced activities of propionaldehyde reductase and hydrocinnamaldehyde reductase, which were previously shown to be catalyzed by the purified GOX0313 oxidoreductase (34). The presence of the GoxR consensus motif TTGATatccATCAA at position -41.5 with respect to the TSS of *pntA1* and the occupancy of this motif by GoxR in the ChAP-Seq experiment suggest that the *pnt* operon is directly activated by GoxR (55). What is the physiological function of the upregulation of the *pnt* operon? Under conditions of oxygen limitation, *G. oxydans* cells face the problem of insufficient NADH reoxidation, because they cannot switch to anaerobic respiration or fermentation. Loss of NADH reoxidation would result in a stop of cytoplasmic carbon source catabolism and growth. A possibility to ensure at least residual NADH oxidation could be the reduction of acetaldehyde to ethanol. Because *G. oxydans* lacks a functional TCA cycle, pyruvate formed by either the PPP or the EDP is converted by pyruvate decarboxylase to acetaldehyde, which is usually further oxidized to acetate by the NADP⁺-dependent acetaldehyde dehydrogenase GOX2018 (18). Under conditions of insufficient NADH reoxidation by NADH dehydrogenase, acetaldehyde could be reduced with NADH to ethanol by the GOX0313 oxidoreductase. Purified GOX0313 protein showed the highest activity for acetaldehyde reduction to ethanol (179 U/mg), whereas the activity for NAD⁺-dependent ethanol oxidation to acetaldehyde was only 20 U/mg (34). We

tested the idea that *G. oxydans* can form ethanol by incubating cells under oxygen-restricted conditions in a buffer containing glucose as a carbon source, and we could detect the formation of small amounts of ethanol over a period of 4 days (Fig. 4). The ΔgoxR mutant also formed ethanol under these conditions but at much lower levels, in agreement with the expectation that the ΔgoxR mutant possesses less GOX0313 activity than the parental strain (Fig. 3).

In addition to the *pnt* operon, GOX0090 was downregulated in the ΔgoxR mutant. The presence of a GoxR binding motif at position -41.5 with respect to the TSS of GOX0090 and the occupancy of this site by GoxR in the ChAP-Seq experiment indicate direct transcriptional activation of this gene by GoxR. The GOX0090 protein is a homolog of YjeF of *E. coli*, which was shown to function as an NAD(P)HX epimerase/dehydratase (35). NAD(P)HX (β -6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide) is a derivative of NAD(P)H that is formed either enzymatically by a side reaction of glyceraldehyde 3-phosphate dehydrogenase or spontaneously, and it functions as an inhibitor of NAD(P)⁺-dependent enzymes, such as glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase (56, 57). *E. coli* YjeF eliminates toxic NAD(P)HX by catalyzing the ADP-dependent dehydration of (S)-NAD(P)X with its C-terminal domain, while the N-terminal domain functions as an epimerase converting (R)-NAD(P)X to (S)-NAD(P)X (35). A similar function can be assumed for the GOX0090 protein of *G. oxydans*, which shows 30% sequence identity to YjeF. Because only the reduced forms, NADH and NADPH, can be hydrated, increased demand for this repair enzyme is expected under conditions of insufficient NAD(P)H reoxidation. Recently, it was reported that YjeF might have a moonlighting function that involves vitamin B₆ (58).

The reporter gene assays and the aldehyde reductase activities suggested that GoxR is active when the cells are cultivated with 15% DO. Two features might be debated as a basis for this result. The first feature relates to the very high activities of the membrane-bound dehydrogenases of *G. oxydans* in oxidizing carbon sources in the periplasm, which results in very high respiration rates. As a consequence, micro-aerobic or anoxic conditions are likely to prevail in the cytoplasm even when the DO level in the medium is still high, allowing the formation of active GoxR. The second feature is that the oxygen sensitivity of GoxR might be lower than that of *E. coli* FNR, due to, for example, the altered spacing of the third and fourth cysteine residues involved in forming the [4Fe-4S] cluster or other sequence alterations that support formation of an active GoxR dimer (45, 59). Further studies are required to compare the cytoplasmic oxygen levels in *G. oxydans* with those in bacteria that do not rapidly oxidize carbon substrates in the periplasm and to determine the oxygen responsiveness of GoxR in comparison to FNR.

In summary, our studies identified GoxR as an FNR-type regulator that regulates expression of genes involved in respiration and redox metabolism in *G. oxydans*, as summarized in Fig. 7. It appears likely that these results are also of relevance for other acetic acid bacteria, because GoxR, the terminal oxidase CioAB, the membrane-bound transhydrogenase PntA1A2B, the oxidoreductase GOX0313, and the NAD(P)HX epimerase/dehydratase GOX0090 (YjeF) are highly conserved in *Acetobacteraceae* strains.

MATERIALS AND METHODS

Materials. Chemicals and biochemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany), Qiagen (Hilden, Germany), Merck (Darmstadt, Germany), Roche Diagnostics (Mannheim, Germany), Fermentas (Thermo Fisher Scientific, Germany), Finnzymes (Thermo Fisher Scientific), or Becton, Dickinson GmbH (Heidelberg, Germany).

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2. The *Escherichia coli* strains were routinely cultivated in lysogeny broth (LB) or on LB agar plates at 37°C (60). For anaerobic cultivation of *E. coli* in sealed serum bottles, a minimal salt medium supplemented with 0.4% glycerol, 40 mM sodium fumarate, 20 mM trimethylamine N-oxide, and 10% (vol/vol) LB was prepared as described (61). When required, kanamycin was added to a final concentration of 50 $\mu\text{g/ml}$. *G. oxydans* 621H and mutant strains derived thereof were cultivated in mannitol medium containing 220 mM (4% [wt/vol]) mannitol, 5 g/liter yeast extract, 2.5 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g/liter $(\text{NH}_4)_2\text{SO}_4$, and 1 g/liter KH_2PO_4 . The initial pH value of the medium was 6.0. *G. oxydans* possesses a natural resistance to cefoxitin; as a precaution to prevent bacterial contaminations,

FIG 7 Model of the GoxR regulon in *G. oxydans* identified in this study. Genes that are activated by GoxR are shown in green boxes, and genes that are repressed by GoxR are shown in red boxes. For the genes in dashed boxes, the *P* values for the Δ goxR/wild-type mRNA ratio were above the standard threshold of 0.05.

cefexitin was added to the medium at a concentration of 50 μ g/ml. When required, kanamycin (50 μ g/ml) was added. Precultures were grown in baffled shaking flasks at 30°C and 140 rpm. For determination of growth parameters, RNA-Seq analysis, and aldehyde reductase assays, *G. oxydans* was cultivated in 250 ml of the same medium in a bioreactor system (DASGIP, Jülich, Germany) composed of four 400-ml vessels, each equipped with electrodes for measuring the DO concentration and the pH. The system enabled these two parameters to be kept constant. The carbon dioxide concentration in the exhaust gas was measured continuously by an infrared spectrometer and the oxygen concentration by a zirconium dioxide sensor. The oxygen availability was kept constant at 15% DO by mixing air, O₂, and N₂. Calibration was performed by gassing with air (100% DO) and 100% N₂ (0% DO). Anaerobic conditions were achieved by gassing with 100% N₂. The agitation speed was kept constant at 900 rpm. Controlling and recording of all data, as well as calculation of oxygen transfer rates and CO₂ transfer rates, were carried out by Fedbatch Pro software (DASGIP, Jülich, Germany). For oxygen limitation experiments, the bacteria were first cultivated at 15% DO to an OD₆₀₀ of 2.5, and then the culture was supplied with a gas mixture composed of 2% O₂ and 98% N₂.

General cloning methods and DNA techniques. For DNA manipulation, standard methods were used (62). For PCR, genomic DNA isolated from *G. oxydans* 621H Δ hdsR was used as the template. Competent *E. coli* cells were prepared with CaCl₂ and transformed as described (63). DNA sequencing was performed by Agowa (Berlin, Germany) or Eurofins (Ebersberg, Germany). Oligonucleotides were synthesized by Biolegio (Nijmegen, Netherlands) and are listed in Table 3.

Construction of plasmids. For heterologous overproduction of the GoxR protein, the *goxR* gene (GOX0984) was amplified from genomic DNA of *G. oxydans* using oligonucleotides 0974_ex_ndel_fw and 0974_ex_stopxho_rev, introducing a NdeI restriction site, including the ATG start codon, and an XhoI site after the stop codon. After digestion with NdeI and XhoI, the PCR product was cloned into pET-TEV, a pET28b derivative carrying a sequence coding for an N-terminal hexahistidine tag followed by a recognition site for TEV protease and a unique NdeI restriction site. The recombinant plasmid pET-TEV-*goxR* was transferred into *E. coli* BL21(DE3) and used to overexpress *goxR*, which resulted in the formation of inclusion bodies. Therefore, a second expression plasmid was constructed based on the expression vector pMal-c. The *goxR* gene, including the TEV recognition site in front of the native start codon, was amplified from the plasmid pET-TEV-*goxR* using oligonucleotides TEV-site-Eco-fw and 0974_ex_stopxba_rev, which introduced EcoRI and XbaI restriction sites. After digestion with EcoRI and XbaI, the PCR product was cloned into pMal-c digested with the same enzymes. The resulting plasmid pMal-c-*goxR* encodes a 69,602-kDa fusion protein of the *E. coli* MBP without signal peptide and GoxR, linked by a TEV cleavage site between the fusion partners.

To identify cysteine residues in GoxR that are involved in the formation of the predicted [4Fe-4S] cluster and therefore are important for GoxR activity, five GoxR variants were constructed. The *goxR* gene (GOX0984) was amplified from genomic DNA of *G. oxydans* using oligonucleotides 0974_ex_ndel_fw and 0974_ex_stopxba_rev, introducing a NdeI restriction site, including the ATG start codon, and an XbaI site after the stop codon. After digestion with XbaI, the PCR product was cloned into pTrc99a restricted with SmaI and XbaI to yield the plasmid pTrc99a-*goxR*. GoxR variants with the amino acid substitutions C14A, C17A, C25A, C31A, and C114A were constructed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Waldbronn, Germany). Plasmid pTrc99a was chosen because it is compatible with the pLacZ (pBBR) reporter plasmid. The pTrc99-based plasmids and selected pLacZ-based plasmid were transferred into a Δ *fnr* derivative of *E. coli* BW25113. This strain was constructed using *E. coli* JW1328 (BW25113 *fnr::Km^r*; Keio Collection [64]) as the parent. By expression of the Flp recombinase from plasmid

TABLE 2 Bacterial strains and plasmids used in this work

Bacterial strain or plasmid	Description ^a	Reference or source
Strains		
<i>G. oxydans</i> 621H	Type strain	DMSZ
<i>G. oxydans</i> Δ hsdR	621H Δ hsdR; hsdR (GOX2567) encodes type I restriction enzyme and is located on plasmid pGOX1	This work
<i>G. oxydans</i> Δ goxR	621H Δ hsdR Δ goxR; goxR (GOX0974) encodes FNR-type transcriptional regulator	This work
<i>G. oxydans</i> 621H::goxR-strep	621H variant encoding GoxR variant with C-terminal Strep-tag at the native genomic locus	This work
<i>E. coli</i> DH5 α	<i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA</i> <i>glnV44</i> Φ 80 Δ (<i>lacZ</i>)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	63
<i>E. coli</i> BL21 Star (DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>gal</i> <i>dcm</i> <i>rne131</i> (DE3)	Invitrogen
<i>E. coli</i> S17-1	Δ <i>recA</i> <i>endA1</i> <i>hsdR17</i> <i>supE44</i> <i>thi-1</i> <i>tra</i> ⁺	75
<i>E. coli</i> BW25113 <i>fmr::kan^R</i>	<i>rrnB3</i> Δ <i>lacZ</i> 4787 <i>hsdR514</i> Δ (<i>araBAD</i>)567 Δ (<i>rhaBAD</i>)568 <i>rph-1</i> <i>fmr::Km^r</i>	64
<i>E. coli</i> BW25113 Δ <i>fmr</i>	<i>rrnB3</i> Δ <i>lacZ</i> 4787 <i>hsdR514</i> Δ (<i>araBAD</i>)567 Δ (<i>rhaBAD</i>)568 <i>rph-1</i> Δ <i>fmr</i>	This work
Plasmids		
pK19mobsacB	Km ^r ; 5.721-kb suicide vector, <i>oriV_{Ec}</i> , <i>oriT</i> , <i>sacB</i>	66
pK19mobsacB-goxR	Km ^r ; 6.656-kb pK19mobsacB derivative containing 0.935-kb PCR fragment comprising fused flanking regions of <i>goxR</i>	This work
pKOS6b	Km ^r ; fluorocytosine-sensitive suicide vector derivative from pAJ63a, <i>upp</i> removed, <i>codBA</i> integrated	67
pKOS6b-goxR-strep	Km ^r ; fluorocytosine-sensitive plasmid for tagging <i>goxR</i> (GOX0974) genomically with C-terminal Strep-tag	This work
pET-TEV	Km ^r ; pET28b derivative for overexpression of genes in <i>E. coli</i> , adding N-terminal decahistidine tag and TEV protease cleavage site to target protein (pBR322 <i>oriV_{Ec}</i> , <i>pT7</i> , <i>lacI</i>)	76
pET-TEV-goxR	Km ^r ; 6.043-kb pET-TEV derivative encoding GoxR with N-terminal decahistidine tag followed by TEV protease cleavage site	This work
pMal-c	Ap ^r ; 6.148-kb P _{tac} -based expression vector for fusion proteins with <i>E. coli</i> MBP without signal peptide	New England Biolabs
pMal-c-goxR	Ap ^r ; 6.871-kb pMal-c derivative encoding MBP-GoxR fusion protein separated by TEV cleavage site	This work
pCP20	Ap ^r , Cm ^r ; 9.400-kb plasmid with temperature-sensitive replication encoding yeast Flp recombinase	65; Yale CGSC, USA
pBBR-K-Ppp3-lacZ (pLacZ)	Km ^r ; 7.416-kb pBBR1-MCS2 derivative for use as promoter probe vector with <i>lacZ</i> as reporter gene, designated pLacZ in this study	DSM, Kaiseraugst, Switzerland
pLacZ _{pntA1}	Km ^r ; 7.629-kb pLacZ _{pPPP3} derivative carrying <i>pntA1</i> (GOX0310) promoter region (314 bp) fused to <i>lacZ</i>	This work
pLacZ _{GOX0090}	Km ^r ; 7.648 kb pLacZ _{pPPP3} derivative carrying the GOX0090 promoter region (333 bp) fused to <i>lacZ</i>	This work
pLacZ _{cioA}	Km ^r ; 7.655 kb pLacZ _{pPPP3} derivative carrying the <i>cioA</i> (GOX0278) promoter region (340 bp) fused to <i>lacZ</i>	This work
pLacZ _{control}	Km ^r ; 7.315-kb pLacZ _{pPPP3} derivative without promoter region in front of <i>lacZ</i>	This work
pTRc99a	Ap ^r , <i>lacI^q</i> ; 4.176-kb expression vector with P _{tac} promoter and pUC18 EcoRI-HindIII polylinker region	77
pTRc99a-goxR	Ap ^r , <i>lacI^q</i> ; 4.897-kb pTRc99a derivative for P _{tac} -based <i>goxR</i> expression	This work
pTRc99a-goxR_C14A	Ap ^r , <i>lacI^q</i> ; 4.897-kb pTRc99a-goxR derivative with GoxR-C14A exchange (TGC→GCC)	This work
pTRc99a-goxR_C17A	Ap ^r , <i>lacI^q</i> ; 4.897-kb pTRc99a-goxR derivative with GoxR-C17A exchange (TGC→GCC)	This work
pTRc99a-goxR_C25A	Ap ^r , <i>lacI^q</i> ; 4.897-kb pTRc99a-goxR derivative with GoxR-C25A exchange (TGC→GCC)	This work
pTRc99a-goxR_C31A	Ap ^r , <i>lacI^q</i> ; 4.897-kb pTRc99a-goxR derivative with GoxR-C31A exchange (TGC→GCC)	This work
pTRc99a-goxR_C113A	Ap ^r , <i>lacI^q</i> ; 4.897-kb pTRc99a-goxR derivative with GoxR-C113A exchange (TGC→GCC)	This work

^aKm^r, kanamycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

pCP20 (65), removal of the chromosomally inserted kanamycin resistance cassette was achieved. This was necessary since kanamycin resistance would have impeded the use of the pLacZ-based reporter plasmids.

Cloning of plasmid-based transcriptional fusions of promoter regions with *lacZ*. The activity of GoxR was tested using reporter plasmids carrying transcriptional fusions of putative target gene promoters with a promoterless *E. coli lacZ* gene encoding β -galactosidase. The pBBR derivative pLacZ (kindly provided by DSM Nutritional Products Ltd., Kaiseraugst, Switzerland) served as the parent

TABLE 3 Oligonucleotides used in this work

Oligonucleotide	Oligonucleotide sequence (5' → 3') ^a	Application
0974_f1_xba_fw	GCTCTAGATGCTGTACCGTCTTCGGC	<i>goxR</i> deletion
0974_f1_fus_rev	<u>CATGCCCTTCGGCGATCATGATGAACCCCGTTCCGTTGCGCTGACCTCGG</u>	<i>goxR</i> deletion
0974_f2_pst_rev	TGCACTGCAGGATTGTTAACGCGGTACGC	<i>goxR</i> deletion
0974_f2_fus_fw	<u>ACGGGGTTCATCATGATCGCCGAAGGCATGGAATAGCCCGCTGTTTCGAC</u>	<i>goxR</i> deletion
0974_region_fw	CTCTGTCAGGACAGACGTCCCG	Region primer
0974_region_rev	GCCTTCAGGAGTCTCTGAACCTCC	Region primer
0974_gene_fw	CTGCAACAGCATTGACGACTGCG	Gene primer
0974_gene_rev	AGGTAATCCGCAATGTCGGTGC	Gene primer
<i>goxR-strep</i> _01_fw	ACAGCTATGACATGATTACG CCCGCCACGATTTCTTTG	Construction of pKOS6b- <i>goxR-strep</i>
<i>goxR-strep</i> _02_rev	CTCGAACTGTGGGTGGGACCA TTCATGCCTTCGGCGATCG	Construction of pKOS6b- <i>goxR-strep</i>
<i>goxR-strep</i> _03_fw	TGGTCCCACCCACAGTTCGAGAAG TAGCCCGCTGTTTCGAGGG	Construction of pKOS6b- <i>goxR-strep</i>
<i>goxR-strep</i> _04_rev	TGCATGCCTGCAGGTCGACT TAAAGTCACCGCGGGTCTTG	Construction of pKOS6b- <i>goxR-strep</i>
pKOS6b_seq_for	TGCTTCCGGCTCGTATGTTG	Sequencing
pKOS6b_seq_rev	GGATGTGCTGCAAGGCGATTAAAG	Sequencing
<i>goxR-strep</i> _seq_fw	AACAGTCAGCCGGATCCTCAG	Sequencing
<i>goxR-strep</i> _seq_rev	CTTCCTGGGACTGCTCTG	Sequencing
0974_ex_ndel_fw	CCCCATATGTCAGCGTCACATAGTCC	pET cloning, pTrc99a cloning
0974_ex_stopxho_rev	GGGCTCGAGCTATTCCATGCCTTCGGCG	pET cloning
TEV-site-Eco-fw	GCGCAATTCGAGAACCTGTATTTTCAGGGCCATATG	pMal-c cloning
0974_ex_stopxba_rev	GGGTCTAGACTATTCCATGCCTTCGGCG	pMal-c cloning, pTrc99a cloning
0090_lacZ_EcoRI_fw	CGGAATTCACCTGAAGCAGGACGGAGTTACG	pLacZ cloning
0090_lacZ_Ndel_rev	GGAATTCATATGGGGCGTTCTCTGCTGGAATC	pLacZ cloning
0278_lacZ_EcoRI_fw	CGGAATTCATCCCGACCATGAAAGAGCG	pLacZ cloning
0278_lacZ_Ndel_rev	GGAATTCATATGGTCGATTGCCTTCTGGGTAGATGG	pLacZ cloning
0310_lacZ_EcoRI_fw	CGGAATTCAAAGAAGGTCCACGAGCGC	pLacZ cloning
0310_lacZ_Ndel_rev	GGAATTCATATGCTGCGATCCTGTGTCTG	pLacZ cloning
0310_Race_sp1	AGGACGATGTCGGCATCGGC	5'-RACE
0310_Race_sp2	GCTGTCGGGGTAGGACGATGC	5'-RACE
0278_Race_sp1	ATGACGAGGCCGGAGACACGCG	5'-RACE
0278_Race_sp2	AAGGTCGAGATAGGCGCTGC	5'-RACE
delta0974_seq	CATCATGTCAGCGTCACATAGTCC	Sequencing
TG40GC_fw	GATGTCCATGATCGAGCCGCCATTGCGTG	Mutagenesis
TG40GC_rev	CCCACGCAATGGGCGGCTCGATCATGGACATC	Mutagenesis
TG49GC_fw	GATCGATGCGCCCATGCCGTGGGCGTCTG	Mutagenesis
TG49GC_rev	CAGACGACGCCACCGCATGGGCGCATCGATC	Mutagenesis
TG73GC_fw	CCGTCGCTGAGTATCGCCAACAGCATTGACGAC	Mutagenesis
TG73GC_rev	GTCGTCAATGCTGTTGGCGATACTCAGACGACGG	Mutagenesis
TG91GC_fw	GCAACAGCATTGACGACGCCGATCTGGCCGTTCTTG	Mutagenesis
TG91GC_rev	CAAGAACGGCCAGATCGGCGCTGCAATGCTGTTG	Mutagenesis
TG337GC_fw	GCAGCGTACGCTGGCCCGCTTCCCCATG	Mutagenesis
TG337GC_rev	CATGGGGAAAGCGGCCAGCGTAGCGCTGC	Mutagenesis

^aUnderlined sequences denote restriction sites, double-underlined sequences denote complementary sequences, and overlaps for Gibson assembly are written in bold letters.

plasmid. The promoter regions of the selected target genes, with a size of about 350 bp, were amplified by PCR and cloned into pLacZ using NdeI and EcoRI restriction sites. For construction of the control plasmid pLacZ_control carrying a promoterless *lacZ* gene, plasmid pLacZ was digested with NdeI and EcoRI, the 3' recessive ends were filled up with Klenow polymerase, and the plasmid was religated.

Transformation of *G. oxydans* by electroporation. The cells were made competent for electroporation by inoculating 60 ml mannitol medium with 1% (vol/vol) of an overnight culture of the strain to be transformed. At an OD₆₀₀ of 0.8 to 1.2, cells from 50 ml of culture were harvested (4 min, 4°C, 8,000 rpm) by centrifugation at 4°C, washed three times with 25 ml of ice-cold 1 mM HEPES buffer (pH 7.0), and then resuspended in 250 μl of 1 mM HEPES buffer (pH 7.0). The suspension was mixed with 20 μl of 75% glycerol per 100 μl of cell suspension, dispensed in 50-μl aliquots, shock frozen in liquid nitrogen, and stored at −80°C.

For electroporation, an aliquot of electrocompetent cells was thawed on ice and then transferred into an electroporation cuvette with 1-mm electrode distance (VWR, Germany). After addition of 100 to 300 ng of plasmid, the cuvette was placed in an electroporation chamber (Bio-Rad Laboratories, USA) and exposed to a pulse of 2 kV. The suspension was immediately mixed with 300 μl of electroporation medium containing 80 g/liter mannitol, 15 g/liter yeast extract, 2.5 g/liter MgSO₄·7H₂O, 0.5 g/liter glycerol, and 1.5 g/liter CaCl₂ (pH 6) and was transferred into a 15-ml Falcon tube for recovery overnight. The cells were then plated at different dilutions on selective solid medium and incubated for 2 to 3 days.

Transformation of *G. oxydans* by conjugation. The plasmid to be transferred into *G. oxydans* was first transferred into chemically competent *E. coli* S17-1. The recombinant *E. coli* S17-1 strain was

cultivated in 100 ml LB with kanamycin to an OD₆₀₀ of 0.6 to 0.8. The *G. oxydans* recipient strain was cultivated in 100 ml mannitol medium with cefoxitin to an OD₆₀₀ of 0.5 to 1. Then, 50 ml of each culture was centrifuged in Falcon tubes and washed twice with 25 ml *G. oxydans* mannitol medium without cefoxitin. After the second washing step, the *G. oxydans* cells were resuspended in the smallest possible volume of mannitol medium and mixed with *E. coli* S17-1 cells that had been treated similarly. The suspension was spotted on a mannitol medium agar plate without antibiotics and incubated at 30°C overnight. The cells were then scraped from the plate with 2 ml mannitol medium, and different dilutions were plated on mannitol medium agar containing kanamycin and cefoxitin (which selects for *G. oxydans* cells harboring the plasmid) and incubated for 2 to 3 days at 30°C.

Marker-free deletion of *goxR*. For marker-free deletion of the *goxR* gene in the *G. oxydans* genome, a two-step homologous recombination method based on plasmid pK19mobsacB (66), which is unable to replicate in *G. oxydans*, was used. Please note that very efficient alternative deletion methods exist for *G. oxydans* (67), which were not yet available when the Δ *goxR* mutant was constructed. In a first step, the upstream and downstream regions of *goxR* were amplified by PCR using the oligonucleotide pairs 0974_f1_xba_fw/0974_f1_fus_rev and 0974_f2_pst_rev/0974_f2_fus_fw. The oligonucleotides 0974_f1_fus_rev and 0974_f2_fus_fw carried complementary 5' ends, allowing fusion of the two PCR products by overlap extension PCR. The resulting 935-bp PCR product was digested with XbaI and PstI and cloned into pK19mobsacB that had been digested with the same enzymes. The resulting plasmid pK19mobsacB-*goxR* was transferred into *G. oxydans* by conjugation. Kanamycin- and cefoxitin-resistant *G. oxydans* clones were isolated on mannitol medium agar plates and incubated overnight in 50 ml mannitol medium supplemented with kanamycin and cefoxitin. The cells were then harvested, washed in mannitol medium with kanamycin, resuspended in 2 ml mannitol medium with cefoxitin but without kanamycin, and incubated for 6 to 10 h at 30°C. This incubation allows a second recombination step, leading either to the wild-type situation or to the desired deletion of *goxR*. The cells were then plated on mannitol medium agar plates containing cefoxitin and 10% (wt/vol) sucrose, the latter being toxic for cells still carrying the levansucrase gene *sacB*. Sucrose- and cefoxitin-resistant colonies that were kanamycin sensitive were analyzed by PCR for the presence or absence of the *goxR* gene. The *goxR* region was amplified with the oligonucleotides 0974_region_fw and 0974_region_rev, which results in a smaller PCR fragment in a Δ *goxR* mutant than in the wild-type strain. With the gene-specific oligonucleotides 0974_gene_fw and 0974_gene_rev, a PCR fragment is obtained only with the wild-type strain and not with the Δ *goxR* mutant. Three Δ *goxR* deletion mutants were selected and stored as glycerol cultures, one of which was used for further studies.

Construction of a *G. oxydans* strain encoding GoxR with a C-terminal Strep-tag. To construct a *G. oxydans* strain genomically encoding a GoxR variant with a C-terminal Strep-tag-II, a two-step homologous recombination system based on the plasmid pKOS6b was used (67). For this purpose, about 500 bp of the 3' terminus of *goxR* without the stop codon was amplified using the oligonucleotides *goxR-strep_01_fw* and *goxR-strep_02_rev*, generating a fragment with overlap to the pKOS6b backbone and including the sequence encoding a Ser-Ala spacer and the Strep-tag (SAWSHPQFEK). The second fragment, with overlap to the Strep-tag-encoding sequence, a stop codon, about 500 bp downstream of *goxR*, and overlap to the pKOS6b backbone vector, was obtained with *goxR-strep_03_fw* and *goxR-strep_04_rev*. The two PCR products and the digested (EcoRI and XbaI) pKOS6b plasmid were assembled using Gibson assembly (68). The resulting plasmid pKOS6b-*goxR-strep* was used to transform *E. coli* DH5 α . Positive clones were selected by colony PCR (pKOS6b_seq_fw and pKOS6b_seq_rev), and isolated plasmids were verified by sequencing. Then pKOS6b-*goxR-strep* was transferred into the *E. coli* strain S17-1 for conjugation into *G. oxydans* as described above. Cefoxitin- and kanamycin-resistant clones were isolated on agar plates and incubated overnight in 1 ml recombination medium (40 g/liter mannitol, 2.5 g/liter yeast extract, 1 g/liter MgSO₄·7H₂O) supplemented with both kanamycin and cefoxitin to a final concentration of 50 μ g/ml. Cells of the overnight cultures were harvested, washed twice with recombination medium, resuspended in 3 ml recombination medium with cefoxitin, and then incubated for ~6 h at 30°C. Positive clones containing the modified *goxR* gene but not the vector backbone were selected on agar plates with cefoxitin and 60 μ g/ μ l 5'-fluorocytosine. Correct integration into the *goxR* locus was confirmed by sequencing of the colony PCR product obtained with the oligonucleotides *goxR-strep_seq_fw* and *goxR-strep_seq_rev*. The resulting strain was named *G. oxydans* 621H::*goxR-strep*.

RNA sequencing and differential gene expression analysis. RNA was isolated using the RNeasy kit (Qiagen) and quality checked using the Agilent TapeStation 2200 system (Agilent Technologies) with RNA ScreenTape according to the manufacturer's instructions. To remove rRNA from all samples, the RiboMinus transcriptome isolation kit for yeast and bacteria (Invitrogen, Carlsbad, CA, USA) was used. rRNA-depleted RNA was precipitated with ethanol overnight at -20°C. The pellet was dissolved in 7 μ l nuclease-free water. Successful depletion was verified with the TapeStation using Agilent high-sensitivity RNA ScreenTape. Sequencing libraries were generated with the NEBNext Ultra II directional RNA library preparation kit for Illumina (New England Biolabs, Frankfurt, Germany) following the protocol for rRNA-depleted RNA using 11 cycles for PCR amplification of adaptor-ligated DNA. For quantification of the final cDNA libraries, the KAPA library quantification kit from KAPA Biosystems (Roche, Unterhaching, Germany) was used following the manufacturer's instructions. The quantitative PCR itself was conducted on a qTOWER v2.2 thermocycler (Analytik Jena, Jena, Germany). Two or four cDNA samples were pooled and then paired-end sequenced (2 $\times$ 75 cycles) on a MiSeq platform (Illumina, Munich, Germany) with the MiSeq reagent kit v3 (150 cycles). Read processing and strand-specific mapping to the *G. oxydans* 621H reference genome (GenBank accession numbers NC_006672.1 to NC_006677.1) were carried out

with the CLC Genomics Workbench (Qiagen, Aarhus, Denmark). Subsequent empirical analysis of differential gene expression for three biological replicates was performed with edgeR (69).

ChAP-Seq analysis for the identification of *in vivo* GoxR binding sites. For preparation of ChAP-Seq samples, the strain *G. oxydans* 621H::*goxR-strep* was used. A preculture was inoculated in 15 ml mannitol medium and incubated for 8 h at 30°C. Subsequently, 1 ml of the first preculture was transferred into 50 ml mannitol medium. After cultivation overnight, this second preculture was used to inoculate the main culture (1 liter in a 5-liter baffled Erlenmeyer flask) to an OD₆₀₀ of 0.5. The culture was incubated at 30°C and 130 rpm. In the late exponential phase (OD₆₀₀ of ~2.8), cells were harvested by centrifugation and washed once in 40 ml phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄ [pH 7.4]). Afterwards, the cell pellet was resuspended in 20 ml phosphate-buffered saline with 1% (vol/vol) formaldehyde and incubated for 20 min at room temperature to cross-link the Strep-tagged GoxR protein to DNA. To stop the cross-linking, glycine was added to a final concentration of 125 mM, followed by incubation for 5 min at room temperature. Afterwards, the cells were washed twice with 40 ml buffer A (100 mM Tris-HCl [pH 8.0], 1 mM EDTA), and the pellets were stored overnight at –80°C. Cell disruption, sonication of the DNA, purification of DNA bound to Strep-tagged GoxR, and the final DNA purification after protein digestion were performed as described (70). The DNA fragments obtained were prepared for sequencing using the NEBNext Ultra II DNA library preparation kit for Illumina following the manufacturer's protocol for DNA fragments without size selection. Adapter-ligated DNA was amplified by using 15 PCR cycles. Quantification of the resulting libraries was performed with the KAPA library quantification kit as described above and sequenced on a MiSeq system (Illumina) using paired-end sequencing with a read length of 2 × 150 bases. Mapping of the sequencing data and peak detection were performed with an analysis pipeline described recently for another transcriptional regulator (71). Briefly, mapping of the reads to the *G. oxydans* 621H reference genome (GenBank accession numbers NC_006672.1 to NC_006677.1) was performed using Bowtie2 (72) in local alignment mode with the following parameters: -D 20 -R 3 -N 0 -L 20 -i S,1,0.50. Based on the genomic read coverage, peaks were detected using convolution with a second-order gaussian kernel. The width of each peak was defined as the distance between two points with a coverage lower than one-half of the maximal peak height. Peak intensities were calculated by normalization of the highest read coverage to the average coverage of nonpeak genomic areas.

Determination of TSSs by 5'-RACE. To determine the TSSs of *cioA* and *pntA1*, the 5'-RACE method was performed with 1 µg RNA isolated from the *G. oxydans* parent strain and a 5'/3'-RACE kit (second generation) according to the protocol of the supplier (Roche Diagnostics, Mannheim, Germany). PCR fragments resulting from amplification with the PCR anchor primer and the gene-specific SP2 or SP3 primers (Table 3) were subjected to agarose gel electrophoresis, isolated, and sequenced. In the case of *cioA* (GOX028) and *pntA1* (GOX0310), two and five sequences, respectively, allowed determination of the TSSs (see Fig. S2 in the supplemental material).

Overproduction and purification of GoxR. The expression plasmid pMal-c-*goxR* was transferred into *E. coli* BL21(DE3) as the expression host. Cultures were grown at 37°C in LB inoculated with 1% (vol/vol) of an overnight preculture. At an OD₆₀₀ of 0.5 to 0.8, expression of the target gene was induced by addition of 0.5 mM IPTG, and the culture was subsequently incubated overnight at 16°C and 120 rpm. Subsequently, 125 ml of the culture was incubated overnight at 300 rpm and 4°C under anaerobic conditions in the presence of 10 mM ferric ammonium citrate and 10 mM L-cysteine hydrochloride. All subsequent steps were performed in an anaerobic chamber. Cells were harvested by centrifugation for 15 min at 4°C. One gram of cells (wet weight) was resuspended in 2 ml buffer A (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol) supplemented with complete protease inhibitor cocktail (Roche Diagnostics), and the cells were disrupted in 2-ml screwcap vials containing glass beads (0.1-mm diameter; 4 g glass beads per g cell wet weight) using a FastPrep disruptor (Fermentas). The cells were disrupted for 20 s and, after chilling for 3 min on ice, for an additional 15 s. The suspension was centrifuged for 20 min at 13,000 rpm in a tabletop centrifuge, and the cell extract was collected in a vial. For purification of the MBP-GoxR fusion protein, the cell extract was loaded onto a 1.5-ml column with amylose resin (New England Biolabs) equilibrated with buffer A. After washing with several column volumes of buffer A, MBP-GoxR was eluted with buffer A supplemented with 10 mg/ml maltose monohydrate, using fraction volumes of 0.5 ml. The purity of the isolated MBP-GoxR protein was checked by SDS-PAGE. Fraction 3 was used to record a UV-visible spectrum from 200 nm to 900 nm using a UV-2450 spectrophotometer (Shimadzu Corp., Kyoto, Japan), because it had the most intense color. For this purpose, the MBP-GoxR fraction was added to a quartz cuvette in the anaerobic chamber and sealed with a rubber stopper. For oxidation of MalE-GoxR, the protein solution was incubated under aerobic conditions with shaking on ice for 7 h before recording of the UV-visible spectrum.

β-Galactosidase assays. The pLacZ derivatives carrying various promoter regions (Table 2) were transferred into the *G. oxydans* parental strain and the Δ*goxR* mutant and cultivated as described in Results. For analysis of the impact of selected cysteine residues in GoxR on repression of the *cioA* promoter, *E. coli* BW213113Δ*fmr* transformed with pLacZ-*cioA* and one of the pTrc99a-*goxR* plasmids was cultivated anaerobically in minimal salt medium. Gene expression was induced by addition of 0.05 mM IPTG, and the cultures were subsequently incubated without shaking at 37°C until they reached an OD₆₀₀ of 0.5 to 0.6. Cells were harvested by centrifugation at 4°C for 10 min and then were used for β-galactosidase assays. The β-galactosidase activity was determined as described (73), by measuring the formation of *o*-nitrophenol from 2-nitrophenyl-β-D-galactopyranoside photometrically at 420 nm and 28°C for *G. oxydans* and *E. coli*.

Determination of NADH-dependent aldehyde reductase activity. The parent strain and the Δ*goxR* mutant were cultivated at 15% DO and 0% DO in a bioreactor system as described above. The

cells were harvested by centrifugation at $8,600 \times g$ and 4°C for 10 min. The cells were washed with 50 mM Tris-HCl (pH 7.5), resuspended in the same buffer, and lysed by two passages through a French pressure cell press (American Instrument Company, Silver Spring, MD, USA) at $1,100\text{ kg/cm}^2$. After ultracentrifugation at $160,000 \times g$ and 4°C for 1 h, the supernatant was obtained and used as the soluble fraction. NADH-dependent aldehyde reductase activity in the soluble fraction was determined spectrophotometrically at 340 nm as described (34); 10 mM propionaldehyde or 1 mM hydrocinnamaldehyde was used as the substrate. Hydrocinnamaldehyde was dissolved in dimethyl sulfoxide at 100 mM prior to the enzyme assay. One unit of enzyme activity was defined as $1\text{ }\mu\text{mol}$ substrate reduced per minute.

Ethanol production under oxygen-depleted conditions. Cells of the parental strain and the ΔgoxR mutant were cultivated in shake flasks with mannitol medium under vigorous shaking. When the cells had reached the stationary phase, they were harvested by centrifugation, washed, and resuspended in 50 mM morpholinepropanesulfonic acid (MOPS) buffer adjusted with KOH to pH 6.0. The density of the cell suspension was adjusted to an OD_{600} of 1. 6 ml of the cell suspension and 6 ml of a 44 mM glucose solution were mixed in a 15-ml Falcon tube. The free space in the tube was flushed with argon gas, and the tube was immediately closed tightly with a cap. The tube was shaken horizontally at 120 rpm at 30°C for the indicated times. Ethanol in the cell-free supernatant of the cell suspensions was determined by gas chromatography using an Agilent 7890A gas chromatograph as described previously (74). Calibration was performed with ethanol solutions from 0.05 to 3.2 mM as an external standard. The detection limit for ethanol was 0.1 mM under the conditions used. Butanol was added to the samples at a concentration of 2 mM as an internal standard. Concentrations were calculated from peak areas using calibration with external ethanol and internal butanol standards.

Data availability. Bacterial strains and plasmids described in this study are available from the corresponding author upon request. The RNA-Seq data were deposited in the GEO database with the accession number [GSE147810](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147810). The ChAP-Seq data were deposited in the GEO database with the accession number [GSE159734](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159734).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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