Microaerobic Lifestyle at Nanomolar O₂ Concentrations Mediated by Low-Affinity Terminal Oxidases in Abundant Soil Bacteria

Daniela Trojan, Emilio Garcia-Robledo, Dimitri V. Meier, Bela Hausmann, Niels Peter Revsbech, Stephanie A. Eichorst, Dagmar Woebken

ABSTRACT High-affinity terminal oxidases (TOs) are believed to permit microbial respiration at low oxygen (O₂) levels. Genes encoding such oxidases are widespread, and their existence in microbial genomes is taken as an indicator for microaerobic respiration. We combined respiratory kinetics determined via highly sensitive optical trace O₂ sensors, genomics, and transcriptomics to test the hypothesis that high-affinity TOs are a prerequisite to respire micro- and nanooxic concentrations of O₂ in environmentally relevant model soil organisms: acidobacteria. Members of the Acidobacteria harbor branched respiratory chains terminating in low-affinity (caq₂-type cytochrome c oxidases) as well as high-affinity (cbb₃-type cytochrome c oxidases and/or bd-type quinol oxidases) TOs, potentially enabling them to cope with varying O₂ concentrations. The measured apparent Km (K_mapp) values for O₂ of selected strains ranged from 37 to 288 nmol O₂ liter⁻¹, comparable to values previously assigned to low-affinity TOs. Surprisingly, we could not detect the expression of the conventional high-affinity TO (cbb₃ type) at micro- and nanomolar O₂ concentrations but detected the expression of low-affinity TOs. To the best of our knowledge, this is the first observation of microaerobic respiration imparted by low-affinity TOs at O₂ concentrations as low as 1 nM. This challenges the standing hypothesis that a microaerobic lifestyle is exclusively imparted by the presence of high-affinity TOs. As low-affinity TOs are more efficient at generating ATP than high-affinity TOs, their utilization could provide a great benefit, even at low-nanomolar O₂ levels. Our findings highlight energy conservation strategies that could promote the success of Acidobacteria in soil but might also be important for as-yet-unrevealed microorganisms.

IMPORTANCE Low-oxygen habitats are widely distributed on Earth, ranging from the human intestine to soils. Microorganisms are assumed to have the capacity to respire low O₂ concentrations via high-affinity terminal oxidases. By utilizing strains of a ubiquitous and abundant group of soil bacteria, the Acidobacteria, and combining respiration kinetics, genomics, and transcriptomics, we provide evidence that these microorganisms use the energetically more efficient low-affinity terminal oxidases to respire low-nanomolar O₂ concentrations. This questions the standing hypothesis that the ability to respire traces of O₂ stems solely from the activity of high-affinity terminal oxidases. We propose that this energetically efficient strategy extends into other, so-far-unrevealed microbial clades. Our findings also demonstrate that physiological predictions regarding the utilization of different O₂ concentrations
based solely on the presence or absence of terminal oxidases in bacterial genomes can be misleading.

**KEYWORDS** terminal oxidase, oxygen, acidobacteria, kinetics, transcriptomics

Oxygen (O₂) has a high redox potential (E°′ = +0.82 V), which, together with its ubiquity, makes it a favorable electron acceptor for energy generation. The concentration of O₂ across numerous microbial habitats can vary from saturation to anoxia (1). It is believed that aerobic microorganisms meet these fluctuating conditions by harboring low- and high-affinity terminal oxidases (TOs), presumably allowing them to use a wide range of O₂ concentrations.

Terminal oxidases, which mediate the final redox reaction in the electron transport chain (ETC) during aerobic respiration, are grouped into three superfamilies: (i) heme-copper oxidases (HCOs), (ii) cytochrome bd-type oxidases, and (iii) alternative oxidases. HCOs are multisubunit complexes and function as cytochrome c or as quinol oxidases, contributing to energy conservation, the generation of a proton motive force, O₂ scavenging, and maintaining redox homeostasis (2, 3). Based on overall amino acid similarities of the catalytic subunits and differences of the proton channels, the HCO superfamily is classified into three families: A (subfamilies A1 and A2), B, and C (4). Family A oxidases have a low affinity for O₂, with a reported Michaelis-Menten constant (Kₘ) for O₂ of 200 nmol O₂ liter⁻¹ (5). HCO families B and C are considered high-affinity TOs with high catalytic activity at low O₂ concentrations but reduced proton-pumping efficiency (6), with Kₘ values for the family C cbb₃-type oxidases of 7 to 40 nmol O₂ liter⁻¹ (7–9). The high-affinity cytochrome bd-type oxidase encoded by the cydAB genes (10–12) has reported Kₘ values of 3 to 8 nmol O₂ liter⁻¹ (13). Cytochrome bd-type oxidases do not pump protons across the membrane but contribute to proton motive force by using electrons from the extracytoplasmic side and protons from the cytoplasmic side (11).

High-affinity TOs are believed to sustain energy conservation at diminishing concentrations by enabling respiration at trace amounts of O₂ (i.e., micromolar O₂ concentrations) (14–16). Although there has been some suggestion that low-affinity TOs are present at micromolar O₂ concentrations in addition to high-affinity TOs (5), it remains unclear if the low-affinity TOs can actively and even solely contribute to respiration at these O₂ concentrations. At nanomolar O₂ concentrations, microorganisms transition from aerobic respiration to anaerobic-based metabolism (substrate-level phosphorylation or anaerobic respiration), referred to as the Pasteur point (17, 18). To the best of our knowledge, gene expression-based investigations of terminal oxidases at nanomolar O₂ concentrations are scarce (e.g., Gong et al. reported expression at O₂ levels of ≤200 nmol [19]), and therefore, it is mostly speculated that the high-affinity terminal oxidases are primarily responsible for energy production at low-nanomolar O₂ concentrations.

In soil, O₂ availability can be spatially and temporally dynamic, depending on the edaphic properties and microbial activity (20, 21). As such, microbial survival in soil is dependent on the ability to adapt to changes in local O₂ conditions. Environmental data and genome surveys suggest that both low- and high-affinity TOs are widely distributed in soils (16). Acidobacteria represent one of the most abundant and phylogenetically diverse phyla in soils worldwide (22–24) and are assigned a central role in carbon mineralization and plant polymeric carbon degradation (25, 26). Genes encoding high- and low-affinity TOs have been identified in several genomes of the phylum Acidobacteria (27), suggesting the capacity to respire across a wide gradient of O₂ concentrations. As respiratory flexibility can be attained through branched respiratory chains that terminate in multiple oxidases with different affinities for O₂ (15), this facet might be key to their ecological success in soil.

Using Acidobacteria as model soil organisms, we explored respiratory kinetics and evaluated their gene expression using whole-transcriptome sequencing and reverse transcription-quantitative PCR (RT-qPCR) across decreasing low-micromolar to nanomolar O₂ concentrations. As such, we could test the hypothesis that at micro-
nanomolar O₂ concentrations, aerobic respiration is mediated by high-affinity TOs. Our data demonstrate that O₂ concentrations down to the nanomolar level can be respired by low-affinity TOs, an unexpected physiological response, suggesting that the ability to respire O₂ under micro- to nanooxic conditions is not exclusively based on the presence and activity of high-affinity TOs.

RESULTS
Distribution of low- and high-affinity terminal oxidases. Five acidobacterial strains were chosen to explore their respiratory kinetics, and of these strains, three were chosen to explore their TO expression patterns across nanomolar O₂ concentrations. All strains harbored branched respiratory chains terminating in multiple oxidases (Fig. 1; see also Data Set S1 in the supplementary material). They differed in their distributions of low- and high-affinity TOs (complex IV) as well as of complexes III (cytochrome bc₁ complex and/or alternative complex III [ACIII]) (Fig. 1; Data Set S1).
Acidobacteriaceae bacterium KBS 83 and Terriglobus sp. strain TAA 43 harbored multiple homologs of only low-affinity TOs; Acidobacteriaceae bacterium KBS 83 encoded three A1 cca₃ HCOs and one A2 cca₃ HCO (Fig. 1a), whereas Terriglobus sp. TAA 43 had one A1 cca₃ HCO and two A2 cca₃ HCOs encoded (Fig. 1b). Terriglobus roseus KBS 63 had two homologs of A2 cca₃ HCOs (Fig. 1c), Edaphobacter sp. strain TAA 166 had one A1 cca₃ HCO and one A2 cca₃ HCO (Fig. 1d), and Acidobacterium capsulatum 161 had one A1 cca₃ HCO encoded (Fig. 1e). In addition to low-affinity TOs, T. roseus KBS 63, Edaphobacter sp. strain TAA 166, and A. capsulatum 161 also harbored high-affinity TOs: T. roseus KBS 63 had a cbb₃ type (C HCO) (Fig. 1c), Edaphobacter sp. TAA 166 had a bd type (Fig. 1d), and A. capsulatum 161 had both types (Fig. 1e).

There was consistent gene synteny for the A1 cca₃ HCO, A2 cca₃ HCO, C cbb₂ HCO, and bd-type quinol oxidases and the adjacent complex III genes among the acidobacterial strains (Fig. 1). Genes for the A1 cca₃ HCO were always located in an operon upstream of the genes encoding the bc complex (described here as a “superoperon”) (Fig. 1). The A2 cca₃ HCO also occurred in a superoperon with the genes encoding ACIII, instead of the bc complex, and were located downstream of the ACIII genes (Fig. 1). Additional, single homologs of either the A1 or A2 cca₃-type oxidases were detected in the genomes of Acidobacteriaceae bacterium KBS 83 (Fig. 1a), Terriglobus sp. TAA 43 (Fig. 1b), and T. roseus KBS 63 (Fig. 1c). T. roseus KBS 63 (Fig. 1c) and A. capsulatum 161 (Fig. 1e) contained cbb₂ operons consisting of genes for cbb₂ subunits N and O as well as an additional cco gene of unknown function. Edaphobacter sp. TAA 166 (Fig. 1d) and A. capsulatum 161 (Fig. 1e) contained both cydA and cydB subunits for the bd-type quinol oxidase.

Assessment of O₂ respiratory kinetics. We determined the O₂ respiration rates and population apparent Km (Kₘ(app)) values for the five acidobacterial strains with differing distributions of high- and low-affinity TOs in exponential phase (non-energy limited) with only O₂-limiting respiration rates (Fig. 2). All strains followed Michaelis-Menten-type kinetics. Acidobacteriaceae bacterium KBS 83 and Terriglobus sp. TAA 43, both harboring only low-affinity TOs, had Kₘ(app) values for O₂ of 166 ± 11 nmol O₂ liter⁻¹ (Fig. 2a) and 250 ± 5 nmol O₂ liter⁻¹ (Fig. 2d), respectively. The maximum population respiration rate (Vₘₜₐₜ) of Acidobacteriaceae bacterium KBS 83 was on average 355 ± 12 nmol O₂ liter⁻¹ h⁻¹, and the maximum respiration rates per cell (Rₘₜₐₜ) progressively decreased over time from 9.8 to 6.8 ± 0.4 fmol O₂ cell⁻¹ h⁻¹ (Fig. 2a; Table S1). The Vₘₜₐₜ of Terriglobus sp. TAA 43 was 998 ± 6 nmol O₂ liter⁻¹ h⁻¹, and the Rₘₜₐₜ was constant at 2.6 ± 0.02 fmol O₂ cell⁻¹ h⁻¹ (Fig. 2d).

For T. roseus KBS 63 and Edaphobacter sp. TAA 166, harboring both low- and either a cbb₃ or bd-type high-affinity TO, the Kₘ(app) values were 113 ± 24 nmol O₂ liter⁻¹ and 288 ± 34 nmol O₂ liter⁻¹, respectively (Fig. 2b and e). The Vₘₜₐₜ values of T. roseus KBS 63 and Edaphobacter sp. TAA 166 (201 ± 35 and 604 ± 69 nmol O₂ liter⁻¹ h⁻¹, respectively) were stable throughout the incubations (Fig. 2b and e). The Kₘ(app) value for A. capsulatum 161, harboring one low-affinity and both types of high-affinity TOs, decreased from 99 ± 14 to 37 ± 2 nmol O₂ liter⁻¹ (Table S2), with a final Kₘ(app) value of 37 ± 2 nmol O₂ liter⁻¹. The Vₘₜₐₜ and Rₘₜₐₜ values decreased for both strains (Fig. 2c). Additionally, the velocity curves saturated rapidly, compared to the other strains (Fig. 2e).

Differential gene expression due to changing O₂ concentrations. Of the five strains, we selected three that encompass different combinations of low- and high-affinity TOs to compare changes in gene expression levels when exposed to different, decreasing O₂ concentrations. Transcriptome analysis of Acidobacteriaceae bacterium KBS 83, T. roseus KBS 63, and A. capsulatum 161 showed that in the course of the time series, 5,121 (93% of all annotated genes), 4,239 (97%), and 3,321 (97%) genes,
respectively, were transcribed at least at one time point across the O₂ concentrations (Table S5).

The decrease from 10 to 0.1 μmol O₂ liter⁻¹ had the greatest impact on the transcriptomes of all three strains, with the highest number of significantly differentially expressed genes observed (Fig. 3a). Among 1,602 (31%) differentially expressed genes of *Acidobacteriaceae* bacterium KBS 83, 16% were upregulated and 15% were downregulated upon the transition from 10 to 0.1 μmol O₂ liter⁻¹ after cells equilibrated for 60 min at each respective O₂ concentration (Fig. 3b). For *T. roseus* KBS 63 and *A. capsulatum* 161, 38% (20% upregulated and 18% downregulated) and 81% (41% upregulated and 40% downregulated), respectively, were differentially expressed upon this transition from 10 to 0.1 μmol O₂ liter⁻¹ (Fig. 3b). Comparatively, there were few to no significant expression changes when transitioning from 0.1 to 0.001 μmol O₂ liter⁻¹ regardless of the equilibration time at the lower O₂ concentration; similar patterns were
observed in the transcriptome of \( T. \) roseus KBS 63 when transitioning from 0.001 to 0 \( \mu \)mol O\(_2\) liter\(^{-1}\) (Fig. 3a). The comparison between 10 and 0.001 \( \mu \)mol O\(_2\) liter\(^{-1}\) revealed the same overall transcription pattern as that for the transition from 10 to 0.1 \( \mu \)mol O\(_2\) liter\(^{-1}\) (Fig. 3). During these incubations, O\(_2\) was decreased in a stepwise manner from 10 \( \mu \)mol O\(_2\) liter\(^{-1}\) to anoxic conditions (\(<0.0005 \mu \)mol O\(_2\) liter\(^{-1}\)) but could no longer be accurately determined. A concentration of 0 \( \mu \)mol O\(_2\) liter\(^{-1}\) indicates no O\(_2\) supply. (b) Breakdown of differentially expressed genes (\( P < 0.05 \)) for 10 versus 0.1 \( \mu \)mol O\(_2\) liter\(^{-1}\) and 10 versus 0.001 \( \mu \)mol O\(_2\) liter\(^{-1}\).

**Fig 3** Impact of decreasing O\(_2\) concentrations on the transcriptomes of Acidobacteriaceae bacterium KBS 83, \( T. \) roseus KBS 63, and \( A. \) capsulatum 161. (a) Heat map depicting the proportions of genes that were differentially expressed (\( P < 0.05 \)) between two O\(_2\) concentrations (micromoles of O\(_2\) per liter). The darker the color, the higher the proportion of genes whose expression has significantly changed between two \( O_2 \) concentrations. All comparisons were done after 60 min at each respective \( O_2 \) concentration, with three exceptions: * depicts differential expression after 10 min, # depicts differential expression after 15 min, and ° depicts differential expression after 50 min. A concentration of 0.001 \( \mu \)mol O\(_2\) liter\(^{-1}\) is defined as apparent anoxia: O\(_2\) was still supplied (3.8 to 10.1 \( \mu \)mol O\(_2\) min\(^{-1}\)) but could no longer be accurately determined. A concentration of 0 \( \mu \)mol O\(_2\) liter\(^{-1}\) indicates no O\(_2\) supply. (b) Breakdown of differentially expressed genes (\( P < 0.05 \)) for 10 versus 0.1 \( \mu \)mol O\(_2\) liter\(^{-1}\) and 10 versus 0.001 \( \mu \)mol O\(_2\) liter\(^{-1}\).

In contrast, strains harboring both low- and high-affinity TOs (\( T. \) roseus KBS 63 and \( A. \) capsulatum 161) consumed all the supplied O\(_2\) at our lowest provided rate (i.e., 5.1 \( \mu \)mol O\(_2\) min\(^{-1}\) [\( T. \) roseus KBS 63] and 10.1 \( \mu \)mol O\(_2\) min\(^{-1}\) [\( A. \) capsulatum 161]). Their O\(_2\) uptake rates were higher than the O\(_2\) inflow rate, thereby creating an apparent anoxic environment below our detection limit of 0.0005 \( \mu \)mol O\(_2\) liter\(^{-1}\) (Fig. 4c and e).

**Transcriptional responses of branching electron transport chain key genes and terminal oxidases to decreasing O\(_2\) concentrations.** We further explored the transcriptional changes of TOs (complexes III and IV) of the ETC by focusing on key functional genes of these complexes (Fig. 4; Data Set S2).

(i) **Acidobacteriaceae bacterium KBS 83.** Continuous expression of two out of the four low-affinity \( \text{caaa}_3 \)-type cytochrome c oxidases, one of the \( \text{bc}_{1}-\text{A1 caa}_3 \) superoperons
Acidobacteriaceae bacterium KBS 83

Terriglobus roseus KBS 63

Acidobacterium capsulatum 161

FIG 4 Respiration dynamics and transcription patterns of complex III and IV genes in the electron transport chain of Acidobacteriaceae bacterium KBS 83, T. roseus KBS 63, and A. capsulatum 161 exposed to decreasing O₂ concentrations. (a, c, and e) Measured O₂ concentrations in cultures of the three strains over time (n= 4 biological replicates/strain) during O₂-limited incubations. O₂ was decreased in a stepwise manner from 10 to 1 to 0.1 to 0.001 to 0 μmol O₂ liter⁻¹. Vertical dashed lines depict the transition time points, while arrows indicate transcriptome sampling points after 60, 10, or 15 min at the respective O₂ concentrations. (b, d, and f) Time-resolved gene expression of complex III and complex IV at 10, 0.1, 0.001, and 0 μmol O₂ liter⁻¹. Heat maps show average transcript per million (TPM) values from biological replicates (n= 3). The last column depicts log₂ fold changes (log₂FC) of transcripts between 10 and 0.001 μmol O₂ liter⁻¹ after 60 min at the respective O₂ concentrations. Downregulation is depicted in blue, and upregulation is in red. Asterisks depict significant differential expression (P < 0.05). Catalytic subunits of terminal oxidases are in boldface type. A concentration of 0.001 μmol O₂ liter⁻¹ is defined as apparent anoxia: O₂ was still supplied (3.8 to 10.1 μmol O₂ min⁻¹) but could no longer be accurately determined. A concentration of 0 μmol O₂ liter⁻¹ indicates no O₂ supply. Data for all replicates, gene locus tags, and further details are listed in Data Set S2 in the supplemental material.
and the ACIII-A2 caa3 superoperon, was observed across all investigated O2 concentrations, even after exposure to 0.001 \( \mu \text{mol O2 liter}^{-1} \) for an extended period of time (Fig. 4b; Data Set S2); similar patterns were observed by RT-qPCR (Fig. S1a). All genes of superoperon ACIII-A2 caa3 exhibited significantly lower expression levels at 0.001 than at 10 \( \mu \text{mol O2 liter}^{-1} \) \((P < 0.05)\), yet the catalytic subunit ctaD of the A2 HCO was consistently highly expressed across O2 concentrations and not significantly downregulated (Fig. 4b). In contrast, ctaD of the A1 HCO complex together with petC of the bc1 complex were significantly upregulated at 0.001 \( \mu \text{mol O2 liter}^{-1} \) \((P < 0.05)\). The transcription level of the electron-receiving subunit II (ctaC) was higher than that of the rest of the bc1-A1 caa3 superoperon and remained high upon transitions to lower O2 concentrations (Fig. 4b); the same responses were observed within the first 10 min after shifts of oxygenation by RT-qPCR (Fig. S1a). We still observed gene expression 15 min after the O2 supply was ceased (Fig. S1a). Even then, the O2 concentration did not fall below 0.01 \( \mu \text{mol O2 liter}^{-1} \) (Fig. 4a), and Acidobacteriaceae bacterium KBS 83 was still expressing its TOs after 3 h at 0.01 \( \mu \text{mol O2 liter}^{-1} \) (Fig. 4b). Of the other complexes IV, only ctaE that encodes subunit III of the single complex IV exhibited high expression levels (Fig. 4b).

(ii) \textit{T. roseus} KBS 63. The expression levels (transcripts per million [TPM]) of the catalytic subunit of the cbb3-type high-affinity TO (ccoN) across the investigated O2 concentrations were low (Fig. 4d; Data Set 2) and too low for reliable quantification by RT-qPCR (Fig. S1b). The catalytic subunits of both low-affinity A2 HCO TOs (ctaD) exhibited the highest expression levels and were transcribed at significantly higher levels \((P < 0.0001)\) at 0.001 than at 10 \( \mu \text{mol O2 liter}^{-1} \) (Fig. 4d). All other genes of the ACIII-A2 caa3 superoperon were also upregulated (Fig. 4b). After a shift to anoxic conditions, the single ctaD gene was still expressed and upregulated (Fig. S1b).

(iii) \textit{A. capsulatum} 161. The cbb3-type high-affinity TO was transcribed at low levels at 10 \( \mu \text{mol O2 liter}^{-1} \) and was significantly downregulated \((P < 0.0001)\) at all subsequent lower concentrations (down to 0.001 \( \mu \text{mol O2 liter}^{-1} \)) (Fig. 4f; Data Set S2). Expression of the cbb3-type high-affinity TO by RT-qPCR was seen from 10 to 0.1 \( \mu \text{m} \text{O2 liter}^{-1} \) but only for 10 min at this concentration as measured by RT-qPCR (Fig. S1c). In contrast, the bd-type TO (cydAB) was expressed at all investigated O2 concentrations (10 to 0.001 \( \mu \text{mol O2 liter}^{-1} \)). The relative abundance of cydA transcripts was very high under all O2 tensions (58-fold higher than that of the rpoB gene) (Data Set S2). RT-qPCR showed a clear and significant \((P \lesssim 0.05)\) upregulation of the catalytic subunit cydA (Fig. S1c). Furthermore, cydA transcription levels were always high, even under anoxic conditions.

The ctaD gene, encoding the catalytic subunit of the low-affinity A1 HCO, was continuously transcribed across all O2 concentrations as detected by transcriptomics and RT-qPCR (Fig. 4f; Fig. S1c). However, the proportion of ctaD transcripts decreased under anoxic conditions (Fig. 4f).

**DISCUSSION**

Members of an abundant soil phylum, the Acidobacteria, respire environmentally relevant micro- and nanomolar O2 concentrations with the use of low-affinity TOs. Respiratory kinetics were determined using highly sensitive optical sensors, which allowed us to study the O2 kinetics with a high degree of accuracy. Our findings extend the current knowledge on O2 kinetics to species outside the Proteobacteria.

Acidobacteria harbor branched respiratory chains terminating in multiple complexes IV with either low or high affinities for O2. Branched ETCs terminating in differing terminal electron acceptors (such as O2, NO3, or NO2) are typically found in bacteria, providing flexibility when exposed to various environmental conditions (14, 15). Enzymatic redundancy in using a single electron acceptor (such as O2) can provide additional flexibility due to varying substrate affinities, allowing the microorganism to respire most efficiently across different concentrations, as seen in organisms living at the oxic-anoxic interface (28–34). This flexibility extends to our investigated soil acidobacterial strains, as many of them have branched ETCs that terminate in multiple complexes IV with either low or high affinities for O2 (Fig. 1). Furthermore, in select strains,
The conventional high-affinity cbb₃-type TO does not actively contribute to the capacity to respire O₂ at nanomolar concentrations. High-affinity TOs are historically believed to enable respiration and provide the capacity for energy conservation at trace concentrations of O₂, a physiology that was shown to be widespread among bacteria and archaea of diverse environments, as suggested by genome surveys (16). Yet in the investigated acidobacterial strains, the cbb₃-type high-affinity TO did not impart the capacity to respire O₂ at nanomolar concentrations. In our experimental setup, strains harboring high-affinity TO genes had the potential to develop low apparent Km values by expressing these TO genes under O₂-limited conditions, as in our incubations, the cells were exposed to multiple oxic-to-anoxic gradients over a 24-h period. Furthermore, our investigated strains harbor the minimal core, the CcoNO protein dyad (41), for the functionality of the enzyme (Fig. 1). Expression of the cbb₃-type oxidase could not be detected below 10 μmol O₂ liter⁻¹ in both strains T. roseus KBS 63 and A. capsulatum 161 (Fig. 4), although they indeed consumed O₂ down to (apparent) anoxia. Compared to reported Km(app) values for O₂ of Proteobacteria strains harboring cbb₃-type oxidases measured by the same method (42), the Km(app) value of T. roseus KBS 63 was high (113 nmol O₂ liter⁻¹) (Fig. 2b). This further provides evidence for the activity of the low-affinity oxidase(s) and suggests that it might be used for respiration in environments with low O₂ concentrations, such as the heterogeneous soil environment. O₂ fluctuations in soil are dynamic, and exposure to low-nanomolar O₂ concentrations might be temporally limited to short intervals (43). Therefore, we hypothesize that the investment in the expression of a less-energy-efficient TO (the high-affinity cbb₃ type) (44, 45) will not provide any competitive advantage for these investigated time intervals. At this time, it is unclear if the cbb₃-type oxidase has lost its function to generate proton motive force in these strains. Alternatively, cbb₃ TO expression in T. roseus KBS 63 and A. capsulatum 161 could be triggered by other factors, such as nutrient limitation or carbon depletion, as recently reported for Shewanella oneidensis (46).

Utilization of acidobacterial bd-type oxidases at nanomolar O₂ concentrations.

The bd-type oxidases are another type of high-affinity TO, which are less efficient at creating the charge gradient for ATP generation as they do not pump protons across the membrane but generate a proton motive force by transmembrane charge separation (12). Expression data showed a clear and significant upregulation of the catalytic subunit cdA gene as O₂ concentrations decreased in A. capsulatum 161 (Fig. 4f; see also Fig. S1c in the supplemental material). This suggests that the bd-type oxidase contributed to the respiratory activity under trace O₂ conditions. In contrast, the cbb₃ type was transcribed only at low levels at 10 μmol O₂ liter⁻¹ and was significantly downregulated (P < 0.0001) at all subsequent lower concentrations (Fig. 4f; Fig. S1c).

However, the use of the bd-type oxidase for respiration activity appears to be strain dependent. In another strain harboring a high-affinity bd-type oxidase (Edaphobacter sp. TAA 166), the expression of cdA could not be detected at any examined O₂ concentration; rather, the low-affinity TOs were expressed across these O₂ concentrations (RT-qPCR data not shown). Here, the bd-type oxidase could be contributing to physiological functions other than respiratory O₂ reductions, such as reactive oxygen species (ROS) stress, iron deficiency, or nitric oxide stress responses (11, 12, 47).

Although the bd-type oxidases are not as efficient at creating a charge gradient, these oxidases have functional and structural characteristics that favor a faster electron
flux than \( \text{cbb}_3 \)-type oxidases (11, 12), which could be advantageous under conditions with plentiful reducing potential stemming from carbon surplus. For instance, they receive electrons directly from the quinol pool and thereby take a shortcut through the branched ETC, bypassing any complexes III (Fig. 1). In support of this conjecture, \( \text{bd} \)-type oxidase genes were found to be more prevalent in environments where carbon is in excess, such as host-associated environments and carbon-rich forest soils compared to carbon-poor agricultural soils (16). As our investigated conditions were a combination of carbon surplus and \( \text{O}_2 \) limitation, we therefore hypothesize that this selected for the utilization of the \( \text{bd} \)-type oxidase compared to the \( \text{cbb}_3 \) type in \( \text{A. capsulatum} \) 161.

The strain expressing the \( \text{bd} \)-type oxidase under low \( \text{O}_2 \) concentrations (\( \text{A. capsulatum} \) 161) was the only one that was inhibited by high \( \text{O}_2 \) concentrations at its maximum respiration rate (\( R_{\text{max}} \)) (Fig. 2c) (>250 nmol \( \text{O}_2 \) liter\(^{-1}\)). Furthermore, its \( K_{\text{m(app)}} \) value decreased over multiple oxic-anoxic shifts \((n = 17)\) within 24 h, indicating a need for less substrate and, therefore, an adaptation to these conditions. This temporal kinetic development was previously observed for marine \( \text{Proteobacteria} \) (42). The final estimated \( K_{\text{m(app)}} \) value of \( \text{A. capsulatum} \) 161 (37 nmol \( \text{O}_2 \) liter\(^{-1}\)) suggests a mixed activity of low- and high-affinity TOs (Fig. 2c), with its high-affinity TO contributing a large portion of the \( K_{\text{m(app)}} \) value. This respiratory kinetic activity of \( \text{A. capsulatum} \) 161 suggests that this strain can use different \( \text{O}_2 \) concentrations due to its enzymes’ \( \text{O}_2 \) affinities. Presumably, this strain has a different strategy to exploit microoxic niches compared to the other investigated strains, which also could be advantageous in the soil when exposed to spatiotemporal gradients and diffusion limitations.

**Acidobacterial low-affinity TOs are used at nanomolar \( \text{O}_2 \) concentrations.**

Acidobacterial low-affinity \( \text{caa}_3 \)-type HCOs are functioning at previously unknown nanomolar \( \text{O}_2 \) concentrations, as shown in the investigated strains (Fig. 4; Fig. S1). The use of low-affinity A HCOs at low concentrations of \( \text{O}_2 \) is energetically favorable, as they have more free energy available for driving proton translocation due to poor \( \text{O}_2 \) binding (44, 45) and a more efficient, and thus favorable, gating for proton leakage (44) than high-affinity TOs. High-affinity C HCOs typically exhibit higher catalytic activity at lower \( \text{O}_2 \) concentrations due to a different redox-driven proton-pumping mechanism that allows an increased electron transfer rate and a faster reduction of \( \text{O}_2 \) (48). Still, these high affinities come with a reduced proton-pumping efficiency (6, 44).

Many of the genes for the \( \text{A2 caa}_3 \), HCO in \( \text{T. roseus} \) KBS 63 were not only expressed across varying \( \text{O}_2 \) concentrations but in some cases also even upregulated at lower \( \text{O}_2 \) concentrations (Fig. 4d; Fig. S1b). A continuous expression of low-affinity \( \text{caa}_3 \)-type TOs at low \( \text{O}_2 \) concentrations was previously reported in aerobic marine bacterial species (19, 49); however, in that study (19), the high-affinity \( \text{cbb}_3 \)-type TO was upregulated at <0.2 \( \mu \text{mol O}_2 \) liter\(^{-1}\). In our study, we did not observe any measurable contribution via transcriptomics or qPCR of the high-affinity \( \text{cbb}_3 \)-type TO in any of the strains at 10 to 0.001 \( \mu \text{mol O}_2 \) liter\(^{-1}\), although we cannot completely rule out the possibility of a minor contribution (undetectable with our current methods) of the \( \text{cbb}_3 \)-type TO. Likewise, it is conceivable that high-affinity \( \text{cbb}_3 \)-type TOs function only at extremely low concentrations of \( \text{O}_2 \) (<1 nmol \( \text{O}_2 \) liter\(^{-1}\)), which we currently cannot establish, maintain, and measure in the laboratory. Nevertheless, it appears that at the low \( \text{O}_2 \) concentrations (down to 1 nmol \( \text{O}_2 \) liter\(^{-1}\)) investigated in this study, \( \text{T. roseus} \) KBS 63 definitely prioritizes the low-affinity TOs. The energetic advantage of the low-affinity TOs might explain the strategy of \( \text{T. roseus} \) KBS 63 to invest in the high expression and upregulation of \( \text{A2 caa}_3 \) HCOs, compared to its \( \text{cbb}_3 \)-type high-affinity TO (Fig. 4d; Fig. S1b).

In contrast, \( \text{Acidobacteriaceae} \) bacterium KBS 83 harbored only low-affinity TOs (\( \text{caa}_3 \) type) and was able to respire at \( \text{O}_2 \) concentrations of 10 \( \mu \text{mol O}_2 \) liter\(^{-1}\) and lower. Below 0.01 \( \mu \text{mol O}_2 \) liter\(^{-1}\), it consumed \( \text{O}_2 \) at a respiration rate lower than the rate at which \( \text{O}_2 \) was supplied, causing concentrations not to reach anoxic conditions (Fig. 4a). However, complete consumption to anoxia was reached during the kinetics.
measurement experiments, reflecting the capacity to respire O₂ at trace concentrations. This difference could be explained by a lower cell density in the incubations for transcriptome analysis, not allowing these incubations to reach anoxia during the time course of the incubations simply due to cell number. Alternatively, O₂ diffusion could explain this discrepancy; this is unlikely as it was not observed in other incubations of the investigated acidobacteria. Efficient energy conservation (generating more ATP/electron) would be a vital survival strategy in times of substrate limitation in environments such as soil. It therefore might be an advantage to use low-affinity TOs even at nanomolar O₂ concentrations as they, despite their lower reaction rate, ultimately drive more charges across the membrane per mole of O₂, making them more efficient in energy conservation.

It appears that the capacity of Acidobacteriaceae bacterium KBS 83 to respire O₂ under low concentrations was limited, as seen by the decreasing $V_{\text{max}}$ and $R_{\text{max}}$ over time (Table S1). Its $K_{\text{m(app)}}$ value (166 nmol O₂ liter⁻¹) is lower than and in contrast to the previously reported $K_{\text{m}}$ value for the $\text{coa}_3$-type oxidase of Pseudomonas aeruginosa (4,300 nmol O₂ liter⁻¹) (8) but in the same range as the one for the low-affinity cytochrome $b_0$ ubiquinol oxidase of Escherichia coli (200 nmol O₂ liter⁻¹) (5). Although it is difficult to compare $K_{\text{m}}$ values across studies as the determined $K_{\text{m}}$ values can differ dramatically depending on the applied approach (8, 50), we want to stress the fact that one has to be careful with historically set benchmarks that propagate in the literature. The determined $K_{\text{m(app)}}$ values of our study represent ecophysiologically relevant estimates as we used whole populations and intact cells as well as highly sensitive optical sensors with an extremely low detection limit.

**Conclusion.** Microorganisms frequently have to cope with changing O₂ tensions; therefore, having the flexibility to use a wide range of O₂ concentrations is beneficial (16). Here, we show that members of a dominant and ubiquitous soil phylum (22, 24, 26), the Acidobacteria, have branched ETCs that terminate in multiple oxidases (high- and low-affinity TOs), providing them with respiratory flexibility and adaptability to environmental changes (14–16). More specifically, their low-affinity TOs are functioning at nanomolar O₂ concentrations, presumably providing a great benefit for soil acidobacteria as they are more efficient in generating ATP than high-affinity TOs (44). We hypothesize that this strategy could be employed by other bacterial clades in soil as well as other habitats. Follow-up work is needed to ascertain if respiration at nanomolar O₂ concentrations allows biomass production or population growth in the long run during exposure to such low O₂ levels. In addition, low O₂ concentrations and nutrient-rich conditions selected for the expression of the high-affinity $bd$-type oxidase rather than the $cbb_3$ type, which presumably provides a more optimal balance of substrate oxidation and ATP production under these conditions. Follow-up studies are needed to elucidate the conditions under which acidobacterial $cbb_3$-type TOs are employed for respiration. Our results extend the current knowledge on the respiratory flexibility of the prevalent Acidobacteria, which could help explain their success in the heterogeneous soil environment.

“Microaerobes” were previously defined as microorganisms that harbor high-affinity TOs in their genomes, either alone or in combination with low-affinity TOs, and use them to respire O₂ in microoxic environments (16). However, “microoxic” or subatmospheric concentrations of O₂ could be anything below 21% (vol/vol) O₂, and within this range, the response of TOs can vary dramatically. In our study, we pushed microoxic to nanooxic conditions and explored the transcriptional response combined with enzyme kinetics to obtain a state-of-the-art assessment of their response to O₂ tension. We detected high- and low-affinity TOs in multiple acidobacterial genomes and respiration at nanomolar O₂ concentrations across the investigated strains. Yet our gene expression data did not indicate any detectable contribution of the $cbb_3$-type high-affinity TOs at these O₂ concentrations; only one strain had contributions from the high-affinity $bd$-type TO. This suggests that the capability for microaerobic respiration in these acidobacteria is not solely due to the presence and associated activity of high-affinity TOs.
Instead, the acidobacterial microaerobic lifestyle seems to also be imparted by low-affinity $caq_{A}$-type TOs that enable them to respire $O_{2}$ at nanomolar concentrations. This illustrates that the presence of a high-affinity TO in a genome is not a prerequisite for microaerobic respiration. To that end, we would like to amend the definition of microaerobe to encompass microorganisms that are capable of respiring $O_{2}$ under microoxic conditions via the utilization of high- or low-affinity TOs. Furthermore, these findings demonstrate that it can be challenging to make predictions on the ecophysiology and lifestyle of microorganisms based solely on their genomic information, even for a process as well studied as aerobic respiration.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Five chemoorganotrophic strains of the family Acidobacteriaceae, Acidobacteriaceae bacterium KBS 83 (DSM 24295), Terriglobus sp. TAA 43 (LMG 30954; DSM 24187), Terriglobus roseus KBS 63 (NRRL B-41598; DSM 18391), Edaphobacter sp. TAA 166 (LMG 30955; DSM 24188), and Acidobacterium capsulatum 161 (ATCC S1196; DSM 1124), were grown in vitamins and salts base (VSB) medium (51, 52) amended with 10 mM glucose as the sole carbon source at pH 6 or 5 (A. capsulatum 161). Additional information on the strains was reported previously (27, 52–54).

**Setup and incubation for respiratory kinetic parameters.** The details of the setup and experimental procedure were previously described (34, 42, 55). Briefly, the incubations were conducted in custom-made 500- or 1,100-ml glass bottles, which had been sequentially rinsed with a solution containing 0.1 M NaOH, 0.1 M HCl, and autoclaved water to prevent contamination. A continuous flow of $N_{2}$ was maintained while filling the bottles with $N_{2}$-purred medium and subsequent sealing with ground-glass stoppers. Exponential-phase acidobacterial cells were injected into these bottles (2 to 3 replicates/strain), while glass-coated magnetic stirrers homogenized the suspension. The $O_{2}$ concentration was optically determined every 20 s by luminescence-based $O_{2}$ sensors (Lumos) with sensor spots (measurement range, 0.5 to 1,500 nmol $O_{2}$ liter $^{-1}$) (56) glued onto the inside of the bottles. Bottles were incubated at room temperature and shielded from light for 24 h. Air-saturated water (4 to 5 ml) was repeatedly injected into the bottles after anoxia was reached by cell respiration, with peak concentrations ranging from 600 to 1,620 nmol $O_{2}$ liter $^{-1}$. One milliliter of the cell suspension was collected and fixed with 1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) to determine cell numbers as described previously (42). After the incubations were completed, $O_{2}$ sensors were calibrated with oxygenated water and sodium dithionite.

**Calculation of kinetic parameters.** $O_{2}$ consumption rates were calculated from linear regression of $O_{2}$ concentrations over time in intervals of 6 min from the highest $O_{2}$ concentration down to anoxia. Kinetic parameters, the apparent half-saturation constant ($K_{m}$) and the maximum respiration rate ($V_{max}$) of the Michaelis-Menten equation, were estimated by performing nonlinear parametric fits on the respiration-versus-$O_{2}$-concentration curves for each replicate. $V_{max}$ and $K_{m}$ were varied iteratively until the best fit was obtained by least-square fits using Solver in Microsoft Excel (57). Maximum respiration rates per cell ($R_{max}$) were calculated by dividing the population respiration rate ($V_{max}$) by cell numbers.

**Michaelis-Menten plots of respiration rates versus $O_{2}$ concentrations were obtained by fitting a Michaelis-Menten model to the data using the equation $V = (V_{max} \times [O_{2}]) / (K_{m} + [O_{2}])$, where $V$ is the rate, $V_{max}$ is the maximum rate (nanomoles of $O_{2}$ per liter per hour), $K_{m}$ is the half-saturation constant (nanomoles of $O_{2}$ per liter), and $[O_{2}]$ is the substrate concentration (nanomoles of $O_{2}$ per liter).

**Transcriptional profiling incubations.** Acidobacteriaceae bacterium KBS 83, T. roseus KBS 63, and A. capsulatum 161 were grown in biological quadruplicates in glass bottles (Schott) containing 1 liter of VSB minimal medium amended with 10 mM glucose under fully aerated conditions. Once cells reached exponential phase, they were transferred into HCl-sterilized and autoclaved-water-rinsed glass bottles equipped with internally preglued sensing spots. Incubations were run for 225 min and split into four discrete, declining $O_{2}$ concentrations (10 $\mu$mol $O_{2}$ liter $^{-1}$, 1 $\mu$mol $O_{2}$ liter $^{-1}$, 0.1 $\mu$mol $O_{2}$ liter $^{-1}$, and 0.001 $\mu$mol $O_{2}$ liter $^{-1}$) down to anoxia (0 $\mu$mol $O_{2}$ liter $^{-1}$) obtained by purging with $N_{2}$-air mixtures (Table S3). $O_{2}$ concentrations were monitored by two Lumos systems with different sensitivity ranges (0.5 to 1,500 and 10 to 20,000 nmol $O_{2}$ liter $^{-1}$) (56). At every time point (Table S3), 30 to 50 ml of the culture was collected for RNA extractions by syringes fixed with a phenol-stop solution (58). The sensor spots were calibrated after the incubations with oxygenated water and sodium dithionite. Additional details can be found in Text S1 (Supplemental Materials and Methods 1) and Tables S1 and S2 in the supplemental material.

**RNA extraction and purification.** Total RNA was extracted from frozen cell pellets using an acidic phenol-chloroform–isooamyl alcohol protocol as described previously (59), with mechanical disruption (FastPrep-24 bead beater; MP Biomedicals, Heidelberg, Germany). The extraction supernatant was purified using standard chloroform–isooamyl alcohol purification, and RNA was precipitated using a polyethylene glycol (PEG) solution and RNA-grade glycogen by centrifugation (21,130 × g for 1 h at 4°C). Coextracted DNA was digested using a Turbo DNA-free kit (Thermo Fisher), and complete DNA removal was verified by failure to obtain quantitative PCR (qPCR) amplification products with the purified RNA template, targeting the rpoB gene encoding the $\beta$ subunit of the DNA-directed RNA polymerase, under the qPCR conditions described in Table S4. A more detailed protocol can be found in Text S1 (Supplemental Materials and Methods 3).
Microaerobic Respiration via Low-Affinity Oxidases

Primer design, cDNA synthesis, RT-qPCR, and data analysis. Specifications of the newly designed primers targeting the catalytic subunits (subunit I) of the TOs are listed in Table S4. See Text S1 (Supplemental Materials and Methods) for details on primer design, cDNA synthesis, reverse transcription-qPCR (RT-qPCR), and data analysis.

Transcriptome sequencing. Triplicate total RNA samples of Acidobacteriaceae bacterium KBS 83, T. roseus KBS 63, and A. capsulatum 161 from selected O2 concentrations and time points were sent to the Vienna BioCenter Core Facilities. RNA was depleted using the New England BioLabs (NEB) Ribo-Zero rRNA removal kit for bacteria. Sequencing was performed on an Illumina NextSeq 550 system, resulting in a total of 36 samples with 8.2 million to 18.2 million 75-nucleotide reads each.

Transcriptome data processing and statistical analyses. Raw reads were trimmed of sequencing adapters and low-quality 3’ ends using BBduk (BBtools v37.61; https://jgi.doe.gov/data-and-tools/bbtools/) with default parameters and error corrected using the Bayes-Hammer module of SPAdes assembler version 3.13.0 (60). Any reads mapping to either SILVA small-subunit (SSU) or large-subunit (LSU) release 132 (61) or the 5S rRNA database (62) with a sequence identity of >70% (performed with BBmap and BBTools; https://jgi.doe.gov/data-and-tools/bbtools/) were removed from the data set. The remaining reads were mapped to the publicly available genomes of the acidobacterial strains (53). The RNA reads per gene were summarized using the featureCounts tool from the Subread package v1.6.2 (63). Based on the generated read count tables, transcripts per million were calculated in R v3.6.0. Differential expression analyses, such as calculations of log2 fold changes of relative transcript abundances and the significance of these changes, were performed in DESeq2 v1.26.0 using default parameters and a P value cutoff of 0.05 (64).

Data availability. The raw transcriptomic reads are available under BioProject accession number PRJNA635786. The code and pipelines used for data analysis are available upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.04 MB.
FIG S1, EPS file, 1.1 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.01 MB.
TABLE S4, DOCX file, 0.02 MB.
TABLE S5, DOCX file, 0.02 MB.
DATA SET S1, XLSX file, 0.02 MB.
DATA SET S2, XLSX file, 0.03 MB.

ACKNOWLEDGMENTS

We thank the Division of Computational Systems Biology for providing and maintaining the Life Science Compute Cluster (LiSC) at the University of Vienna. We are grateful to Lars B. Pedersen at Aarhus University for his technical assistance and to Sergey Borisov at the Technical University of Graz for the production of optical oxygen sensors.

This work was funded by an Austrian Science Fund (FWF) project grant (grant number P26392-B20 to D.W. and S.A.E.), an ERDF Operational Programme and the Regional Government of Andalusia (project reference FEDER-UCA18-107225 to E.G.-R.), and the Dr. Anton Oelzelt-Newin’sche Stiftung of the Austrian Academy of Sciences (ÖAW). Support for the kinetic measurements and flow cytometry counts was obtained from the Poul Due Jensen Foundation.

REFERENCES


