Aerobic carboxydotrophy under extremely haloalkaline conditions in *Alkalispirillum/Alkalilimnicola* strains isolated from soda lakes

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INTRODUCTION

CO is a low-potential and very toxic electron donor which can be utilized as an energy source for aerobic lithoautotrophic growth only by a few highly specialized bacteria that possess a CO-insensitive respiratory chain. Such bacteria are known as carboxydrotrophs (Meyer et al., 1990; Mörsdorf et al., 1992). Recent work by Hardy & King (2001), Dunfield & King (2004) and Tolli et al. (2006) has shown that the actual diversity of carboxydrotrophic bacteria able to proliferate at low CO concentrations is substantially broader than previously anticipated. Recently, a large diversity of the [Mo–Cu] CO-dehydrogenase (CODH), a key enzyme of the CO-oxidizing system in aerobic carboxydobacteria, has been detected in various habitats by two degenerate primer pairs that specifically target the *coxL* gene, encoding the large catalytic subunit of CODH (King, 2003; Dunfield & King, 2004; King & Weber, 2007). This has substantially broadened the diversity of bacteria (both within the Proteobacteria and the Firmicutes) capable of utilizing CO as an electron donor compared with those previously recognized on the basis of cultivation at high CO concentrations. In contrast to carboxydrotrophs, actually growing on CO, many of the latter can only oxidize CO without autotrophic growth and are called ‘carboxydovores’. Most probably, they belong to the lithoheterotrophs.

A very recent example points to the possibility of aerobic CO oxidation under extremely haloalkaline conditions.
The genome sequence of a halokaliphilic gammaproteobacterium, Alkalilimnicola ehrlichii, from the haloalkaline Mono Lake, CA, USA, indicated the presence of a putative coxl gene, which was confirmed by PCR. Further physiological testing revealed the potential of this bacterium, as well as the type strain Alkalilimnicola halodurans and its closest relative Alkalilsirillum mobilis, to aerobically consume CO at various concentrations, but without autotrophic growth (Hoef et al., 2007). The absence of autotrophic growth in this case cannot be explained, as Alkalilimnicola can grow lithoautotrophically with other electron donors and has a complete set of RuBisCO genes. Accordingly, although the potential for aerobic CO oxidation under highly alkaline conditions has already been proven, the possibility of a carboxydotrophic mode of growth under these doubly extreme conditions remained unclear.

In this paper, we describe a group of Gammaproteobacteria that is capable of growth with CO under extremely haloalkaline conditions. All five carboxydotrophic isolates obtained from soda lake sediments belonged to the Alkalilsirillum/Alkalilimnicola group.

**METHODS**

**Samples.** Top 5–8 cm sediment samples were collected from 10 hypersaline soda lakes in Kuhinda Steppe (Altai, south-west Siberia, Russia) in 2008, with the pH and salinity of the brines ranging from 10.0 to 10.6 and from 60 to 300 g l\(^{-1}\), respectively. In addition, sediments from eight hypersaline alkaline lakes in Wadi Natrun (Lybian desert, Egypt; collected in 2000) with a pH and salinity ranging from 9.0 to 10.1 and from 180 to 300 g l\(^{-1}\), respectively, were used. Before use, the samples were kept at 4 °C. The individual samples from each area were pooled together in equal proportion to make two mixed-sediment inocula.

**Enrichment, isolation and cultivation of pure cultures.** A purely mineral medium based on sodium carbonate/bicarbonate strongly buffered at pH 10 and with a total Na\(^+\) content from 0.6 to 4 M (Sorokin et al., 2006a) was employed for enrichment and routine cultivation. Ammonium (4 mM) served as the nitrogen source. The concentration of Mo in the trace metal solution (Piening & Lippert, 1966) was increased to 40 μ g l\(^{-1}\). Routine incubations with CO as the electron donor were performed in 100 ml serum bottles capped with black butyl rubber and containing 50 ml mineral liquid medium. The gas phase contained 2–20 % O\(_2\) and 2–50 % CO, with the balance argon. The enrichment was inoculated with the fine fraction of the sediments (2 %, v/v) after removal of coarse sandy material by low-speed centrifugation. The incubation was performed at 30 °C statically. The culture was monitored by CO analysis in 0.1 ml gas-phase samples. When more than 50 % of the CO had been consumed, the enrichments (without the residual sediment) were subcultured several times at 1 : 100 dilutions and then serially diluted in 20 ml tubes with 5 ml liquid medium of the same composition placed into closed 3.5 l jars with the same CO/O\(_2\) content used in the positive seed cultures. The highest positive dilutions were also subjected to a second serial dilution or directly plated onto solid medium prepared by 1 : 1 mixing of the liquid soda base with 4 % washed agar at 50 °C. The plates were incubated under the same conditions as the serially diluted tubes. After a month of incubation, the dominant colony types were isolated into 5 ml liquid medium in tubes and grown in closed jars. The colonies which resulted in positive liquid cultures (determined by turbidity) were subjected to another round of solid-medium purification, and eventually, after confirmation of their purity by colony morphology, microscopy and denaturing gradient gel electrophoresis (DGGE), were characterized further.

The dynamics of growth were studied using 200 ml cultures in 1 l butyl rubber-capped bottles with a gas phase containing 10–20 % CO and 5 % O\(_2\). The bottles were incubated at 30 °C with gentle shaking at 100 r.p.m. When CO and O\(_2\) were depleted, the gas phase was replaced. The biomass growth was routinely monitored by measuring OD\(_{590}\) and cell protein (Lowry et al., 1951) after centrifugation of 1–2 ml culture. A large quantity of cells for enzymic analysis was produced in 20 l bottles with 5 l medium and several replacements of the gas phase during growth. The influence of pH on growth and activity (see below) with CO in pure cultures was investigated in media containing 0.6 M total Na\(^+\) either as NaCl (0.1 M HEPES, pH <8.5) or as sodium carbonate/bicarbonate (pH 8.5–11.0). The effect of sodium on growth and activity was examined at pH 10 using a carbonate/bicarbonate buffer system containing 0.1–4.0 M total Na\(^+\).

**Activity of washed cells.** Cultures were grown under defined conditions and the cells were harvested in late exponential phase by centrifugation, washed and resuspended at 20 mg protein ml\(^{-1}\) in sodium carbonate buffer, 0.6 M Na\(^+\), pH 10. Respiration rates with different electron donors were measured in a 4 ml final volume in the same buffer at a cell density of 0.1 mg protein ml\(^{-1}\) using an oxygen electrode (Yellow Springs Instruments). CO and H\(_2\) were introduced as saturated solutions in the same buffer maintained at 30 °C at a final concentration of 0.2 mM; sulfide, formate and acetate were used at a final concentration of 0.1 mM. In some cases, the CO-utilizing activity of washed cells (0.5 mg protein ml\(^{-1}\)) under different conditions was measured directly by analysing CO consumption from the gas phase.

**Enzymic activities.** Cells were disrupted by sonication and the extracts were separated into soluble and membrane fractions by ultracentrifugation at 144 000 g for 2 h. CODH and formate dehydrogenase (FDH) activities in the cell fractions were assayed spectrophotometrically according to Lorite et al. (2000) using a combination of phenazine methosulfate (PMS) and nitrotetrazolium blue (NTB) as artificial electron acceptors. The same method was employed for the in-gel activity staining. Briefly, native 8 % polyacrylamide gels were cut into two parts after completion. One part was stained for protein standards and the other part was washed and then incubated anaerobically in closed jars for 30 min in 0.1 M HEPES, pH 8/0.3 M NaCl/PMS + NTB in the gas phase. The membrane fraction was run in the presence of 0.1 % of the detergent β-d-dodecyl maltoside. Protein electrophoresis under denaturing conditions was done according to Laemmli (1970) using 5–20 % polyacrylamide gels. Sulfide-quinone reductase (SQR) activity in membranes was measured at pH 9 with 0.2 mM each of sulfide and decyl-ubiquinone. The 1 ml reaction mixture was incubated anaerobically in 1.5 ml HPLC screw-capped flasks for 30 min, and the disappearance of sulfide was analysed in 0.1 ml samples taken every 5 min. In controls, decyl-ubiquinone was omitted. Cytochrome c oxidase activity in membranes was measured spectrophotometrically at pH 8 using tetramethyl-p-phenylenediamine (TMPD) (1 mM) as substrate.

**Analyses.** CO and O\(_2\) concentrations in the gas phase were measured by a gas chromatograph (Varian CP 3800) equipped with a MolSieve capillary column (1.2 m x 1 mm; 13 x 80/100 mesh; 50 °C) and a thermal conductivity detector (TCD) (200 °C), with N\(_2\) as a carrier gas (2 ml min\(^{-1}\)). Formate was analysed by anionic chromatography HPLC [column HPX-87-H (Bio-Rad) at 60 °C; UV/IR detector; carrier 5 mM H\(_2\)SO\(_4\), 0.6 ml min\(^{-1}\)].
The cytochrome composition in cell fractions was analysed spectroscopically using a UV/visible diode array HP 8453 spectrophotometer. Formate (1 mM) and CO (0.4 mM) were tested as natural reductants, and ascorbate (1 mM) and dithionite (a few crystals) as high- and low-potential artificial reductants, respectively.

Phase-contrast microphotographs were obtained using a Zeiss Axiosplan Imaging 2 microscope. For electron microscopy, the cells were fixed in glutaraldehyde (final concentration 3 %, v/v) in 0.5 M NaCl, and after removal of the fixative stained with 2 % (w/v) uranyl acetate, and cells were imaged with a Jeol-100 electron microscope.

**DNA analysis.** Genomic DNA for total analysis was extracted by the phenol/chloroform method (Marmur, 1961). G+C content determination and DNA–DNA hybridization were performed by the thermal denaturation/reassociation technique (Marmur & Doty, 1962; De Ley et al., 1970) using Escherichia coli as a standard. Genomic DNA for PCR was extracted from the cell pellet using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories), following the manufacturer’s instructions. The 16S rRNA gene was amplified using general bacterial primers GM3(f) and GM4(r) (Schäfer & Muyzer, 2001). To amplify the cbbL gene fragment (800 bp), encoding the RuBisCO large subunit form I, a specially designed primer pair and protocol were used (Spiridonova et al., 2004). Detection of the cbbL gene, encoding the catalytic subunit of CODH, was performed according to Dunfield & King (2004) with primers OMFP-O/Br (type I) and BMSr-O/Br (putative). Genomic DNA from Oligotropha carboxydovorans was used as a positive control. The PCR products were purified from agarose using the QIAquick Gel Extraction kit (Qiagen). Phylogenetic trees were reconstructed using the TRECON W package (Van de Peer & De Wachter, 1994). The sequences obtained in this study were determined commercially (Macrogen, Korea).

**RESULTS**

**Enrichment, isolation and identification of pure cultures**

Primary enrichments from soda lake sediments at pH 10 using variable salt, CO and O₂ concentrations resulted in five positive cultures in which at least 50 % of the CO was consumed within a month of incubation. In general, this demonstrated that aerobic CO consumption was possible in soda lake sediments at CO and O₂ contents in the gas phase below 20 and 5 %, respectively, and an Na⁺ concentration below 2 M. Further combinations of dilution series and plating eventually allowed the isolation of a single bacterial pure culture from each of the five enrichments that was capable of growth with CO on purely mineral medium (Table 1). The bacteria were represented by motile vibrio-shaped cells of variable length (Supplementary Fig. S1). When CO was replaced with acetate, all strains accumulated a large amount of polyhydroxyalkanoates (PHA) (confirmed by Nile blue staining).

16S rRNA gene sequence analysis placed the ACO isolates in the Alkalispirillum (four strains)/Alkalilimnicola (one strain) cluster in the family Ectothiorhodospiraceae of the Gammaproteobacteria (Fig. 1a), where the potential to consume (but not to grow with) CO has been demonstrated in Alkalilimnicola ehrllichii (Hoef et al., 2007). It may be that low oxygen and CO concentrations are critical to achieve carboxydrotrophic growth in this group of haloalkaliphiles. Our previous work under variable denitrifying conditions also resulted in a selection of several Alkalispirillum/Alkalilimnicola members from soda lake sediments (Sorokin et al., 2006b). Two out of three of those earlier isolates, namely Alkalispirillum sp. AGDZ and Alkalispirillum sp. ALPs2, turned out to be able to grow with CO (20 % CO/5 % O₂). On the other hand, an H₂-utilizing strain, Alkalilimnicola sp. AHN1, from the same habitat neither consumed nor grew on CO. From this, we may conclude that the carboxydrotrophy under microoxic conditions is an inherent property of the genus Alkalispirillum (six confirmed isolates). Whether this also applies to the genus Alkalilimnicola (Alkalilimnicola ehrllichii and one of the ACO isolates) remains to be seen.

DNA–DNA hybridization data were also consistent with the 16S rRNA gene-based clustering of the ACO isolates. Strains ACO1, 2, 3 and 4 had DNA similarity above 65 % between each other, with the type strain Alkalispirillum mobile, and with the previously described denitrifying isolates Alkalispirillum AGDZ and ALPs2, while strain ACO5 showed only 30–35 % similarity with the Alkalispirillum ACO strains and obviously belonged to the genus Alkalilimnicola.

**Table 1.** Isolation of pure cultures of haloalkaliphilic carboxydrotrophs from soda lakes

<table>
<thead>
<tr>
<th>Source</th>
<th>Condition (pH 10)*</th>
<th>CO concn (%)</th>
<th>O₂ concn (%)</th>
<th>Na⁺ concn (M)</th>
<th>Strain</th>
<th>Cell morphology</th>
<th>DNA G+C (mol%)</th>
<th>NCCB† no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kulunda steppe</td>
<td></td>
<td>20</td>
<td>5</td>
<td>0.6</td>
<td>ACO1</td>
<td>Bean-shaped rod</td>
<td>65.6</td>
<td>100201</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>5</td>
<td>2.0</td>
<td>ACO2</td>
<td>Short vibrio</td>
<td>66.0</td>
<td>100202</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>5</td>
<td>0.6</td>
<td>ACO4</td>
<td>Long vibrio</td>
<td>65.5</td>
<td>100203</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>5</td>
<td>0.6</td>
<td>ACO5</td>
<td>Short vibrio, chains</td>
<td>64.5</td>
<td>100204</td>
</tr>
<tr>
<td>Wadi Natrun</td>
<td></td>
<td>10</td>
<td>5</td>
<td>2.0</td>
<td>ACO3</td>
<td>Short vibrio</td>
<td>65.8</td>
<td>100205</td>
</tr>
</tbody>
</table>

*Negative results were obtained when the following concentrations were used: CO >20 %; O₂ >5 %; Na⁺ >2 M.
†Netherlands Culture Collection of Bacteria.
Analysis of the **cbbL** gene, encoding the form I large subunit of RuBisCO (the key enzyme of the Calvin–Benson cycle of autotrophic CO₂ assimilation) showed that it was universally present in the CO-utilizing isolates. The phylogeny of this gene was consistent with the clustering on the basis of the 16S rRNA gene (Fig. 1b). This gene has also been detected in the genome of *Alkalilimnicola ehrlichii* (Hoeft et al., 2007) and in the denitrifying *Alkalipirillum/Alkalilimnicola* isolates from soda lakes (Tourova et al., 2007).

**Growth physiology**

Strain ACO3 was able to grow with CO at concentrations up to 10% in the gas phase; ACO2, 4 and 5 up to 20%; and
ACO1 up to 30% after a gradual adaptation. The CO-growing cultures were inhibited by oxygen concentrations above 5% in the gas phase at the beginning of growth and tolerated up to 10% when the cultures reached sufficient density (above 20 mg cell protein l⁻¹). Despite being active denitrifiers (with acetate), the ACO strains were unable to use CO as electron donor under denitrifying conditions at concentrations of 1–20% (with nitrate or N₂O as acceptors). Furthermore, in contrast to most of the known aerobic carboxydotrophs (King & Weber, 2007), and closely related *Alkalilimnicola ehrlichii* (Hoeft *et al.*, 2007) and *Alkalilimnicola AHN1* (Sorokin *et al.*, 2006b), the ACO strains failed to grow with H₂, under either microoxic or denitrifying conditions. On the other hand, all of them grew aerobically with formate as the sole carbon and energy source at full oxygen (atmospheric air, 20% O₂) with growth rates similar to growth on CO. The maximum experimental growth rate for growth on CO and formate in different ACO strains varied from 0.015 to 0.022 h⁻¹; heterotrophic growth with acetate was 10 times faster, with rates from 0.15 to 0.20 h⁻¹. The maximal experimental growth yields with CO, formate and acetate determined for strain ACO1 at pH 10 and 0.6 M total Na⁺ were 1.05–1.4 mg cell protein mmol⁻¹ (five different experiments), 1.2 mg cell protein mmol⁻¹ (single experiment) and 10.0–10.5 mg cell protein mmol⁻¹ (two experiments), respectively. With CO alone, the CO consumption was parallel to biomass growth (Fig. 2a). Under mixotrophic conditions with CO and acetate, a much higher biomass was reached, but the growth rate resembled that of carboxydotrophic cultures, indicating suppression of acetate-dependent energy metabolism by CO (Fig. 2b).

**Influence of pH and salinity on growth and activity**

With respect to their pH response, the ACO isolates belonged to the obligate alkaliphiles. Autotrophic growth with CO was possible within a pH range from 8.0 to 10.5, with an optimum at around 9.5 (Fig. 3a). The respiration of washed cells grown at pH 10 with CO had a much broader pH range and was still active up to pH 11.7, which is the highest pH for activity among the known haloalkaliphilic chemolithoautotrophs (Sorokin & Kuenen, 2005). Sulfide oxidation by the cells grown with CO (see below) and acetate oxidation by heterotrophically grown cells of strain ACO1 also had a highly alkaline pH optimum at 9.5–10.0, but these activities were definitely less alkalinotolerant than the CO respiration (Fig. 3a). At pH 10, the ACO strains grew immediately with CO in soda brines at a salt content of up to 2.5 M total Na⁺, and after adaptation at 2 M at up to 3.5 M Na⁺ (Fig. 3b). The same salt limit was observed for heterotrophic growth with acetate, except that adaptation was not necessary and that two strains (ACO3 and 4) tolerated salt-saturating conditions (4 M Na⁺). Concerning their salinity optimum, however, the ACO strains definitely belonged to a moderately salt-tolerant type, with growth and activity maxima around 1 M total Na⁺.

![Figure 2](http://mic.sgmjournals.org)  
**Fig. 2.** Growth dynamics of strain ACO1 under lithoautotrophic conditions with 20% CO (a) (results of two independent experiments) and (b) in mixotrophic culture (5 mM acetate + 20% CO) in comparison with heterotrophic culture on acetate (5 mM). Symbols: ●, biomass growth in carboxydotrophic and mixotrophic cultures; ○, biomass growth in heterotrophic culture with acetate; ▼, CO consumption.

**Metabolic activity of resting cells**

The CO-oxidizing capacity was fully expressed only when CO was used as the sole substrate and was inhibited in cells grown with either formate or acetate (Fig. 4). Acetate metabolism was also repressed in the presence of CO, which corresponded to the results of growth experiments. On the other hand, the CO-grown cells respired formate with the same activity as CO, although the activity was 40% lower than in cells grown with formate. While the same phenomenon has been shown in the neutrophilic carboxydrotroph *Oligotropha carboxydovorans* (formerly known as *Pseudomonas*) (Meyer & Schlegel, 1978), the formate oxidation activity induced in CO-grown cells of *Oligotropha* was 10 times lower than the CO-oxidizing activity. Furthermore, the cells of all ACO strains grown with either CO or formate also exhibited a significant sulfide-dependent
respiration in contrast to acetate-grown cells. While the ability to oxidize sulfide is known for *Alkalispirillum*/*Alkalilimnicola* (Oremland et al., 2002; Sorokin et al., 2006b), the high level of formate-oxidizing activity in the CO-grown cells of all ACO strains is somewhat unexpected.

**Enzymic activity in cell fractions**

Four different enzyme activities were detected in the cell-free extract and its fractions obtained from the ACO1 cells grown with CO. In the membrane fraction, CODH, FDH, SQR and cytochrome c oxidase were detectable. In the soluble fraction, only CODH was present (Table 2). The CODH in membranes had a lower pH optimum than the FDH, but both were only moderately alkalitolerant as compared with the whole-cell respiration, with pH optima at 8.5 (CODH) and 9.0 (FDH). This might be the result of either the intracellular localization of these two dehydrogenases (i.e. the internal side of the cell membrane) or an acidic shift in the pH profile of the enzymes under *in vitro* conditions with artificial electron acceptors.

Further evidence on the presence of two different enzyme systems responsible for the oxidation of CO and formate in ACO1 was obtained by comparing the protein expression of cells grown with different substrates and by in-gel activity staining (Fig. 5). Comparison of total cell extracts from the cells grown with CO, formate and acetate identified two closely located polypeptide bands with apparent masses above 100 kDa uniquely expressed during growth on CO (white arrows in Fig. 5a). Another band with a mass of 56 kDa was present both in CO- and formate-grown cells but not in the acetate-grown cells (Fig. 5a). Further comparison of different cell fractions demonstrated that the soluble fraction of the CO-grown cells contained only one high-molecular-mass band unique for CO, while both of them were present in the membranes (Fig. 5b). Analysis of the membrane fraction also made it clear that the CO- and formate-grown cells shared two unique polypeptides with apparent masses of 56 and 15 kDa (Fig. 5b). These observations led to the conclusion that the two high-molecular-mass bands with masses >100 kDa, expressed in the CO-grown cells, belonged to a CODH, while the 56 and 15 kDa bands, present in the membranes of both CO- and formate-grown cells, belonged to an FDH. Activity staining of cell fractions from the CO-grown ACO1 cells (Fig. 5c) demonstrated the presence of two different high-molecular-mass complexes with CODH activity in soluble and membrane fractions, with apparently higher activity in the membranes, and a single FDH complex in the membranes. All three complexes had different molecular masses, indicating that the CODH and FDH are different enzymes in ACO1.

Overall, these data corresponded well to the results of activity measurements on the level of whole cells and in the cell-free extracts discussed above. On the other hand, the high molecular mass of the two polypeptide bands specific for CO-grown cells did not correspond to what is known for the classical CODH in other aerobic carboxydrotrophs, which have a mass for the large catalytic α-subunit in the range of 75–88 kDa. That the enzyme from ACO1 might differ from the classical CODH was also evident from the fact that the two primer pairs specific for the coxL gene in all known aerobic carboxydrotrophs (King & Weber, 2007) did not result in any amplification when the genomic DNA of ACO strains was used as a template. Attempts to vary the amplification conditions were not successful.
Cytochrome reduction in cell fractions of strain ACO1

Addition of CO and formate to the whole-cell extract from ACO1 cells grown on CO resulted in a rapid reduction of cytochromes c\textsubscript{551} and b\textsubscript{557} (Supplementary Fig. S2). When the extract was separated into soluble and membrane fractions, only CO was able to act as a reductant for cytochromes. These results indicated that for both CODH and FDH, the initial accepting cytochromes were soluble. In contrast to the neutrophilic carboxydotrophs, the soluble cytochrome pool accepting electrons from the CODH in alkaliphilic strain ACO1 included cytochrome c. The fact that there was almost the same degree of reduction of soluble cytochromes with CO and ascorbate indicates that these cytochromes are high-potential (≥ +100 mV). In the membranes, difference spectra showed the presence of two CO-reacting cytochromes, b\textsubscript{558} and b\textsubscript{562}, which might be part of the cytochrome c oxidase cbb\textsubscript{3}. CO-difference spectra showed the presence of a high concentration of soluble CO-binding cytochrome b\textsubscript{558}, which most probably acts as a CO carrier.

**Table 2.** Enzymic activity in cell-free extracts from cells of ACO1 grown with CO

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Na\textsuperscript{+} concn (M)</th>
<th>Membranes Activity [nmol (mg prot\textsuperscript{−1} min\textsuperscript{−1})]</th>
<th>Soluble fraction Activity [nmol (mg prot\textsuperscript{−1} min\textsuperscript{−1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CODH</td>
<td>8.5</td>
<td>0.5</td>
<td>1000</td>
<td>210</td>
</tr>
<tr>
<td>FDH</td>
<td>8.5–9.0</td>
<td>0.5</td>
<td>560</td>
<td>0</td>
</tr>
<tr>
<td>SQR</td>
<td>9.5</td>
<td>0.3</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>8.0</td>
<td>0.5</td>
<td>800</td>
<td>70</td>
</tr>
</tbody>
</table>

*Average from two independent sets of measurements.*

DISCUSSION

This work has demonstrated that growth with CO as the electron donor is possible under extremely haloalkaline conditions and that carboxydotrophy under such extreme conditions is common among the facultatively autotrophic, haloalkaliphilic Gammaproteobacteria of the genera *Alkalispirillum*/*Alkalilimnicola*, which are characterized by a very versatile metabolism. Most probably, *Alkalilimnicola ehrlichii*, for which the potential to oxidize CO has been shown previously, is also capable of growth with CO, but the conditions that allow such growth (oxygen and CO concentrations) remain to be determined. For the strains isolated in this study, carboxydotrophy was possible only at relatively low oxygen and CO concentrations.

An important question which remains unclear is the relevance of CO and its oxidation for soda lakes. This work was more focused on the study of pure cultures. However, there are two lines of evidence that indirectly indicate that CO oxidation in soda lakes might be important. One comes from the work with *Alkalilimnicola ehrlichii*, in which the potential to consume CO at p.p.m. concentrations has been demonstrated (Hoeft et al., 2007). Another is the indication of significant CO emission from hypersaline mats (Hoehler et al., 2001). Further environmental study is necessary to clarify the situation.

In general, the alkalispirilla are remarkably salt tolerant when grown heterotrophically; for example, with acetate. Surprisingly, the same was true for their carboxydotrophic growth. Among the known lithoautotrophs isolated from soda lakes, so far only sulfur-oxidizing members of the genus *Thioalkalivibrio* are capable of lithoautotrophic growth in saturated soda brines containing 4 M total Na\textsuperscript{+} (Sorokin et al., 2006a). Therefore, despite a very low growth rate with CO, this energy source is equivalent to sulfide/thiosulfate in providing enough energy for bacteria to cope with extremely haloalkaline conditions.
One of the most interesting physiological aspects of the carboxydrotrophy in the novel isolates is the linking of the CO- and formate-oxidizing systems. The fact that both are expressed only during growth on CO might be interpreted in two ways: formate is either a co-substrate for the CODH or an intermediate of CO oxidation, and therefore growth with CO leads to the expression of FDH. The first possibility is not supported by the enzymic measurements that show the presence of two separate enzyme systems for CO and formate oxidation. We also could not find any evidence for formate accumulation during CO oxidation. So, at present, the reason for the triggering of high FDH activity in CO-grown cells of the alkaliphilic isolate remains unclear.

As there is a rapid reduction of the soluble (periplasmic) cytochrome pool in the presence of CO and formate, the CODH and FDH must be exposed to the outside of the cell membrane. Together, the enzyme activity measurements, protein expression profiles and cytochrome reduction data suggest the schematic organization of the CO/formate-oxidizing system in the alkaliphilic isolate ACO1 given in Fig. 6. CO reacts with the reduced soluble cytochrome $b_{558}$ in the periplasm and is transported to either a soluble or a membrane-bound CODH, while formate reacts with a membrane FDH. Both dehydrogenases pass the electrons to the soluble periplasmic cytochrome $c_{552}$/cytochrome $b_{557}$ complex, which finally delivers the electrons to the cytochrome $c$ oxidase, which may be of the $cbb_3$ type. Sulfide may be oxidized directly through a membrane-bound SQR.

Concluding, the ability to grow with CO under extremely haloalkaline conditions (pH up to 10.4 and salt up to 3.5 M total Na$^+$) has been demonstrated for a group of Gammaproteobacteria belonging to the genera *Alkalispirillum* and *Alkalilimnicola* from hypersaline alkaline lakes. The carboxydrotrophic growth occurs at relatively high pH and high salt concentrations, making these isolates unique among known carboxydrotrophs. The schematic organization of the CO/formate-oxidizing system in the alkaliphilic isolate ACO1 is provided in Fig. 6.

### Fig. 5
Total protein profiles in cell fractions of strain ACO1 grown at pH 10 with different substrates. (a) Comparison of total proteins expressed during growth with different substrates under denaturing conditions (5–20% gradient); (b) comparison of total proteins in cell fractions from CO-grown cells under denaturing conditions (5–20% gradient); (c) in-gel activity staining of CODH and FDH in cell fractions from the CO-grown cells under non-denaturing conditions (7.5%). Arrows and arrowheads indicate overexpressed polypeptides. Membrane preparations under non-denaturing conditions were solubilized with 0.1% (w/v) β-D-dodecyl maltoside. Total protein loading was 30 μg per lane. sol, Soluble fraction; membr, membrane fraction.

### Fig. 6
Tentative scheme for inorganic substrate oxidation in haloalkaliphilic strain ACO1 based on enzyme activity measurements and cytochrome reduction data. cyt., Cytochrome.
low oxygen and CO concentrations. The bacteria are facultative chemolithoautotrophs able to utilize CO (but not \( H_2 \)), formate and sulfide as electron donors, and presumably the Calvin–Benson cycle for inorganic carbon assimilation. The system of CO oxidation includes a soluble CO-binding cytochrome \( b \), two different high-molecular-mass CODH complexes that donate electrons to a high-potential soluble cytochrome \( bc \), and a membrane cytochrome \( c \) oxidase, most probably of the \( cbb_3 \) type.

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