

Cyanobacterial Sulfide-Quinone Reductase: Cloning and Heterologous Expression

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The gene encoding sulfide-quinone reductase (SQR; E.C.1.8.5.), the enzyme catalyzing the first step of anoxygenic photosynthesis in the filamentous cyanobacterium *Oscillatoria limnetica*, was cloned by use of amino acid sequences of tryptic peptides as well as sequences conserved in the *Rhodobacter capsulatus* SQR and in an open reading frame found in the genome of *Aquifex aeolicus*. SQR activity was also detected in the unicellular cyanobacterium *Aphanothece halophytica* following sulfide induction, with a V_{\max} of 180 μmol of plastoquinone-1 (PQ-1) reduced/mg of chlorophyll/h and apparent K_m values of 20 and 40 μM for sulfide and quinone, respectively. Based on the conserved sequences, the gene encoding *A. halophytica* SQR was also cloned. The SQR polypeptides deduced from the two cyanobacterial genes consist of 436 amino acids for *O. limnetica* SQR and 437 amino acids for *A. halophytica* SQR and show 58% identity and 74% similarity. The calculated molecular mass is about 48 kDa for both proteins; the theoretical isoelectric points are 7.7 and 5.6 and the net charges at a neutral pH are 0 and -14 for *O. limnetica* SQR and *A. halophytica* SQR, respectively. A search of databases showed SQR homologs in the genomes of the cyanobacterium *Anabaena* PCC7120 as well as the chemolithotrophic bacteria *Shewanella putrefaciens* and *Thiobacillus ferrooxidans*. All SQR enzymes contain characteristic flavin adenine dinucleotide binding fingerprints. The cyanobacterial proteins were expressed in *Escherichia coli* under the control of the T7 promoter. Membranes isolated from *E. coli* cells expressing *A. halophytica* SQR performed sulfide-dependent PQ-1 reduction that was sensitive to the quinone analog inhibitor 2*n*-nonyl-4-hydroxyquinoline-*N*-oxide. The wide distribution of SQR genes emphasizes the important role of SQR in the sulfur cycle in nature.

Of the many organisms performing plant-type oxygenic photosynthesis, only cyanobacteria can facultatively shift to anoxygenic, bacterium-type photosynthesis with sulfide (H_2S) as the electron donor in a photosystem I-dependent reaction (3, 15, 26). This shift, first discovered in the cyanobacterium *Oscillatoria limnetica* (11), occurs after 2 h of incubation in the presence of sulfide and light and requires protein synthesis (23). The induced cells perform sulfide-dependent CO_2 fixation (10, 11, 15, 23), H_2 evolution (5), or N_2 fixation (4), depending on the growth and physiological conditions.

The discovery of anoxygenic photosynthesis in *O. limnetica* formed the basis for the understanding of an important and unique trait of cyanobacteria. In marked contrast to other plant-type phototrophs, which are sulfide sensitive, these organisms grow and form mats in anaerobic, sulfide-rich niches characteristic of many natural habitats and polluted water (25, 26, 38). The unique capacity to shift between the two types of photosynthesis has been suggested to represent a primitive relic in the evolution of photosynthesis (25).

Photooxidation of sulfide coupled to CO_2 reduction is not unique to cyanobacteria. Sulfide is the most widely used electron donor among photolithotrophic bacteria (7, 8, 17). The transfer of electrons from sulfide directly into the quinone pool was proposed and supported by the inhibition exerted by quinone analogs as well as energetic considerations (6, 8, 42).

Tracking of the inducible factor that enables photosynthetic

sulfide oxidation in *O. limnetica* led to the discovery of sulfide-quinone reductase (SQR; E.C.1.8.5.), a novel enzyme that transfers electrons from sulfide into the quinone pool (36). The SQR was solubilized from membranes of sulfide-induced *O. limnetica* cells and purified in an active form (2). The isolated active SQR is a hydrophobic membrane enzyme composed of a single polypeptide with an apparent molecular mass of 57 kDa (as determined by sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE]). It was shown to have high affinities for sulfide ($K_m = 8 \mu\text{M}$) and quinone ($K_m = 31 \mu\text{M}$ for plastoquinone-1 [PQ-1]), to contain a flavin cofactor, and to be sensitive to quinone analogs and KCN (2, 34). The N-terminal sequence was found to contain the characteristic features of an NAD or flavin adenine dinucleotide (FAD) binding domain (2, 44).

SQR recently has been proven to be widely spread among anoxygenic phototrophs. SQR activity has been detected in purple “nonsulfur” bacteria (*Rhodobacter capsulatus*) (35), purple sulfur bacteria (*Allochrochromatium vinosum*) (34), green sulfur bacteria (*Chlorobium*) (37), and green gliding (“nonsulfur”) bacteria (*Chloroflexus aurantiacus*) (34); in the nonphotosynthetic chemoautotrophs *Paracoccus denitrificans* (31), *Wolinella succinogenes*, and *Aquifex aeolicus*; and in the mitochondria of the sulfide-tolerating marine worm *Arenicola marina* (34).

The SQR of the purple bacterium *R. capsulatus* was isolated and purified, its gene was cloned, sequenced, and functionally expressed in *Escherichia coli* (33) and, recently, it was shown that the enzyme is essential for the sulfide-dependent growth of *R. capsulatus* (32). The *R. capsulatus* SQR polypeptide consists of 427 amino acids and has a molecular mass of 47 kDa

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TABLE 1. Strains and plasmids used

Strain or plasmid	Characteristics or history	Source or reference
Strains		
<i>Oscillatoria limnetica</i>	Filamentous	15
<i>Aphanothece halophytica</i>	Unicellular	15
<i>Escherichia coli</i>		
JM109	e14 ⁻ (McrA ⁻) <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1</i> Δ(<i>lac-proAB</i>) [F' <i>traD36 proAB lacI^qΔM15</i>]	Stratagene
XL1 Blue MRF'	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^qΔM15 Tn10</i> (Tet ^r)] ^c	Stratagene
SOLR	e14 ⁻ (McrA ⁻) Δ(<i>mcrCB-hsdSMR-mrr</i>)171 <i>sbcC recB recJ uvrC umuC::Tn5</i> (Kan ^r) <i>lac gyrA96 relA1 thi-1 endA1 λ^R</i> [F' <i>proAB lacI^qΔM15</i>] ^c Su ⁻ (nonsuppressing)	Stratagene
BL21 (DE3)	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>gal λ</i> (DE3)	39
Plasmids		
pBS	Multicopy cloning and sequencing vector; confers Amp ^r	Stratagene
pBS-OL	pBS with <i>EcoRI</i> insert of <i>O. limnetica</i> <i>sqr</i> region	This work
pBS-AH	pBS with <i>EcoRI</i> insert of <i>A. halophytica</i> <i>sqr</i> region	This work
pT-OL	pBS with <i>O. limnetica</i> <i>sqr</i> PCR product	This work
pT-AH	pBS with <i>A. halophytica</i> <i>sqr</i> PCR product	This work
pT7-7	Expression vector	40
pTOLSqr	pT7-7 with <i>O. limnetica</i> <i>sqr</i> ORF	This work
pTAHsqr	pT7-7 with <i>A. halophytica</i> <i>sqr</i> ORF	This work

and a net charge of +1. The amino acid sequence of its N terminus shows high similarity (48% identity and 72% similarity) to the amino acid sequence of the *O. limnetica* N terminus (2), including the FAD binding fingerprint. The complete protein sequence contains two additional FAD binding motifs (33). The *Rhodobacter* and the cyanobacterial enzymes are rather divergent. For example, they are expected to differ in their quinone binding sites. The natural quinone acceptor for SQR is most probably ubiquinone in *R. capsulatus* but plastoquinone in the cyanobacterial system (34).

Recently, a gene encoding a mitochondrial polypeptide that exhibits SQR activity was cloned from the fission yeast *Schizosaccharomyces pombe*. This enzyme, HMT2 (heavy metal tolerance), was proposed to function in the detoxification of endogenous sulfide (43). HMT2 shares high similarity with sequences of unknown functions from the genomes of nematodes, fruit flies, mice, rats, and humans and only low similarity (~20%) with *R. capsulatus* SQR (43). In addition to demonstrating further the distribution of SQR in eukaryotes, this recent publication suggests two possible roles for SQR—utilization and detoxification of sulfide—raising the question of whether one type of enzyme or more types are involved.

We have previously shown three functions in which SQR can be involved in cyanobacteria: (i) anoxygenic photosynthetic growth in *O. limnetica* (23); (ii) anaerobic respiration in *O. limnetica* (24); and (iii) detoxification of sulfide in *Aphanothece halophytica*, which survives but does not grow in the presence of sulfide (15). In contrast to *O. limnetica*, in which the SQR has been purified and biochemically characterized, in *A. halophytica* the sulfide-interacting enzyme has not yet been identified. In the present work, we describe some biochemical properties of *A. halophytica* SQR as well as the cloning and expression of the *sqr* genes of both *O. limnetica* and *A. halophytica*.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The list of strains and plasmids used is provided in Table 1. *O. limnetica* was grown in aerobic growth medium (3) at 37°C as described previously (1). *A. halophytica* was grown in the same medium supplemented with 10 μg of vitamin B₁₂ per liter in batches of 100 to 500 ml at 35°C without shaking. *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco, Detroit, Mich.) or minimal medium A (12) at 37°C, unless otherwise

indicated. Antibiotics were added to final concentrations of 100 μg/ml (ampicillin), 50 μg/ml (kanamycin), or 12.5 μg/ml (tetracycline).

Induction of anoxygenic photosynthesis. *O. limnetica* was induced with sulfide as described previously (1). To induce anoxygenic photosynthesis in *A. halophytica*, cells of 5-day-old (exponential-growth-phase) cultures were harvested, washed and resuspended in anaerobic growth medium (3), and incubated in stoppered bottles flushed with N₂. 3(3,4-Dichlorophenyl)-1,1-dimethylurea (10 μM) and NaHCO₃ (2.5 mM) were added, and cells were induced by the addition of 1.25 mM Na₂S. After 2 h of incubation at 35°C under cool-white fluorescent lamps (150 μE/m²/s), cells were washed in anaerobic growth medium and either rapidly frozen in liquid nitrogen and stored at -80°C or immediately assayed for SQR activity.

Spectroscopic assay of SQR. SQR activity was measured as sulfide-dependent PQ-1 reduction recorded at 292 nm minus 266 nm in an SLM-Aminco DW-2000 dual-wavelength spectrophotometer as previously described (1).

Tryptic peptides of *O. limnetica* SQR. SQR was purified from *O. limnetica* cells as previously described (2). Samples containing purified SQR were separated by SDS-PAGE and Coomassie blue stained. The gel was destained in 50% acetonitrile–200 mM ammonium bicarbonate, dried, and rehydrated with 100 mM ammonium bicarbonate containing modified trypsin (sequencing grade; Promega). After overnight incubation at 37°C, the resulting peptides were eluted from the gel, redissolved in 0.1% trifluoroacetic acid, and separated by reversed-phase high-pressure liquid chromatography on a Vydac C₁₈ column (1 by 150 mm) with a linear gradient of 4 to 60% acetonitrile in 0.1% trifluoroacetic acid. The peptides were collected, and their amino acid sequence was determined (at the Protein Research Center, The Technion, Haifa, Israel) with a model 494A Peptide Sequencer (Applied Biosystems Division, Perkin-Elmer).

Solubilization of *A. halophytica* SQR. Frozen induced cells were thawed and washed (i) with Turks Island salt solution prepared in double strength (15) and (ii) with buffer containing 20 mM sodium HEPES (pH 7.9), 0.3 mM sodium EDTA, 5 mM KCl, 5 mM MgCl₂, 0.5 M glycine betaine, and 0.1 M sucrose. The washed cells were resuspended in the buffer, after the addition of bovine serum albumin (1 mg/ml) and lysozyme (3 mg/ml) to a final concentration of 25 μg of chlorophyll/ml, and incubated for 25 min at 35°C in the dark. The partially lysed cells were sonicated, and thylakoid membranes were prepared as previously described (1). Thylakoid membranes were washed and SQR was solubilized as previously described for *O. limnetica* SQR (2).

Gel electrophoresis and Western blotting. SDS gel electrophoresis was carried out as described by Laemmli (19). Western blotting with polyclonal antibodies raised against denatured *O. limnetica* SQR (2) was carried out by use of an alkaline phosphatase detection system as previously described (30).

Isolation of cyanobacterial DNA. Exponentially growing cyanobacterial cells in 100-ml batches were harvested and washed with 30 ml of buffer containing 50 mM Tris (pH 8.0) and 2 mM EDTA. The pellet was resuspended in 2 ml of buffer–0.8 ml of sterile glass beads (212 to 300 μm; Sigma G9143). An equal volume of phenol-chloroform mixture was added, and the cells were broken by four cycles each of 30 s of vigorous vortexing and 1 min of incubation on ice. The lysate was centrifuged at 12,000 × g for 10 min, the upper phase was collected, and the extracted DNA was washed with chloroform and precipitated with ethanol as described previously (30).

A. halophytica	MA-H-----IVIVGGGEGGLSAAYELKHLHG-	26
O. limnetica	MA-H-----VAVIAGLAGLPTAYELRHILPR-	26
Anabaena	MA-H-----IVIVGAGLGGCLPTAYELRHILPK-	26
R. capsulatus	MA-H-----IVVLGAGLGGAIMAYELREQVRK-	26
T. ferrooxidans	MA-H-----VVILGAGTGGPAAYEMKEALGGS-	26
S. putrefaciens	MA-K-----IIIVGAGLGGVSVAFELKQRLPS-	26
A. aeolicus	MAKH-----VVVLGGGEGGLATAYELRNLMF--	26
S. pombe	MLTLNSTIKSVTGSFQASMLARFASTH-----KVLVGGGSGAGLSVAHQIYNKFSKY	54
A. vinosum (FCC)	---MTLNRRDFIKTSGAAVAAVGILGPPHLAFGAGRKKVVVGGGEGGATAAKYIKLADPS-	57
A. halophytica	-----KHKITLISDETFETFIIPSLPWVAFNLRERLEDVQLPLAFITLARQGNW	73
O. limnetica	-----QHRVTLISDKPNEFTFTESLPWVAFDLTPLEERVQLDVGGKLLKGRNDW	73
Anabaena	-----QHQVTVISETPTVETFTIPSLPWVAMGLTSLSTQVSLQORLRQKGNW	73
R. capsulatus	-----EDKVTVITTKDPMYHEVPSNPWVAVGWRDRKETVVDLPTMARKNDIF	73
T. ferrooxidans	-----GHEVTLISANDYEQFVPSNPWVGVGWERDDTAFPIRHYVERKGFHF	73
S. putrefaciens	-----DCEIGVISEGTDQFQVPSNPWVALGERTREDISLPIGITYLAKRKISY	73
A. aeolicus	-----DLKITLISDRPYGFTFAFPHLAWGWRKFPEDISVPLAFILPKFNHF	73
S. pombe	RFANDQGRDTSLKPCGIGIVDGAKYHYIQGWTLTGAGSSVAKTRRELASLVPADKFKL	114
A. vinosum (FCC)	-----IEVTHIEPNTDYITCYLSNEVIGEDSKLESIKHGVDGUR-AHGQOV	102
A. halophytica	QHGRVTGLDPNCKRVSVEGEDITFDVYDYLVTTCASLAYHLMSEIGPE-EGMTQSVONAH	132
O. limnetica	IHGKVNHDIPENKTLVAGQOT-LEYDYVVVATGPELATDAIAGLGE-NGVTQSVONPHH	131
Anabaena	ILGRVTDYLNPNOKISLGE-QSISDYDYLITATGAELALDAVAGLGPD-EGMTQSVONPH	130
R. capsulatus	IPVAAKRLEPAENRVELENGQSVSDQIVLATGPELAFDEIEGEGF-EGEOSTICHDH	131
T. ferrooxidans	IAQSAEQIDAEQNITLADGNTVHYDYLILATGPKLAFENVPSDEH-EGEPVQSICTVDH	132
S. putrefaciens	SGEGVTHIDPLNKKLTLGDGTTNNYDYLICTGPKLAFENVPSGDF-EGEOSTICHDH	131
A. aeolicus	IHEKAESHDPDANTVITQSGKKIEVDYLVLIATGPKLAFVFG-AEQ-----ENSTSICTAH	128
S. pombe	HPEFVKSLEHRENKIVTQSGQETSKYDYLVAAGCTYTDGRIKGLTEALDDPNTFVVITYS	174
A. vinosum (FCC)	VHDSATGIDDPKRLKTAGCAEFYDRCVAPGTEIITYDKIEGYS-----EBAAKLPHAWK	159
A. halophytica	AEMARDANDEFLE--NPGHLLVGAVPG--ASCGPAYEFALLADYALRQEGKRQDVE-IT	187
O. limnetica	ALMAKEANQKFLQ--DPGELVVGAVPG--ASCFGPAYEFALLADYVLRKGRDRDVP-IT	186
Anabaena	AIKAFQANQNFIL--APGRLVVGALPK--TSCFGPAYEFTLLADYVLRKQGLRECVS-IT	185
R. capsulatus	ABAACAADFRCF--NPGHLLVGAAG--ASCFGPAYEFTFILTALRRKRKIRDKVP-MT	186
T. ferrooxidans	AERAPAEYQALIR--EPGEIVIGAMAG--ASCFGPAYEFTMIVASDLKRRGMRDKTISFT	188
S. putrefaciens	AEKAYECFQQLIA--KPGPVVVGALQG--ASCFGPAYEFTVLSLESILRKHKVRQHIE-IT	186
A. aeolicus	ALETQKKLQELYA--NPGEVVIGALPG--VSCFGPAYEFALMLHMLKRRGIRDKVP-MT	183
S. pombe	EKYADAVYFWIEK-TKSENALFTQ-BSGVLKCAQAPQKIMMMEDYVWRHRKVRSNID-VS	231
A. vinosum (FCC)	AGEQTAILRKOLEDMADGGTVILAPPAAPFRCPGPFYERASOVAYVYKAKHKPKSKV--II	217
A. halophytica	FITSEPPYVGHGIGGMANSK-LVTELMKCRNDWVEN--AEIAETKEDVHKLTDGRE--	242
O. limnetica	FVTEPEPYVGHGIGGMANSAE-LVTDLLENKGRVRLFN--TAVKEIHFEEHLDSDGEG--	241
Anabaena	FVTEPEPYVGHGIGGMANSAE-LVTKFMAERGVVEVIEN--VAVTATEANQTHLNGGRV--	240
R. capsulatus	FVTEPEPYVGHGIGGLDVEGDTKG-ILEGNLRDKHKKMTS--TRIKRVFPGKVVVEVTEG	243
T. ferrooxidans	FITSEPPYVGHGIGGVDGSKG-ILTKGLKEGHEAYTN--CKYTKVDNRRKYVTVQDEKG	245
S. putrefaciens	FVTEPEPYVGHGIGGVDGSKS-LMEHEFRERSKMKCN--AKTTFHSGIAYVOELDRKG	243
A. aeolicus	FITSEPPYVGHGIGGIGASKR-LVEDLFAERNMDWIAU--VAVKATPPDKVIYEDLNGN-	239
S. pombe	FYTGMPIL--FSVKRYSALLRQNEQLHRVKNYKDE--LVEVKGSEKAVFKNLNDG	287
A. vinosum (FCC)	LDSSQTFSS---KQSQFSKWERIYGFGTENAMLEWHFPGDPAVVKVDGGMVAFG--	272
A. halophytica	-----FFFNYSMLPPFRGAQFLKEV-FGLTDEK-GFVPLVDTY-QHEPVPSIYSAG-	291
O. limnetica	-----LPEKVMILPPFRGPAFLREA-PELANPK-GFVPTNTY-QHEKYSVYSAG-	290
Anabaena	-----LPEFANSMILPPFRGPRFRVQVPLSNOD--GFIEVLPY-RHEPYASHIYAVG-	289
R. capsulatus	TVKPEKELPFGVAMLPFRGKALMGI-EGLVNR-GFVIVDQHQ-QNEFTKIVFAAG-	299
T. ferrooxidans	ETIKEMVLVFKGMIPASKGVFAVAGV-EGLQNDG-GFVLVDEHQ-FEKKIVANIFAAG-	301
S. putrefaciens	EVEFQHTLPEFLAMFLPPEKCSAAIABV-PELQNEK-GFVLTDIFQ-RSERYPETHAAG-	299
A. aeolicus	-----THEVPAKFTMFMPSQGGPEVVASAGDKVANPANKMIVNRCF-QNPPYKNIYFGV-	293
S. pombe	-----LFEPEFDLLHAVPSMRSEHFIKSS--DLADKS-GFVAVDQSTTQSTKFPVYFAIG-	339
A. vinosum (FCC)	-----DEFKADVINLIPFORAGKIAQIA--GLTNDG-GWCEVDIKTFESSIHKGEVIED	324
A. halophytica	VITQLAAPETEVEPLCAPKTGMTESMAMAVAHNITARELGEINARPYKPSLEATGMADFG	351
O. limnetica	VIVETNPPEKTELPGVVPKTMTEAMGMVAHNIAIKLGVSKAKVQPTLEAICTADFG	350
Anabaena	VVVEHKPSEVTEPLGVPKTMTEAMGMVAHNITALELGVFSAPPVPTLDAICFADFG	349
R. capsulatus	VCVAIPPVGFGPVPCGVPKTGMIESMVTATAHNIGRIVRELEPDEY-GSNVAVGLADFG	358
T. ferrooxidans	IATAIPPVETTEVPTCAPKTGMIESMVAAVHNIAKADLEGRKGEQTMGTWNAVCFADMG	361
S. putrefaciens	VCVAIPVPLENPQYRLAYPKQVLMIESMTTAVDNLILANLKG-EPPQTHPTNATGLADMG	358
A. aeolicus	VVTAIPPEIKETIETGVPKTMIESMVAVAHNIVNDIRN-NPKDYARELSAICTADFG	352
S. pombe	DCSGLP-----TSKTYAAILTAQAPVMVHNLSFVNG-KNLTASYNGYTSQPLLTG	388
A. vinosum (FCC)	ACIANP-----MPKSGYSANSGKRVAAAVVALKKG--EEPGETSEYLNITGYSILA	372
A. halophytica	DNGITETIAAPVVDPSVGERRHATALRGLVWNWAKNAFENYFLAKMRGTAVPWFKGL	411
O. limnetica	DNGIVEVADPVLDEPKTGPERRAITKRGKVSWSKTAPEFTFFLSKMRGLAVPWFERGL	410
Anabaena	NSGILFLANPVLPLDLAGRRRAVALSGAVVITWAKAAFERIFLAKMRGTAVPWFKLLAL	409
R. capsulatus	DKGIATFVACQIPE-P-----RNVNWSQGWVHWAKGCFERYFMKELRGTSETFYEKAA	413
T. ferrooxidans	DRGAATFALPOLK-P-----RKVDVFAYGRVHVHAKVAFERYFIRKMKGVSIPIVVRPDR	416
S. putrefaciens	DKGAATFVALPQNE-P-----RANWNTSMGWVHWAKTAFERYFLKVRVNGTSEPTIYERYVL	313
A. aeolicus	EDAGFEFADVPIE-P-----RERVITKMGKWAHIFKTAPEKRYFLKVRVNGNLAPESEKVL	407
S. pombe	YGKLIILAEFLYKQEE-----KESFGFRSRELDQTVPRRMVYHLKQFFPFVYWNBAVRNG	443
A. vinosum (FCC)	PAYGISVAAYIRNA-----DGSATISVPSDGGVTPVDAPDWVLEREVQ-VYYSYNNIVH	427

<i>A. halophytica</i>	Y-LRLTIVTPISETPTQQDLTSIKG 437
<i>O. limnetica</i>	R-FMGLSLVVEPLDTRTETGNQAFASKS 436
<i>Anabaena</i>	K-L-LGLSLVAVL ----- 420
<i>R. capsulatus</i>	K-L-LGLIDKLVKVGK ----- 427
<i>T. ferrooxidans</i>	SVGQ ----- 419
<i>S. putrefaciens</i>	G-SLGLHRTFGPK ----- 410
<i>A. aeolicus</i>	EIFLKVHPVIELCKDCEGAPGSR ----- 430
<i>S. pombe</i>	KWYGSRGLIPEHFFVN ----- 459
<i>A. vinosum</i> (FCC)	DTFG ----- 431

FIG. 1. Sequence alignment of the known SQR enzymes and putative homologs. Multiple amino acid sequence alignment of the known SQR enzymes of *A. halophytica*, *O. limnetica*, and *R. capsulatus* (33) as well as the putative close homologs from *A. aeolicus* (13), *S. putrefaciens* (sequence number >4287 from the genome of *S. putrefaciens* in the TIGR bank), *T. ferrooxidans* (sequence number >62 from the genome of *T. ferrooxidans* in the TIGR bank), *Anabaena* PCC7120 (sequence number C279 from the genome of *Anabaena* in CyanoBase), and the distant homologs from *S. pombe* as well as the flavoprotein subunit of *A. vinosum* flavocytochrome *c* (FCC). Residues that are identical among at least four of the sequences are indicated by black shading. The conserved FAD binding domains are indicated by overlining. The two fully conserved cysteines are marked by asterisks.

Cloning of SQR genes. Based on tryptic peptides and conserved regions, fully degenerate primers were prepared for PCR amplification with genomic DNA as a template. For *O. limnetica*, the forward primer, 5'-CCIACIGCITA(CT)GA(AG)CT-3' (P1), was designed on the basis of the previously identified (2) N-terminal polypeptide stretch P¹⁵TAYEL²⁰ (Fig. 1), with inosine in the fully degenerate positions. The reverse primer, 5'-TG(AGCT)CC(AGCT)AC(AG)TA(AGCT)GG(CT)TC-3' (P2), was designed on the basis of the peptide H¹⁹⁶GVYPE¹⁹¹, which is fully conserved among *R. capsulatus* SQR (33), and an open reading frame (ORF) from *A. aeolicus* (13) (Fig. 1). This reverse primer was also used for *A. halophytica*, together with the forward primer, 5'-TT(CT)GG(AGCT)CC(AGCT)GC(AGCT)TA(CT)GAATT(CT)-3' (P3), which was designed on the basis of the conserved sequence F¹⁶⁰GPAYEF¹⁶⁶ (Fig. 1). The PCR was performed with Q-biotaq enzyme (Quantum) by use of an Eppendorf Mastercycler 5330 apparatus, with various annealing temperatures (touchdown PCR [29]), starting at 48°C and reducing the temperature by 2°C every 10 cycles down to 38°C. Specific PCR products were purified, cloned into T-vector that was freshly prepared as described previously (21), and sequenced.

Genomic DNA libraries were constructed using a Lambda ZAPII/predigested vector/Gigapack cloning kit (Stratagene). ³²P-labeled probes were used to screen the libraries. Plasmids (pBS-OL from the *O. limnetica* library and pBS-AH from the *A. halophytica* library) were excised from positive plaques. Sequencing of DNA was conducted by use of an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer).

Sequence analysis and polypeptide alignments. Sequence analysis was done using Wisconsin Package version 9.0 (Genetics Computer Group, Madison, Wis.). Preliminary sequence data were obtained from The Institute for Genomic Research website at <http://www.tigr.org> and the CyanoBase website at <http://www.kazusa.or.jp/cyano/search.html>. Polypeptide alignments were done using ClustalW (<http://www2.ebi.ac.uk/clustalw>) and refined by visual inspection.

Expression of SQR in *E. coli*. For expression of the cyanobacterial SQRs in *E. coli*, the genes were amplified from the respective plasmids by PCR using the following primers: 5'-CGGAATTCATATGGCACACGTTGAGTTAT-3' and 5'-TCCCCGGGGGACATCTCTATTCCCTAGTCCC-3' for *O. limnetica* SQR and 5'-CCGGAATTCATATGGCACATATCGTAATTG-3' and 5'-CGCGGATCCGCGCAATAACTTAAACCGACTGG-3' for *A. halophytica* SQR. The amplified fragments were purified and ligated to freshly prepared T-vector, resulting in pT-OL and pT-AH, respectively. An *Nde*I-*Sma*I fragment from pT-OL or a *Nde*I-*Bam*HI fragment from pT-AH was inserted into the *Nde*I-*Sma*I or *Nde*I-*Bam*HI restriction sites of pT7-7, resulting in pTOLSqr or pTAHsqr, respectively. Plasmid amplifications were done with *E. coli* JM109.

For expression of SQR, *E. coli* BL21(DE3) was transformed with pTOLSqr or pTAHsqr and grown in LB broth at 37°C. Expression was induced at an optical density at 600 nm of 0.6 by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and further incubation for 3 h. Expression levels were determined by SDS-PAGE analysis.

Membranes were prepared from *E. coli* cells as described previously (33) and either tested for SQR activity or frozen in liquid nitrogen. For solubilization of SQR, membranes (containing 100 to 300 μg of protein) were resuspended in 1.15 ml of buffer containing 60 mM choline chloride, 4.5 mM Tris-Cl (pH 8.0), 110 mM sucrose, 20% glycerol, 100 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), and 1.25% *n*-dodecyl-β-D-maltoside (DM). After 30 min of incubation on ice, the suspension was centrifuged for 25 min at 435,000 × *g*. The supernatant solution was collected (DM sup). The pellet was resuspended in 1 ml of lysis buffer (50 mM Tris-Cl [pH 8.0], 100 mM NaCl, 10 mM EDTA) containing 0.5% Triton X-100. Incubation and centrifugation were repeated as above. The supernatant solution was collected (Triton sup). The pellet was resuspended in 1 ml of lysis buffer containing 8 M urea and incubated and centrifuged as above. The

supernatant solution was collected (Urea sup). The pellet was resuspended in 500 μl of lysis buffer (Urea pellet). The presence of SQR was determined by both Coomassie blue staining and Western blotting after separation of the proteins by SDS-PAGE.

RESULTS AND DISCUSSION

Cloning of *Oscillatoria* *sqr*. The cloning of *O. limnetica* *sqr* was based on the following amino acid sequence information: (i) the N-terminal sequence (2); (ii) four sequences of tryptic peptides that were obtained from purified *O. limnetica* SQR; and (iii) information obtained from the complete genome sequence of the hyperthermophilic chemolithotrophic bacterium *A. aeolicus*, which belongs to the first branching-off lineage within the phylogenetic tree of bacteria (13). In the genome of this bacterium, an ORF was identified that encodes a protein with 38% identity and 57% similarity to the *R. capsulatus* SQR, a remarkable homology in view of the evolutionary distance between the two species (45) (see Fig. 6). Alignment of the two sequences revealed four fully conserved regions that were used for the cloning of *O. limnetica* *sqr*. Based on all of the amino acid sequences, fully degenerate primers were designed and used in different combinations in touchdown PCR as described in Materials and Methods to obtain a specific probe for cyanobacterial *sqr*. A 546-bp fragment that was obtained by PCR with primer P2 (based on a *Rhodobacter-Aquifex* conserved domain) and primer P1 (based on part of the N-terminal sequence) was found to contain a region coding for one of the tryptic peptides of *O. limnetica* SQR (D¹⁶⁰RVPIT¹⁶⁶; Fig. 1). This fragment was then used as a probe for screening an *O. limnetica* genomic library. The DNA of several positive clones was sequenced and found to contain an ORF that encodes a protein that includes all the amino acid sequences of *O. limnetica* SQR tryptic peptides. The GenBank accession number for the *O. limnetica* SQR gene is AF242368.

SQR activity in *A. halophytica*. Cells of *A. halophytica* were previously shown to be capable of anoxygenic sulfide-dependent photosynthesis but incapable of growth on sulfide. Like that in *O. limnetica*, the anoxygenic reaction in *A. halophytica* requires sulfide induction (15). Therefore, we looked for SQR activity in *A. halophytica* cells that had been induced for 2 h in the presence of 1.25 mM sulfide and light. Unlike the situation in *O. limnetica*, in which SQR activity was observed only in

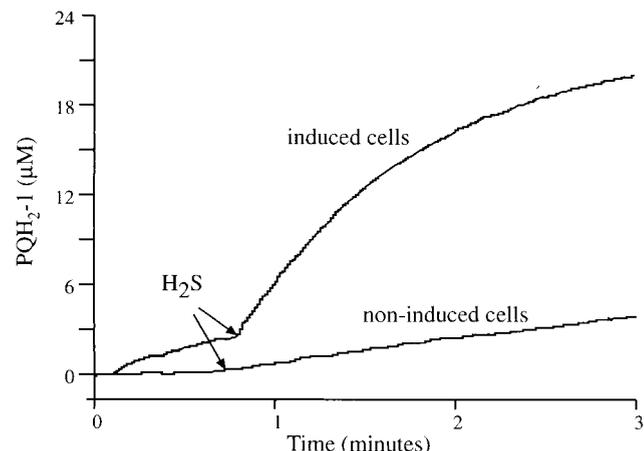


FIG. 2. SQR activity in *A. halophytica*. The assay mixture contained 10 mM potassium HEPES (pH 7.4), 10 mM MgCl₂, 10 mM KCl, 30 μM PQ-1, and cells containing 3 μg of chlorophyll per ml. The reduction of PQ-1 was detected at A₂₉₂ minus A₂₆₆. Where indicated, the reaction was started by the injection of 60 μM Na₂S. PQH₂-1, reduced PQ-1.

	ah	ol	rc	aa	sp	tf	scp	av
<i>O. limnetica</i> (ol)	58							
<i>R. capsulatus</i> (rc)	40	74						
<i>A. aeolicus</i> (aa)	40	59	42					
<i>S. putrefaciens</i> (sp)	42	56	44	38				
<i>T. ferrooxidans</i> (tf)	37	55	42	45	39			
<i>S. pombe</i> (scp)	20	36	38	46	40	50		
<i>A. vinosum</i> (av)	25	38	38	40	56	66	17	
<i>Anabaena</i> PCC7120	59	75	80	60	58	57	40	20

FIG. 3. Identity and similarity of SQR sequences. ah, *A. halophytica*. The number in the upper left corner of each box is the percent identity (from a Blast search), and the number in the lower right corner is the percent similarity (from a Blast search).

thylakoids isolated from sulfide-induced cells, a high rate of PQ-1 reduction (180 μ M PQ-1 reduced/mg of chlorophyll/h) was measured in intact induced cells of *A. halophytica* (Fig. 2). On a chlorophyll basis, this rate was about 50% higher than that reported previously for *Oscillatoria* thylakoids. Attempts to further increase the accessibility of the substrates (sulfide and PQ-1) by permeabilization of the cells with toluene, mild lysozyme treatment, or sonication had no effect.

To assess whether *A. halophytica* SQR is membrane bound, thylakoid membranes were prepared from sulfide-induced *A. halophytica* cultures by lysozyme treatment and mild sonication (1). Thylakoids were then washed with 5 mM sodium EDTA, and the SQR was solubilized with 10 mM sodium cholate and 25 mM DM as previously described for *O. limnetica* (2). Most of the SQR activity measured in intact cells or thylakoids was retained in the solubilized preparation (data not shown). The solubilized SQR had K_m values of 20 and 40 μ M for H_2S and PQ-1, respectively, and was specifically inhibited by the qui-

none analog 2*n*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) in the submicromolar range (data not shown).

Cloning of *A. halophytica* *sqr*. Alignment of the amino acid sequence of cloned *O. limnetica* SQR with those of *R. capsulatus* and *A. aeolicus* confirmed the conserved domains (Fig. 1). Based on these domains, a few more degenerate primers were constructed and used in touchdown PCR to obtain a probe specific for *A. halophytica* *sqr*. Primers P2 and P3 yielded a fragment of the expected size (111 bp). The nucleotide sequence of this fragment was 77% identical to that of *O. limnetica* *sqr*. This fragment was used as a probe for cloning the entire gene from the *A. halophytica* genomic library. The DNA of a few positive clones was sequenced and found to contain an ORF (GenBank accession no. AF242369) that encodes a protein that is 58% identical and 75% similar to *O. limnetica* SQR (Fig. 3).

ORF downstream of *sqr*. Downstream of each cyanobacterial *sqr* gene, a small ORF (beginning 3 bp after the termination codon of *A. halophytica* *sqr* or 195 bp downstream of the termination codon of *O. limnetica* *sqr*) encoding a 91-amino-acid peptide or a 100-amino-acid peptide was found (GenBank accession no. AF242371 and AF242370, respectively). The two ORFs share 63% identity and 69% similarity. A BLAST search for the proteins revealed highest scores (about 35% identity and 60% similarity) for several transcription factors from different sources, mostly bacteria and cyanobacteria. Since in both cyanobacterial systems SQR is an induced protein and regulatory proteins are often located in the vicinity of their targets of regulation, these small proteins may play a role in the regulation of SQR.

Expression of SQR in *E. coli*. Since no molecular tools exist for either *O. limnetica* or *A. halophytica*, we expressed the cyanobacterial *sqr* genes in *E. coli*. Both genes were cloned into the pT7-7 vector under the control of the T7 promoter, yielding pTOLSqr and pTAHsqr, which contained the *O. limnetica* *sqr* and *A. halophytica* *sqr* genes, respectively (Table 1). *E. coli* BL21(DE3) cells containing a chromosomal T7 RNA polymerase gene inducible by IPTG were transformed with these plasmids. IPTG induction gave major bands of the expected ap-

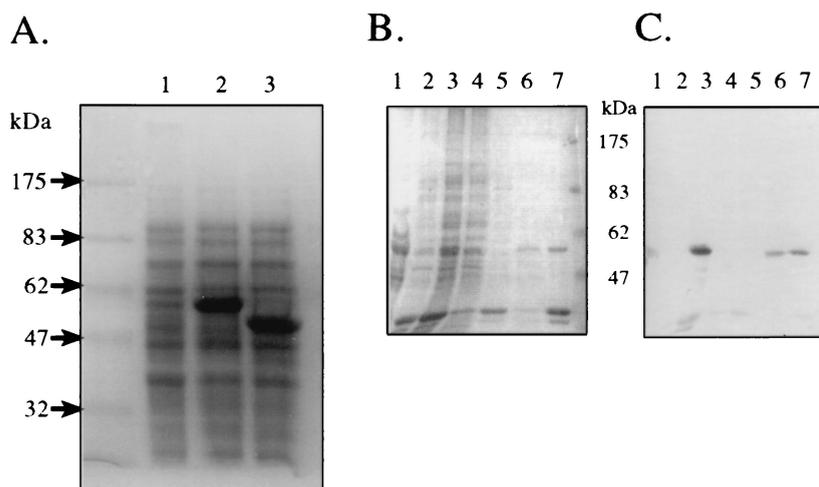


FIG. 4. Overexpression of cyanobacterial SQR in *E. coli* (A) and solubilization (B) and antibody recognition (C) of overexpressed *O. limnetica* SQR. (A) Cells of *E. coli* BL21(DE3) transformed with either pTOLSqr or pTAHsqr were grown at 37°C in LB medium. At an optical density at 600 nm of 0.6, IPTG was added to 0.5 mM. After 2 h, 0.5-ml cultures were centrifuged, resuspended in 2% SDS loading buffer, and boiled for 5 min. Samples of 15 μ l were loaded on the gels. Lane 1, noninduced BL21(DE3)/pTOLSqr; lane 2, induced BL21(DE3)/pTOLSqr; lane 3, induced BL21(DE3)/pTAHsqr. (B and C) Coomassie blue staining (B) and Western blotting (C) with polyclonal antibody raised against denatured *O. limnetica* SQR (2). Lane 1, *O. limnetica* membranes (containing 2 μ g of chlorophyll); lane 2, noninduced BL21(DE3)/pTOLSqr membranes; lane 3, as in lane 2 but IPTG induced; lane 4, DM sup; lane 5, Triton sup; lane 6, Urea sup; lane 7, Urea pellet (see Materials and Methods). All samples loaded in lanes 2 to 7 were derived from membranes containing 40 μ g of protein.

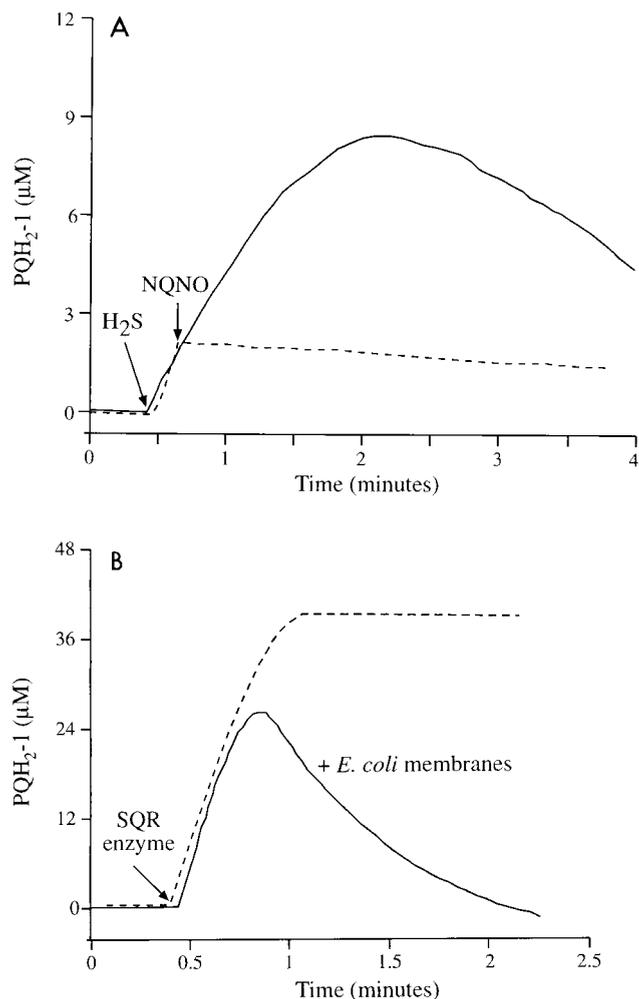


FIG. 5. SQR activity in membranes of *E. coli* expressing *A. halophytica* SQR (A) and kinetics of PQ-1 reduction and reoxidation in the presence of *E. coli* membranes (B). (A) Membranes of induced BL21(DE3)/pTAHsqr (60 μg of protein) were incubated under anaerobic conditions in an assay mixture containing 10 mM potassium HEPES (pH 7.4), 10 mM MgCl₂, 10 mM KCl, and 50 μM PQ-1. The reaction was started by the addition of 60 μM Na₂S. NQNO (1 μM) was added where indicated. (B) An assay mixture containing 60 μM H₂S was incubated under anaerobic conditions in either the presence (—) or the absence (---) of noninduced *E. coli* membranes (60 μg of protein). Partially purified SQR obtained from *O. limnetica* thylakoids (40 μg of chlorophyll) was added where indicated. PQH₂-1, reduced PQ-1.

parent sizes (52 kDa for *A. halophytica* SQR and 57 kDa for *O. limnetica* SQR) on SDS-PAGE (Fig. 4A). In both cases, after cell disruption, these induced proteins were found in the membrane fraction (Fig. 4B). Polyclonal antibodies raised against denatured *O. limnetica* SQR (2) reacted with the major 57-kDa band derived from *E. coli* cells transformed with pTOLsqr specifically (Fig. 4B and C). The antibodies did not cross-react with the band corresponding to *A. halophytica* SQR (data not shown).

Using standard induction conditions, the overexpressed SQR accumulated in the cells in inclusion bodies in an inactive form, as indicated by the fact that the enzyme was solubilized from the *E. coli* membranes only in the presence of 8 M urea (30) (Fig. 4B and C, lane 6) and not by detergent treatments (DM and Triton X-100; Fig. 4B and C, lanes 4 and 5, respectively). With minimal medium A (rather than LB broth) and a

lower growth and induction temperature (30°C rather than 37°C), SQR activity could be detected in membrane preparations of *E. coli* cells expressing *A. halophytica* SQR (Fig. 5A). SQR activity was not detected in membranes prepared from noninduced *E. coli* cells (data not shown). The estimated specific activity of the heterologously expressed *A. halophytica* SQR enzyme was 3.0 μmol of PQ-1 reduced/mg of SQR protein/min or 142 mol of PQ-1 reduced/mol of SQR/min. The calculation is based on the measured SQR activity in the induced *E. coli* membranes and on the estimated protein content of the distinct band (52 kDa) in the same membrane preparation. This activity of the heterologously expressed membrane-bound *A. halophytica* SQR is comparable with the activities of the purified *O. limnetica* SQR (1.9 μmol of PQ-1 reduced/mg of protein/min) (2) and the *R. capsulatus* SQR expressed in *E. coli* (4.0 μmol of decyl-UQ reduced/mg of protein/min) (33). SQR activity was completely inhibited by 1 μM quinone analog NQNO (Fig. 5A), as expected for cyanobacterial SQR activity (1, 2).

As shown in Fig. 5A, in the heterologous expression system, the quinol (PQH₂-1) is reoxidized. The apparent reoxidation of the quinol results from a competitive quinol oxidant that was observed in the *E. coli* preparations. This unidentified oxidant was apparent also when *E. coli* membranes were added to the assay mixture of purified *O. limnetica* SQR (Fig. 5B). It was found to be insensitive to either anaerobiosis or KCN (up to 0.2 mM) but was lost upon boiling of the *E. coli* membranes. This oxidant is membrane bound, as it cannot be washed away but can be extracted by detergents (e.g., DM). Hence, the calculated activity of the expressed *A. halophytica* SQR is underestimated.

Cyanobacterial SQRs. The two SQR polypeptides deduced from the gene sequences consist of 436 (*O. limnetica* SQR) and 437 (*A. halophytica* SQR) amino acid residues, with molecular weights of 47,744 and 48,205, respectively. Both values are significantly smaller than the apparent molecular weights obtained by SDS-PAGE (Fig. 4A). Aberrant mobility in SDS-PAGE is a property characteristic of membrane proteins (41). Indeed, cyanobacterial SQR behaves as an integral membrane protein, being solubilized in the presence of the detergent DM (2). Structure prediction analysis with PREDATOR (www.embl-heidelberg.de/cgi/predator.serv.pl) predicts 25% α-helix and 18% β-sheet structures for *O. limnetica* SQR and 38% α-helix and 9% β-sheet structures for *A. halophytica* SQR. However, hydropathy analysis with the Kyte-Doolittle hydrophobicity scale did not predict any membrane-spanning α helix. The theoretical isoelectric points are 7.7 for *O. limnetica* SQR and 5.6 for *A. halophytica* SQR, and the net charges at a neutral pH are 0 and -14, respectively. Both cyanobacterial enzymes contain, at their N termini, all 11 fingerprint residues of the βαβ ADP binding site motif characterizing the NAD or FAD binding proteins, with the exception that the 10th fingerprint residue is shifted by one position (44). Two additional segments that were suggested to be involved in FAD binding in the *R. capsulatus* SQR (33) and other enzymes (14) are partially conserved in the cyanobacterial enzymes (Fig. 1).

Although the two cyanobacterial species display different phenotypes in a sulfide-rich environment—*O. limnetica* growing anaerobically (23) and *A. halophytica* only surviving (15)—they share the same initial step in the sulfide oxidation pathway, which is catalyzed by the SQR enzyme. The results presented in this work show that the two enzymes are similar: the K_m values of *A. halophytica* SQR for sulfide and PQ-1 are 20 and 40 μM, respectively, while those of purified *O. limnetica* SQR are 8 and 31 μM, respectively; both enzymes are inhibited by NQNO in the submicromolar range; and both enzymes

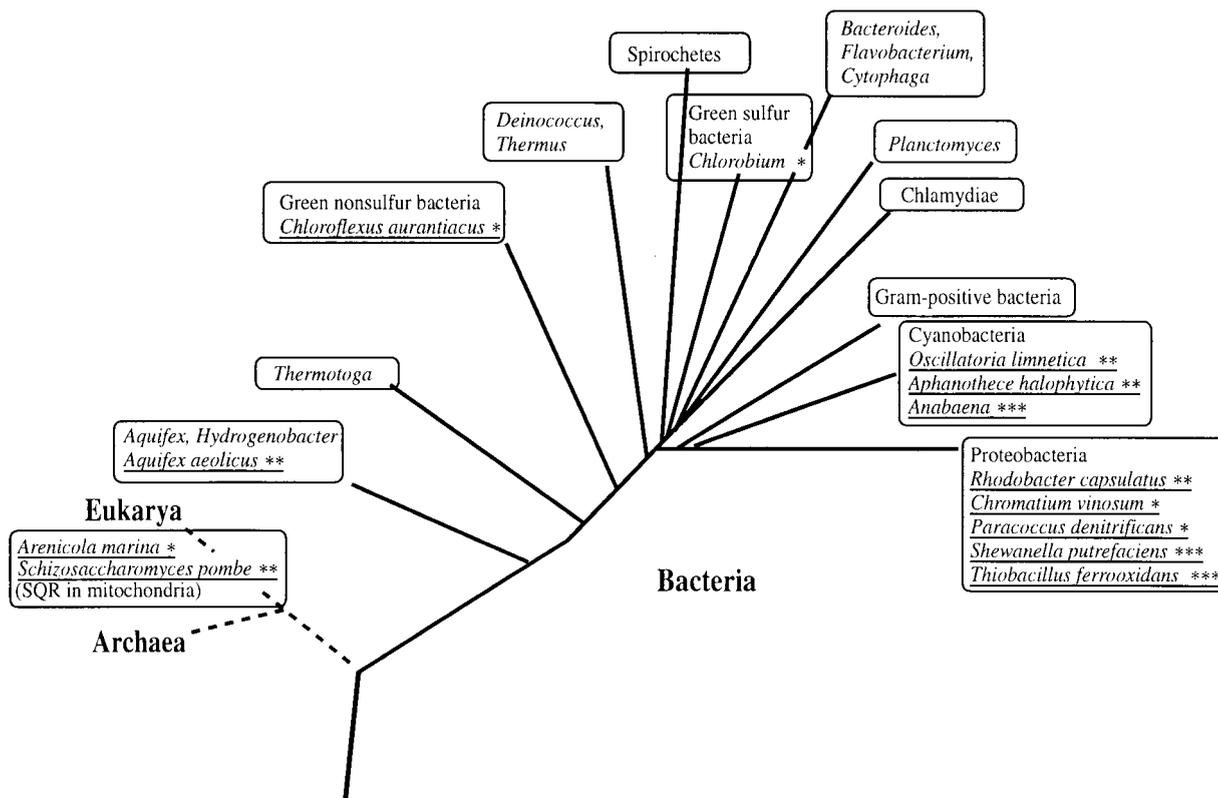


FIG. 6. Distribution of SQRs. Schematic demonstration of the phylogenetic distribution of the SQRs known so far. Species that are already known to possess SQR are underlined. *, SQR detected only by membrane **; both membrane activity and cloned SQR genes; ***, SQR-like ORFs.

are solubilized by the same protocol, previously described for *O. limnetica* SQR (2). Cloning of the cyanobacterial SQRs shows that the two enzymes are highly homologous. Hence, rather than being due to a difference in the SQR mechanism, the different growth phenotypes exhibited by *O. limnetica* and *A. halophytica* could be due to different SQR activity levels in cells, resulting from different expression levels. However, the data showing similar SQR activities are not in favor of this possibility. It therefore seems more likely that the different phenotypes are the outcome of other physiological parameters. For example, the two organisms may differ in unsaturated fatty acid composition and metabolism. *O. limnetica* possesses monounsaturated fatty acids that can be synthesized by the desaturation of long-chain fatty acids under aerobic as well as anaerobic conditions. *A. halophytica* possess polyunsaturated fatty acids that are synthesized only in an oxygen-dependent process (22), a possible reason for its obligate aerobic growth.

Alignment of SQRs and implications. The cloning of *O. limnetica* *sqr* is the first molecular genetic work reported for *O. limnetica*. In *A. halophytica*, one gene, *dnaK* (D84421), had been cloned prior to *sqr* (20). In addition to these cyanobacterial *sqr* genes, the previously cloned *R. capsulatus* *sqr* gene (33), and the *sqr*-like ORF identified in the genome of *A. aeolicus* (13), a data bank search revealed two new *sqr*-like ORFs among the unfinished sequences of the TIGR bank (Fig. 3). An ORF with 42% identity and 56% similarity to *O. limnetica* *sqr* was found in the genome of *Shewanella putrefaciens* (sequence number >4287), and another ORF, with 38% identity and 55% similarity, was found in the genome of *Thiobacillus ferrooxidans* (sequence number >62). These two species

are chemolithotrophic proteobacteria that can utilize sulfide as an electron donor. An additional *sqr*-like ORF was identified in the genome of the filamentous cyanobacterium *Anabaena* PCC7120 and recently published in CyanoBase (sequence number C279). The *Anabaena* ORF is 64% identical and 80% similar to *O. limnetica* *sqr* (Fig. 3). It has been previously suggested that sulfide is formed in *Anabaena variabilis* by the reduction of sulfate in vegetative cells and translocated into heterocysts, where it is utilized (16). The occurrence of SQR in *Anabaena* may suggest the role of sulfide as an electron donor in heterocysts, where photosystem II is known to be inactive.

Alignment of the deduced amino acid sequences from these seven prokaryotic genes allows the identification of conserved domains with possible functional or structural significance (Fig. 1). (i) All seven SQR genes encode proteins with FAD binding fingerprints at their N termini (Fig. 1) as well as two other FAD binding domains that were first recognized in *R. capsulatus* SQR (33). (ii) Recently, based on crystallographic data for several quinone binding proteins and sequence analysis, a structural element for the quinone binding site has been suggested (28). It is composed of a helical stretch that flanks one side of the quinone headgroup and contains a triad of close contact residues. The central residue of the triad in all cases is a conserved histidine which hydrogen bonds to one carbonyl of the quinone. The second residue, four amino acids upstream of the histidine, is an aliphatic amino acid that is in close proximity to the hydrophobic side chain of the quinone (28). Three or four residues downstream of the histidine, there is a residue that is conserved within homologous proteins but that is different between various quinone binding sites (28). In

the SQRs there are two histidines (H¹³¹ and H¹⁹⁶ in *O. limnetica* and H¹³² and H¹⁹⁷ in *A. halophytica*) that are fully conserved (Fig. 1). The first conserved histidine is located in a putative helical stretch (predicted by PREDATOR) with a conserved C(X)₃H(X)₃A sequence and is therefore a possible candidate for the quinone binding site. (iii) In the FAD binding pocket of flavocytochrome *c* of *A. vinosum*, there are a cysteine known to covalently bind flavin and two additional cysteines that form a disulfide bridge (9). Alignment of the SQRs with the flavoprotein subunit of flavocytochrome *c* shows that the cysteine residue that covalently binds FAD in flavocytochrome *c* is not conserved in the SQRs, while the two cysteines that form the disulfide bridge in flavocytochrome *c* (9) are fully conserved in the SQRs (C¹⁵⁹ and C³⁴⁵ of *O. limnetica* and C¹⁶⁰ and C³⁴⁶ of *A. halophytica*). A third fully conserved cysteine (C¹²⁷ or C¹²⁸ in *O. limnetica* or *A. halophytica*, respectively) is not conserved in flavocytochrome *c*. (iv) Glycine has been suggested to play a structural role in many proteins (18). Sixteen glycine residues are conserved in all seven SQRs (Fig. 1).

Most interestingly, the first eukaryotic SQR gene (*hmt2*) was recently cloned from the fission yeast *S. pombe* (43). The *hmt2*⁺ gene was cloned by complementation of a cadmium-hypersensitive *S. pombe* mutant. Purified HMT2 (overexpressed in *E. coli*) exhibited SQR activity (43). Figure 3 shows the high homology shared by all prokaryotic SQRs, as opposed to the low homology to *S. pombe* SQR. In addition, all SQRs, both eukaryotic and prokaryotic, share a low homology with the flavoprotein subunit of *A. vinosum* flavocytochrome *c* (Fig. 1 and 3) (9). Flavocytochrome *c* has previously been considered a sulfide oxidase in prokaryotes. However, its inactivation in *A. vinosum* was reported not to affect sulfide oxidation in this system (27). Although the homology is low, the two previously mentioned cysteines that form the disulfide bridge adjacent to the flavin in flavocytochrome *c* are also conserved in the eukaryotic SQR (Fig. 1). It is worth mentioning that the affinity of the eukaryotic SQR for its substrates was reported to be very low (K_m of 2 mM for both sulfide and coenzyme Q₂) (43), unlike the high affinity measured for the prokaryotic SQRs (K_m values in the micromolar range [34]). It is possible that domains that are conserved among the prokaryotic SQRs but not in the eukaryotic SQR are involved in improving the affinity of the enzyme for its substrates.

The distribution of all the SQRs known so far, including those from cloned genes and the *sqr*-like ORFs and those detected only by membrane activity, is summarized in Fig. 6. The wide distribution emphasizes the important role of SQR in the sulfur cycle in nature.

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