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Disruption of *sucA*, Which Encodes the E1 Subunit of α-Ketoglutarate Dehydrogenase, Affects the Survival of *Nitrosomonas europaea* in Stationary Phase

Norman G. Hommes, Elizabeth G. Kurth, Luis A. Sayavedra-Soto, and Daniel J. Arp*

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331-2902

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Although *Nitrosomonas europaea* lacks measurable α-ketoglutarate dehydrogenase activity, the recent completion of the genome sequence revealed the presence of the genes encoding the enzyme. A knockout mutation was created in the *sucA* gene encoding the E1 subunit. Compared to wild-type cells, the mutant strain showed an accelerated loss of ammonia monooxygenase and hydroxylamine oxidoreductase activities upon entering stationary phase. In addition, unlike wild-type cells, the mutant strain showed a marked lag in the ability to resume growth in response to pH adjustments in late stationary phase.

Nitrosomonas europaea is a nitrifying bacterium which has traditionally been considered an obligate chemolithoautotroph that obtains its carbon predominantly from CO_2 and its energy and reductant predominantly from the oxidation of NH_3 (13). The range of carbon sources capable of supporting growth was recently extended to include fructose and pyruvate (7). The demonstration of growth on reduced carbon compounds has caused a reevaluation of the barriers to heterotrophic growth in *N. europaea*. In particular, the absence of detectable α-ketoglutarate dehydrogenase activity in *N. europaea* and in other lithotrophs has been mentioned as a key factor in obligate autotrophy (9, 14, 20). For example, null mutations of α-ketoglutarate dehydrogenase in the facultative methylotroph *Methylobacterium extorquens* AM1 resulted in the inability to grow on substrates other than C_1 compounds (16, 19). A number of autotrophic and methanotrophic bacteria appear to lack genes encoding α-ketoglutarate dehydrogenase (20). However, despite the absence of measurable activity, the genes *sucA*, *sucB*, and *lpd*, encoding α-ketoglutarate dehydrogenase subunits E1, E2, and E3, respectively, were revealed in the genome sequence of *N. europaea* (2). All the other genes required for a complete tricarboxylic acid (TCA) cycle were present as well.

In heterotrophs the TCA cycle provides metabolic intermediates for biosynthesis and reductant for ATP synthesis. The oxidation of organic acids to CO_2 coupled to the reduction of cofactors requires the complete TCA cycle, while the provision of intermediates does not. For organisms that can switch between these modes, the α-ketoglutarate dehydrogenase step is often the regulated step. For example, the expression of α-ketoglutarate dehydrogenase can be affected by both the oxygen status of the cell (11, 15) and the carbon sources available (1, 3, 16). In obligately lithotrophic bacteria such as *N. europaea*, which generate energy by the oxidation of inorganic substrates and lack α-ketoglutarate dehydrogenase activity, biosynthetic precursors are thought to be generated by a branched TCA pathway in which the reactions leading from oxaloacetate to succinate occur in a reductive direction. Required anaplerotic intermediates can be generated by alternative pathways such as the glyoxylate shunt, the γ-aminobutyrate shunt (6), or a bypass using 2-oxoglutarate ferredoxin oxidoreductase (12), which do not appear to be present in *N. europaea*.

Given the longstanding observation that α-ketoglutarate dehydrogenase activity is not detectable in *N. europaea*, combined with the interest surrounding the role of the TCA cycle in the metabolism of lithotrophs, we have generated a mutant strain with the gene for the E1 subunit of α-ketoglutarate dehydrogenase (*sucA*) inactivated. The mutant strain was examined for potential phenotypes relating to growth, starvation, transcription, enzymatic activity, and regulation.

Expression and mutagenesis of *sucA*. Since there is no measurable α-ketoglutarate dehydrogenase activity in *N. europaea* extracts (9) despite the presence of the genes which encode the enzyme, we tested whether the *sucA* gene, encoding the E1 subunit, was transcribed by performing reverse transcription-PCR (RT-PCR). RNA was extracted from late-exponential-phase cells using the Aurum Total RNA minikit (Bio-Rad Laboratories) following the manufacturer's directions, and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories). RT-PCR showed strong *sucA* product formation (Fig. 1, lane 1) relative to the control sample (no reverse transcriptase) (Fig. 1, lane 2), indicating that the gene for the α-ketoglutarate dehydrogenase E1 subunit was indeed transcribed. Quantitative RT-PCR (qRT-PCR) experiments confirmed this result (see below). The *sucB* gene, encoding the E2 subunit, was likewise shown to be expressed (data not shown). The *lpd* gene, encoding the E3 subunit, is located in the pyruvate dehydrogenase operon and encodes the E3 subunit for both enzymes.

Having determined that the *sucAB* genes for α-ketoglutarate dehydrogenase are expressed, the role of α-ketoglutarate de-

* Corresponding author. Mailing address: Department of Botany and Plant Pathology, Oregon State University, 2082 Cordley, Corvallis, OR 97331-2902. Phone: (541) 737-1294. Fax: (541) 737-5310. E-mail: arpd@science.oregonstate.edu.

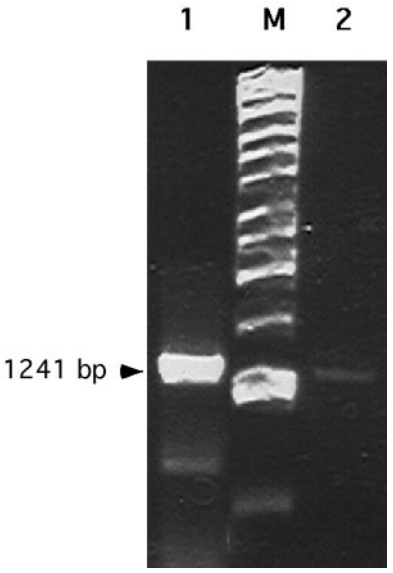

FIG. 1. RT-PCR of the gene for α-ketoglutarate dehydrogenase (*sucA*). The reaction was done with reverse transcriptase (lane 1) and without reverse transcriptase (lane 2) as a control for DNA contamination. Lane M, molecular weight ladder.

hydrogenase in the cell was tested by inactivating *sucA* and observing what phenotypes might result. A copy of *sucA* was amplified by PCR from wild-type genomic *N. europaea* DNA. In order to inactivate *sucA*, a DNA cassette (Kan) containing the neomycin/kanamycin-phosphotransferase gene (including its own promoter and terminator sequences) was inserted into a BamHI site in the gene (Fig. 2A). The constructs were electroporated into *N. europaea*, and kanamycin-resistant mutants were isolated as previously described (8). Southern hybridizations, using *sucA* and *kan* probes, confirmed that the Kan cassette was correctly recombined into the chromosomal *sucA* gene (Fig. 2B). qRT-PCR amplification with primers for *sucA* using RNA from the mutant strain did not produce a product, confirming that transcription of *sucA* was disrupted (not shown).

Growth of the *sucA* mutant. The growth of the *sucA* mutant strain was compared to that of wild-type *N. europaea* cells. For growth experiments, cells from early-stationary-phase cultures grown on $(NH_4)_2SO_4$ and Na_2CO_3 (4) were harvested by centrifugation and washed three times in buffer (2 mM $MgSO_4$, 50 mM Na_2HPO_4 [pH 8.0]). Sterile 160-ml culture bottles were prepared with 25 ml *N. europaea* medium containing $(NH_4)_2SO_4$ (25 mM) and Na_2CO_3 (3.9 mM). The culture bottles were sealed with gray butyl rubber stoppers (Supelco, Bellefonte, PA) and fastened with aluminum crimp seals. *N. europaea* cell suspensions were injected into the bottles, typically to an initial optical density at 600 nm of 0.006 ($\sim 10^7$ cells/ml). The bottles were incubated with shaking in the dark at 30°C. Growth was monitored by measuring the optical density at 600 nm. Protein assays (Biuret) were done as described previously (5).

Comparing the growth curves of wild-type and mutant strains, we found that during the exponential phase of growth, *sucA* mutant cells grew at the same rate as, and to similar final densities of, wild-type cells (Fig. 3). Thus, the loss of *sucA* had no apparent effect on cells during active growth. In contrast, during stationary phase, significant differences between the two strains were seen relating to (i) the response to pH shifts in the media and (ii) the activity of enzymes in the nitrification pathway.

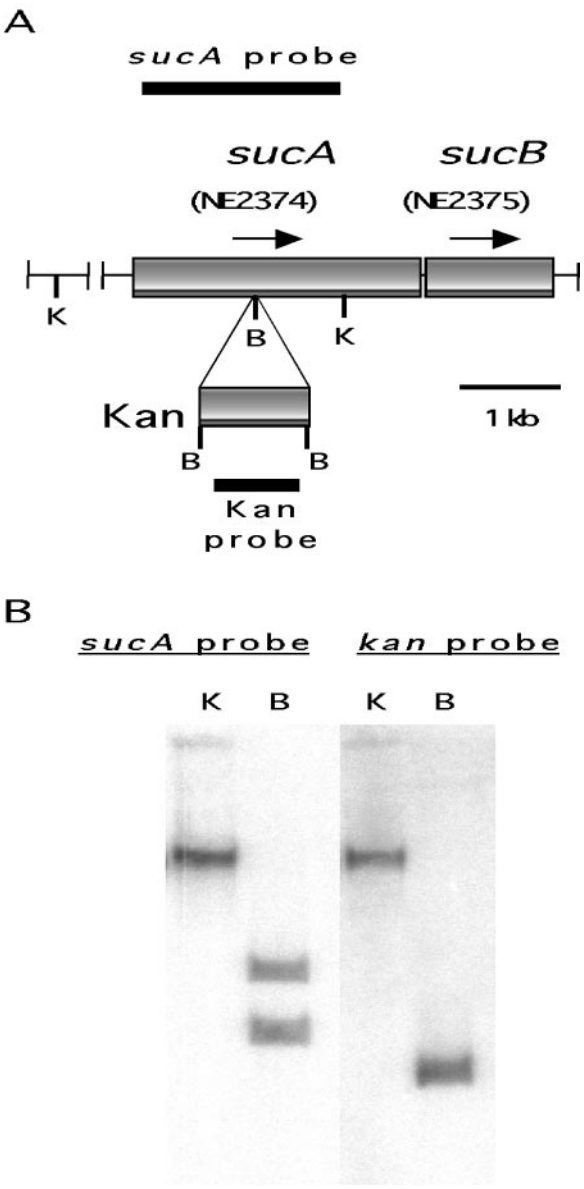

FIG. 2. (A) Physical map of the genes for α-ketoglutarate dehydrogenase (*sucAB*). The site of insertion of the kanamycin cassette is indicated. The locations of the hybridization probes are indicated by heavy bars. B, BamHI; K, KpnI. (B) Southern hybridization of *N. europaea* genomic DNA from the *sucA* mutant strain. The DNA was digested with KpnI or BamHI and probed with a *sucA* probe or a *kan* probe.

pH effects. As *N. europaea* cells grow into stationary phase, they acidify the medium. The decrease in pH shifts the equilibrium away from NH_3 (the energy-generating substrate) and towards NH_4^+ (not a substrate) and may cause a more general cellular response to pH. We tested whether wild-type and

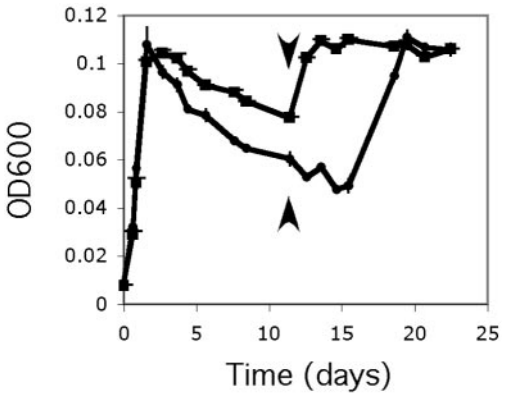

FIG. 3. Growth curves of *N. europaea* wild-type (■) and *sucA* mutant (●) strains. Arrowheads indicate the point of pH adjustment upward to 7.8. The growth curves are representatives of three samples. OD_{600}, optical density at 600 nm.

mutant strains in late stationary phase might differ in their abilities to resume growth when the pH of the growth medium was adjusted upward to 7.8 (the pH of the cultures ranged from 6.0 to 6.4). When the pH was adjusted in stationary phase, 11 days after inoculation, the wild-type cells resumed growth within 24 h while the mutant cells showed a distinct lag of up to 4 days in the resumption of growth (Fig. 3). This result suggests that the two strains have different physiological states in late stationary phase. The loss of *sucA* apparently reduces the ability of the mutant cells to adapt to the challenges of stationary phase. This weakened state is manifest in the lag in the resumption of growth following the upward pH shift. In addition, the mutant strain usually, though not always, lost cell density at a higher rate in stationary phase than did wild-type cells (Fig. 3), which may also reflect the weakened state of the mutant strain.

O_2 consumption rates. The previous experiments showed that resumption of growth in stationary phase was affected by the *sucA* knockout mutation. Since growth of *N. europaea* on ammonia is dependent on ammonia monooxygenase (AMO), which catalyzes the oxidation of NH_3 to NH_2OH, and hydroxylamine oxidoreductase (HAO), which catalyzes the oxidation of NH_2OH to NO_2^- for energy generation, the activity of these enzymes was measured at different times during the growth cycle. Specific O_2 consumption rates were measured in wild-type and mutant cells taken from late exponential phase (day 2), from stationary phase (day 5), and from late stationary phase (day 7). At each time point, the $(NH_4)_2SO_4$-dependent, NH_2OH-dependent, and lactate-dependent O_2 consumption rates were measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) as described previously (10). Washed cells were resuspended in buffer (50 mM KH_2PO_4, 2 mM $MgSO_4$ [pH 7.0]) and added to the electrode chamber to an optical density at 600 nm of 1.7 (~0.22 mg protein). The assay was initiated by the addition of $(NH_4)_2SO_4$ (10 mM), NH_2OH (10 mM), or lactate (20 mM). The rate of oxygen uptake (nmoles · mg protein^{-1} · min^{-1}) was calculated based on a concentration of 230 μM O_2 in air-saturated buffer at 30°C (18).

Significant differences between the two strains were seen. In wild-type cells, the $(NH_4)_2SO_4$-dependent rate, which requires AMO and HAO activities, gradually declined to about 33% of the maximal rate between the second and seventh day after inoculation. The NH_2OH-dependent rate, which requires HAO activity, remained fairly constant over the time course in wild-type cells. In the mutant strain, the $(NH_4)_2SO_4$-dependent activity dropped to near zero by the fifth day (Table 1), and the NH_2OH-dependent rate dropped more than 90% by the fifth day. A low rate of O_2 consumption was also detected with lactate; this rate was higher in wild-type cells than in the *sucA* mutant strain. With substrates other than NH_3, the observed rates of O_2 consumption were not inhibited by the addition of allylthiourea, an inhibitor of AMO, precluding the possibility that the observed rates were due to the oxidation of residual NH_3. Thus, while there were relatively minor differences between the two strains in AMO- and HAO-dependent O_2 consumption rates during exponential growth, there were major differences between the strains in AMO and HAO activities during stationary phase. The lack of AMO activity during late stationary phase could account for the delay in the resumption of growth in the mutant strain in the pH shift experiments described above.

The metabolic connection between *sucA* and AMO is unclear. There is no evidence that *sucA* is required for energy generation from NH_3 via AMO activity. In addition, the primary energy storage compounds used by *N. europaea* during stationary phase are thought to be polyphosphates (17), the utilization of which should not require α-ketoglutarate dehy-

TABLE 1. Specific O_2 consumption rates of wild-type *N. europaea* and *sucA* mutant cells incubated in a Clark-type oxygen electrode[a]

Strain	Substrate (concn)	O_2 consumed [nmol · min^{-1} · mg^{-1} of protein (± SD)]		
		Late-log-phase cells (day 2)	Stationary-phase cells (day 5)	Late-stationary-phase cells (day 7)
WT	$(NH_4)_2SO_4$ (10 mM)	1,081 (79)	499 (31)	389 (17)
	NH_2OH (1 mM)	320 (7.5)	272 (20)	289 (17)
	Lactate (20 mM)	8.2 (0.6)	13.8 (0.5)	5.2 (1.5)
	None	16 (2.4)	7.2 (1.6)	18 (5.2)
sucA mutant	$(NH_4)_2SO_4$ (10 mM)	1,261 (3.4)	1.8 (1.5)	0.0
	NH_2OH (1 mM)	390 (20)	32 (1.6)	27 (8)
	Lactate (20 mM)	8 (2.8)	1.4 (0.4)	1.1 (0.4)
	None	21 (3.9)	3.4 (0.6)	8.8 (2.1)

[a] The O_2 consumption rate in the presence of substrate represents the average increase in rate over the endogenous rate upon the addition of substrate over three replicate runs. WT, wild-type *N. europaea*.

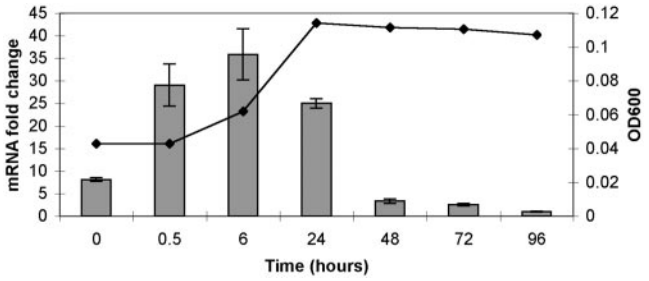

FIG. 4. Quantitative RT-PCR using RNA extracted from wild-type *N. europaea* cells harvested from stationary phase and inoculated into fresh growth medium. mRNA was extracted at 0, 0.5, 6, 24, 48, 72, and 96 h. Primers for α-ketoglutarate dehydrogenase (*sucA*) and 16S rRNA were used. The data were normalized to the 16S rRNA values for each sample. The *n*-fold expression levels are relative to the amount of *sucA* RNA in the 96-h sample. The scale for the optical density at 600 nm of the cultures is on the right-hand *y* axis.

drogenase. One possibility might be that *sucA* expression allows the generation of a small amount of reductant from the oxidation of organic compounds in the cell. The observed lactate-dependent O_2 consumption would be consistent with this idea. The availability of reductant during stationary phase might have several potential benefits. First, this reductant may serve to keep AMO reduced, thereby increasing its stability under the low-NH_3 conditions in stationary phase. Second, AMO must be in a reduced state to initiate NH_3 oxidation. Thus, under conditions of limited NH_3 availability, such as may exist in late stationary phase, AMO may be dependent on alternative sources of reductant to initiate NH_3 oxidation. Lastly, the degradation or maintenance of AMO may be under the control of regulatory systems that are sensitive to the redox or energy status of the cell. The regulatory systems could be affected by the presence of an active α-ketoglutarate dehydrogenase.

Quantitative PCR. Since the predominant phenotypes associated with the *sucA* mutation seem to be associated with stationary phase, we considered the possibility that *sucA* transcription in *N. europaea* might be induced during this time. The levels of *sucA* transcript were quantified by qRT-PCR. Primers for 16S rRNA were used as controls. Wild-type *N. europaea* cells were harvested from stationary phase, washed, and placed in fresh medium. qRT-PCR was done in triplicate using RNA extracted from cells over a 4-day time course (0.0, 0.5, 24, 48, 72, and 96 h) following the manufacturer's directions (Bio-Rad Laboratories). Cycle threshold values were calculated automatically by MyiQ software using the PCR baseline-subtracted curve fit analysis mode. Relative quantification was calculated using Gene Expression Macro v1.1 (Bio-Rad Laboratories). Mean cycle threshold values were normalized to reference gene 16S and calibrated to the sample with the smallest mRNA abundance. The results of the qRT-PCR showed that the amount of *sucA* mRNA fluctuated significantly over the time course (Fig. 4). There was a rapid induction of *sucA* mRNA following the addition of fresh medium, with the highest value obtained at 6 h. Thereafter, the amount of *sucA* mRNA declined to a low, though still detectable, level after 4 days. The data from these experiments indicate that the *sucA* gene was expressed primarily during exponential growth despite the observation that the predominant effect of a *sucA* knockout occurs during stationary phase. Perhaps the low level of *sucA* mRNA that is present during stationary phase may be sufficient for the needs of the cell. Alternatively, if α-ketoglutarate dehydrogenase is produced during exponential phase, it may persist into stationary phase.

Historically, *N. europaea* and other nitrifiers have been known for their obligate lithoautotrophy. In recent years, however, *N. europaea* has proven to have greater metabolic capability than previously thought, since it is able to use alternative reduced carbon compounds, such as fructose or pyruvate, as carbon sources for growth. Now the discovery that the genes for α-ketoglutarate dehydrogenase are expressed and enhance survivability during stationary phase suggests that *N. europaea* is able to derive some amount of energy and reductant from reduced carbon compounds via the activity of α-ketoglutarate dehydrogenase. Having an alternative source of maintenance energy and reductant would greatly enhance the ability of *N. europaea* to survive conditions of low NH_3, a substrate which is often in short supply.

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