[NiFe] Hydrogenase from *Alteromonas macleodii* with Unusual Stability in the Presence of Oxygen and High Temperature[▽][†]

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Hydrogenases are enzymes involved in the bioproduction of hydrogen, a clean alternative energy source whose combustion generates water as the only end product. In this article we identified and characterized a [NiFe] hydrogenase from the marine bacterium *Alteromonas macleodii* "deep ecotype" with unusual stability toward oxygen and high temperature. The *A. macleodii* hydrogenase (HynSL) can catalyze both H_2 evolution and H_2 uptake reactions. HynSL was expressed in *A. macleodii* under aerobic conditions and reached the maximum activity when the cells entered the late exponential phase. The higher level of hydrogenase activity was accompanied by a greater abundance of the HynSL protein in the late-log or stationary phase. The addition of nickel to the growth medium significantly enhanced the hydrogenase activity. Ni treatment affected the level of the protein, but not the mRNA, indicating that the effect of Ni was exerted at the posttranscriptional level. Hydrogenase activity was distributed ~30% in the membrane fraction and ~70% in the cytoplasmic fraction. Thus, HynSL appears to be loosely membrane-bound. Partially purified *A. macleodii* hydrogenase demonstrated extraordinary stability. It retained 84% of its activity after exposure to 80°C for 2 h. After exposure to air for 45 days at 4°C, it retained nearly 100% of its activity when assayed under anaerobic conditions. Its catalytic activity in the presence of O_2 was evaluated by the hydrogen-deuterium (H-D) exchange assay. In 1% O_2, 20.4% of its H-D exchange activity was retained. The great stability of HynSL makes it a potential candidate for biotechnological applications.

Biological hydrogen production mediated by hydrogenases or nitrogenases is an attractive solution to generate a renewable energy carrier. Since the process catalyzed by nitrogenases requires ATP, hydrogenases may be more efficient for the large-scale production of H_2 as an alternative energy storage molecule. Hydrogenases can catalyze the reversible reduction of protons to molecular H_2 according to the equation $2H^+ + 2 e^- \leftrightarrow H_2$. Depending on the energy demands of the cell, a hydrogenase catalyzes either H_2 production to dissipate excess reductant or H_2 oxidation to capture the energy in H_2 (2, 3, 19). Hydrogenases can be found in a wide variety of microbes, including bacteria, archaea, and unicellular eukaryotes (48). Such microbes may contain one or multiple hydrogenases found in the cytosol, the periplasm, or the cell membrane (47). In addition to its important role in microbial energy metabolism, hydrogenase activity is also involved in other cellular processes, such as methanogenesis, nitrogen fixation, and pathogenesis (47). However, despite its importance to microbial processes, much remains to be understood about the molecular mechanisms for hydrogenase synthesis, assembly, and regulation of gene expression.

Hydrogenases are divided into three distinct groups: [NiFe] hydrogenases, [FeFe] hydrogenases, and [Fe] hydrogenases (44, 48). The [NiFe] hydrogenase represents the largest known group of the hydrogenases (48). Its core enzyme is a heterodimer composed of a large and small subunit and is involved in H_2 evolution and uptake reactions *in vivo*. [NiFe] hydrogenases were crystallized, and the architecture of the catalytic site has been elucidated (50). An intrinsic characteristic of a [NiFe] hydrogenase is the reversible inactivation by O_2, while the [FeFe] hydrogenase is irreversibly inactivated by O_2 (4). [NiFe] hydrogenases are thus more stable to O_2 exposure than [FeFe] hydrogenases.

[NiFe] hydrogenase-mediated processes have been explored for biotechnological applications (32, 46). Photosynthetic and fermentative microbial systems are being developed to use water or carbon sources as feedstock for H_2 production (32, 41, 43, 46), in which electrons generated from photosynthesis or oxidation of carbohydrates are driven to the hydrogenases for reduction of protons to H_2. [NiFe] hydrogenases could also be applied to *in vitro* electrochemical apparatuses (23), such as H_2 fuel cells, in which the hydrogenases are used as bioelectrocatalysts for proton reduction and H_2 oxidation. For a successful biohydrogen production/oxidation system, the [NiFe] hydrogenase needs to be thermostable, tolerant to O_2, and catalytically active in O_2. Due to the limitations of existing hydrogenases, their application on the industrial scale is not yet successful. Efforts are needed to identify [NiFe] hydrogenases with better stability and catalytic activities. In the present study we identified and characterized a [NiFe] hydrogenase from *Alteromonas macleodii* and examined its O_2 tolerance, thermostability, and catalytic activity.

A. macleodii is a heterotrophic marine bacterium present in surface and deep ocean waters. *A. macleodii* strain "deep ecotype" (AltDE) was isolated from the deep Mediterranean Sea (27). Whole-genomic sequence analysis shows that this

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TABLE 1. Bacterial strains used in this study

Strain	Genotype and phenotype[a]	Reference
A. macleodii "deep ecotype" (AltDE)	Wild type	27
A. macleodii strain 107 (ATCC 27126)	Wild type	7
T. roseopersicina GB2131	*hupSL*Δ::Gm, *hoxH*Δ::Em; Gm^r Em^r	36

[a] Gm^r, gentamicin resistance; Em^r, erythromycin resistance.

bacterium contains the gene cluster of a putative [NiFe] hydrogenase (HynSL) (21). According to *Bergey's Manual of Systematic Bacteriology*, AltDE is defined as an aerobic organism, and it is not known to ferment (14). The presence of hydrogenase genes in the genome may indicate the availability of energy-rich H_2 that the organism may be adapted to exploit in its native environment (14). It may also reflect a lifestyle of the bacterium in proximity to anaerobic conditions in the ocean depths and near the pelagic brine lakes of the Mediterranean basin (40).

The putative [NiFe] hydrogenase HynSL from AltDE displayed 60% identity to the large subunit and 64% to the small subunit of the stable [NiFe] hydrogenase (HynSL) from the purple sulfur bacterium *Thiocapsa roseopersicina* (12, 20, 30, 54). In a previous study, we identified an *Alteromonas* [NiFe] hydrogenase from the Sargasso Sea, which is 99% identical to HynSL in AltDE (30). The expression of its genes cloned from the Sargasso Sea in the foreign host *T. roseopersicina* generated an active hydrogenase capable of producing H_2 (30). The goal of the present study was to determine whether the *A. macleodii* [NiFe] hydrogenase HynSL is naturally expressed in AltDE, whether it is active, and how it is regulated during the growth cycle. To investigate how environmental factors such as oxygen, nickel, and iron influence the turnover rates of the hydrogenase, we determined activity of HynSL and examined its expression under various growth conditions. We further studied its cellular localization in AltDE cells, examined its thermostability and O_2 stability, and measured its catalytic activity in the presence of O_2.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the present study are listed in Table 1. *A. macleodii* was grown aerobically in marine broth or agar (Difco) at 27°C. For anaerobic growth, *A. macleodii* cells grown aerobically were transferred into flasks containing fresh medium supplemented with 40 mM KNO_3 as the electron receptor, sealed with rubber stoppers, and then flushed with argon to remove oxygen. To assess the effect of nickel and iron on the expression of HynSL, *A. macleodii* cells were cultured in marine broth supplemented with $NiCl_2$ (10, 50, or 100 μM) and/or EDTA-Fe (50 μM). *T. roseopersicina* was grown anaerobically under illumination in Pfennig's mineral medium or on Pfennig's medium plates as described previously (30). Antibiotics for *T. roseopersicina* were used at the following concentrations: streptomycin, 5 μg ml^{-1}; gentamicin, 5 μg ml^{-1}; and erythromycin, 50 μg ml^{-1}.

Purification of *A. macleodii* and *T. roseopersicina* hydrogenases. Aerobically grown AltDE cells or anaerobically grown *T. roseopersicina* cells were resuspended in a sonication buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 mM dithiothreitol (DTT). The cell suspensions were sonicated twice (each for 2 min) in an ice-water bath by using a Branson Sonifier 250 ultrasonic cell disruptor as described previously (30). The slurry was centrifuged at 16,000 × g for 20 min, and the supernatant was recovered for column chromatography that was performed at room temperature and under ambient air. DEAE 52-cellulose (Whatman) column was equilibrated with the sonication

buffer and used to load the crude cell extracts. After the column was washed with the elution buffer it was eluted with a linear gradient of 0 to 0.6 M NaCl. Eluted fractions were collected and assayed for H_2 evolution activities to identify the fractions containing the hydrogenases. Protein concentration was determined as previously described (30).

Preparation of *A. macleodii* subcellular fractions. AltDE cells were resuspended in 10 ml of solution A (20 mM Tris-HCl [pH 7.5], 1 M mannitol, 1 mM DTT, 0.5 mM EDTA) supplemented with lysozyme (0.4 mg/ml; Sigma) and then incubated at 37°C for 30 min. The efficiency of cell-wall degradation and spheroplast formation was monitored by microscopy. The spheroplast suspension was centrifuged at 16,000 × g for 10 min, and the supernatant was collected as the periplasmic fraction. The pellet was washed with the solution A and then centrifuged at 16,000 × g for 10 min. Recovered spheroplasts were resuspended in 10 ml of solution B (20 mM Tris-HCl [pH 7.5], 1 mM DTT, 0.5 mM EDTA, 1 mg of RNase/ml, 1 mg of DNase/ml) and incubated at 37°C for 30 min. Cell debris was removed by centrifugation at 10,000 × g for 20 min. The supernatant, containing the cytoplasm and the total-membrane fractions, was further centrifuged at 100,000 × g for 3 h, and the supernatant was harvested as the cytoplasmic fraction. The pellet was washed using solution B, and the membrane fraction was recovered by centrifugation at 100,000 × g for 1 h and then resuspended in 10 ml of solution B. The activities of alkaline phosphatase, malate dehydrogenase, and NADH oxidase in the periplasm, cytoplasm, and membrane fractions were determined, respectively, as previously described (17).

PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (38). Coomassie blue staining of proteins in the gels was carried out by using SimplyBlue SafeStain reagent (Invitrogen). Native PAGE using alkaline polyacrylamide gels (pH 10) was performed as described previously (52). Western blotting was performed with polyclonal rabbit antibodies specific for *T. roseopersicina* HynL as the primary antibodies.

Hydrogenase activity assays. *In vitro* H_2 uptake and evolution assays were performed as previously described (30). H_2 uptake activity staining (1, 42) was performed after hydrogenase samples were separated by native PAGE. The gel was incubated in 50 mM potassium phosphate buffer (pH 7) supplemented with 2.5 mM oxidized benzyl viologen (BV) in a sealed bottle. After flushing the bottle with H_2 for 30 min, it was incubated at 55°C for 5 min. After blue bands appeared, 2,3,5-triphenyl tetrazolium chloride (200 μg/ml) was added to fix the staining. A duplicate gel was run simultaneously for Western blotting to determine the positions of the hydrogenases in the gel.

O_2 stability and thermostability assays. Partially purified *A. macleodii* hydrogenase was used for O_2 stability and thermostability assays. To determine thermostability, the hydrogenase samples were treated for 2 h at 70°C or 80°C. The treated samples, along with the untreated sample (used as a control), were then subjected to *in vitro* H_2 evolution activity assay. O_2 stability was examined by determining the H_2 evolution activities of hydrogenase samples on the day of purification and after being exposed to air at 4°C for 14 and 45 days, respectively.

H-D exchange assay. Hydrogen-deuterium (H-D) exchange experiments were performed as previously described (29). Briefly, 0.4 ml each of the partially purified enzyme (~0.55 mg of protein/ml) was incubated in the presence of 20 mM potassium phosphate buffer (pH 7) and deuterium oxide (D_2O; Sigma) in a glass vial sealed with a rubber stopper. After the vessel was flushed out with 10% H_2 balanced with argon, various amounts of O_2 were added. The reaction mixtures were incubated at 37°C for 2 h, and the concentration of H-D in the headspace of the vials was determined by using an Omnistar quadrupole mass spectrometer (Pfeiffer).

RNA extraction and RT-PCR. Each RNA sample was extracted from 1 ml of *A. macleodii* cell culture by using an RNeasy kit (Qiagen) and then treated with a DNA-Free Turbo kit (Ambion) according to the manufacturer's instructions. The RNA samples (500 ng/each) were denatured and run on a 1% agarose gel containing morpholinepropanesulfonic acid buffer and formaldehyde (38). The gel was then stained with ethidium bromide to visualize rRNA that was used as an internal control for RNA quantitation. Reverse transcription-PCR (RT-PCR) was performed as described previously (51) using *hynL*-specific primers (5′-GTA TGG CAT CGG TGC GTG CT and 5′-CTA GCT GCC CAG ACT CAA CC), *hynS*-specific primers (5′-GGC AAT GCG AGA AGA CCC TC and 5′-CAG AGC AAC CAA GAC ACG G), or primers at the junction between *hynS* and *hynL* (5′-CGT CTT TTG GCG GGA TCC C and 5′-GTA AAA TCA GTT CAA TTC CC). The non-RT control reaction, containing all of the components except the reverse transcriptase, was performed as described above.

RESULTS

Examination of *A. macleodii* [NiFe] hydrogenase HynSL in AltDE. Genomic sequencing of AltDE (21) revealed a gene cluster (*hynD/hupH/hynS/hynL/hypC/hypA/hypB/hypD/hypF/hypE*) coding for a putative [NiFe] hydrogenase and its accessory proteins. This hydrogenase, previously named HyaAB (30), is now renamed HynSL to be consistent with the consensus in the hydrogenase community (48). Two structural genes of the *A. macleodii* hydrogenase HynSL, renamed *hynS* (MADE_00346, 999 bp) and *hynL* (MADE_00347, 1,878 bp), encode a 332-amino-acid small subunit (HynS) and a 625-amino-acid large subunit (HynL), respectively. The *hynS/hynL* genes of an *Alteromonas* [NiFe] hydrogenase from the Sargasso sea show 99% identity to their counterpart genes in AltDE and encoded an active hydrogenase in *T. roseopersicina* (30). Thus, we sought to determine whether the *A. macleodii* [NiFe] hydrogenase HynSL is naturally expressed in AltDE and whether it is active.

To determine whether AltDE contains an active hydrogenase, we performed H_2 evolution and uptake activity assays using crude cell extracts from AltDE cells grown under its normal aerobic growth condition. As expected, no activities were detected in the negative control, *A. macleodii* strain 107 (Alt107, ATCC 27126), a strain that naturally does not contain any hydrogenase genes (21). Whereas AltDE displayed both H_2 evolution (16.4 ± 0.7 nmol H_2 mg of protein^{-1} min^{-1}) and H_2 uptake (24.82 ± 2.5 nmol H_2 mg of protein^{-1} min^{-1}) activities at 55°C. These results indicate that AltDE expressed functional hydrogenases. The hydrogenase activity was compared to those at lower temperatures. We observed that the H_2 evolution and H_2 uptake activity at 55°C was approximately two and five times higher than at 37°C, respectively, suggesting that the enzyme is more active at higher temperatures. We partially purified the *A. macleodii* hydrogenase from AltDE cells through column chromatography. The hydrogenase activity was eluted as a single peak from the DEAE 52 cellulose column at ~0.4 M NaCl (Fig. 1A), which is consistent with the fact that no hydrogenase gene homologs other than *hynS* and *hynL* genes were identified in the genome. Although *A. macleodii* hydrogenase was not purified to homogeneity from this chromatography step, an ~10-fold increase in purity was reached in the purified fraction (fraction 38), as evidenced by the fact it had a specific activity that was ~10 times higher (55.45 ± 7.38 nmol of H_2 mg^{-1} min^{-1} in the purified fraction versus 5.53 ± 0.71 nmol of H_2 mg^{-1} min^{-1} in original crude extract).

We further evaluated the partially purified *A. macleodii* hydrogenase by using the H_2 uptake activity staining method. The purified hydrogenase sample was separated on two identical native PAGE gels, along with a positive control from *T. roseopersicina* strain GB2131 (a mutant strain carrying only the *T. roseopersicina* hydrogenase HynSL). We applied one native PAGE gel for in-gel H_2 uptake activity staining and another gel for Western blotting by using antibodies (anti-TrHynL) against the large subunit of *T. roseopersicina* HynSL, which can specifically recognize the *A. macleodii* large subunit HynL (30). A single activity band was detected in the positive control strain GB2131 in the native gel stained for H_2 uptake activities in the presence of BV and H_2 (Fig. 1B). At the position correspond-
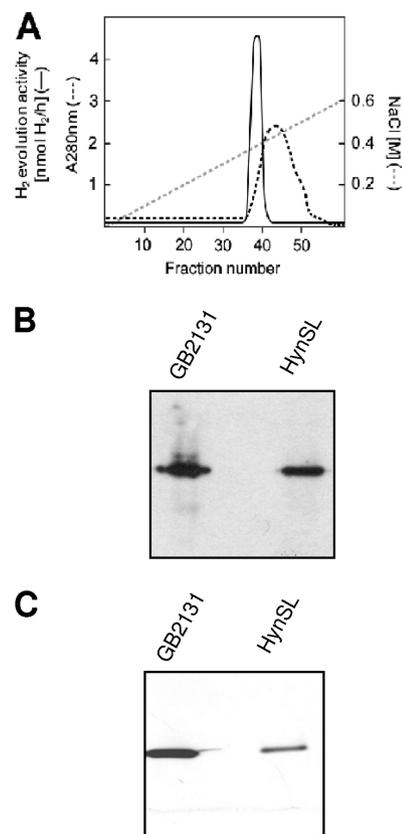

FIG. 1. Purification and identification of *A. macleodii* hydrogenase HynSL from AltDE cells. (A) A_{280} and hydrogenase activity profiles in protein fractions eluted from the DEAE column. The NaCl concentrations (gray dashed line), A_{280} values (black dashed line), and total hydrogenase activities (solid line) in eluted fractions are indicated separately. (B) H_2 uptake activity staining in a native polyacrylamide gel. The gel was incubated in the presence of BV and H_2 for the activity staining. (C) Western blotting to detect the purified AltDE hydrogenase in a duplicate native polyacrylamide gel. Anti-TrHynL antibodies were used for immunodetection of *A. macleodii* HynL. AltDE, crude extract from aerobically grown AltDE cells; HynSL, partially purified *A. macleodii* hydrogenase (fraction 38 eluted from the DEAE column in panel A); GB2131, a *T. roseopersicina* strain used as a positive control. Portions of crude extracts (40 µg) and of partially purified *A. macleodii* HynSL (5 µg) were loaded into each lane for in-gel H_2 uptake activity staining and Western blotting assays.

ing to this activity band, a specific *T. roseopersicina* HynSL band (Fig. 1C) was detected on the duplicate native gel subjected to Western blotting with anti-TrHynL, as expected. A single activity band was also detected in the purified AltDE hydrogenase sample in the stained activity gel (Fig. 1B), and its position matched exactly the position of the specific *A. macleodii* HynSL band detected on the Western blotted gel (Fig. 1C). These results indicate that a hydrogenase from the native PAGE gel had in-gel H_2 uptake activity, and it corresponded to *A. macleodii* hydrogenase HynSL. Overall, our results demonstrate that the *A. macleodii* hydrogenase HynSL is a functional hydrogenase in AltDE.

Effect of oxygen on hydrogenase expression in AltDE. The presence of oxygen has been shown to affect expression of hydrogenase genes, and in most microorganisms hydrogenase expression is limited to microaerobic or anaerobic conditions
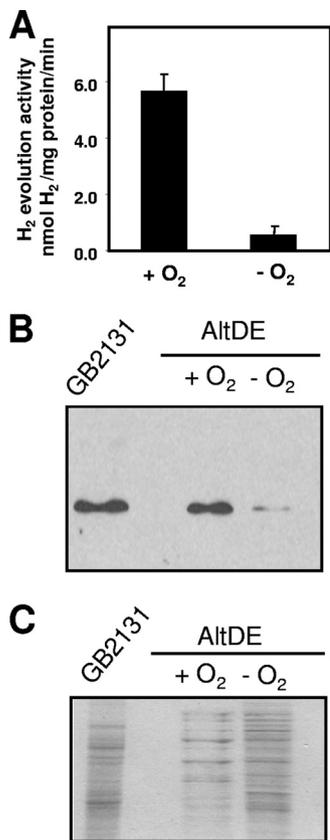
FIG. 2. Effect of O_2 on *A. macleodii* hydrogenase abundance and its activity in AltDE. (A) Examination of H_2 evolution activity in crude cellular extracts from AltDE cells. The AltDE cells were grown in marine broth medium for 18 h under aerobic conditions ($+O_2$) or incubated under anaerobic conditions ($-O_2$). The bars represent average values $\pm$ the standard deviations (SD) of three independent experiments. (B) Immunodetection of HynL in crude cellular extract of AltDE after native PAGE. Crude extracts from the panel A were loaded in the gel, and protein blots were probed with anti-TrHynL antibodies. Crude extract from *T. roseopersicina* GB2131 was loaded as a positive control. (C) SDS-PAGE gel stained with Coomassie brilliant blue. Portions (40 μg) of total protein were loaded into each lane for Western blotting and Coomassie blue staining.

(49). Therefore, we sought to investigate how oxygen regulates *A. macleodii* HynSL in AltDE by performing *in vitro* H_2 evolution assays on crude cell extracts from anaerobically and aerobically grown cells. In AltDE cells grown anaerobically in the medium supplemented with KNO_3, no hydrogenase activity was detected, whereas in AltDE cells anaerobically incubated in the medium without KNO_3, hydrogenase activity was detected, but it was only ~10% of the activity that we observed in cells incubated under aerobic conditions (Fig. 2A). We next examined the level of HynSL protein in the protein extracts after aerobic or anaerobic incubation. Western blotting with anti-TrHynL antibodies after native PAGE of crude cell extracts revealed that the differences in activity were also paralleled by protein accumulation (Fig. 2B). Coomassie blue staining of a duplicate gel after SDS-PAGE confirmed that equivalent total protein was loaded to each lane (Fig. 2C). These results illustrate that contrary to most organisms, the accumulation of active *A. macleodii* HynSL is enhanced in the presence of molecular oxygen.

Effect of metal treatment on hydrogenase expression in AltDE. Nickel and iron are essential elements for the assembly and maturation of [NiFe] hydrogenases (10, 11, 53). We first sought to investigate whether adding extra nickel to the growth medium enhances the activity of the *A. macleodii* hydrogenase HynSL under aerobic growth conditions. Different concentrations (10, 50, and 100 μM) of $NiCl_2$ were tested. Our result shows that the addition of nickel increased the H_2 evolution activity in AltDE in direct proportion to the concentration of $NiCl_2$ added to the medium (see Fig. S1 in the supplemental material). However, the addition of $NiCl_2$ did not affect the growth rate of AltDE cells during the 22-h period (see Fig. S2 in the supplemental material). We further examined the effect of nickel on AltDE cells in different growth phases. The AltDE cells, after being treated with or without $NiCl_2$ for 3, 6, 9, 13, 18, and 22 h, were collected, and hydrogenase activities were determined. Based on the results (Fig. 3A), we have two main observations. First, the addition of $NiCl_2$ (100 μM) increased the H_2 evolution activity in AltDE at all of the time points examined (Fig. 3A). Second, in the absence of added $NiCl_2$, the hydrogenase activity increased consistently along the growth curve of AltDE, until cell growth reached the stationary phase (between 13 and 18 h) (Fig. 3A). This increasing hydrogenase activity in untreated cells was paralleled by a strong increase in HynL protein abundance, as evidenced by Western blotting on cells collected at 3-, 6-, 9-, and 18-h time points (Fig. 3B). These results suggest that additional hydrogenase was synthesized or that it was assembled or matured in the late log or stationary phase.

In contrast to the effect of nickel, the addition of Fe-EDTA (50 μM) did not affect the level of hydrogenase activity (Fig. 3A). Additional iron salts, including ferric sulfate, ferric citrate, ferric ammonium citrate, and ferric nitrate were tested to determine whether other forms of iron might be more accessible to the cells and affect hydrogenase activity. No increase in the hydrogenase activity was observed in response to these iron salts (see Fig. S3 in the supplemental material).

To understand the mechanisms involved in the increased *A. macleodii* HynSL activity in metal-treated cells, we examined HynSL expression at the translational level after the AltDE cells were treated with nickel and iron. The in-gel H_2 uptake activity staining coupled with Western blotting was performed by using two identical native PAGE gels. Only cells treated for 18 h were used for examination since the lower in-gel H_2 uptake activity at 3-, 6-, and 9-h time points could not be detected due to the limitation of the method. Our result (Fig. 3C) shows that H_2 uptake activity was increased after the $NiCl_2$ treatment, whereas the activity stayed the same after the Fe-EDTA treatment, which is consistent with our observation from the H_2 evolution activity assay. Western blotting (Fig. 3D) detected higher levels of HynL in cells with Ni-only or Ni+Fe treatments, whereas the Fe-only treatment did not change the level of HynL. These results suggest that the higher levels of hydrogenase activities are due to increased levels of the active *A. macleodii* HynSL enzyme.

To understand the mechanisms involved in the increased HynSL protein accumulation in Ni-treated cells, we examined *hynSL* gene transcription. First, RT-PCR amplification of
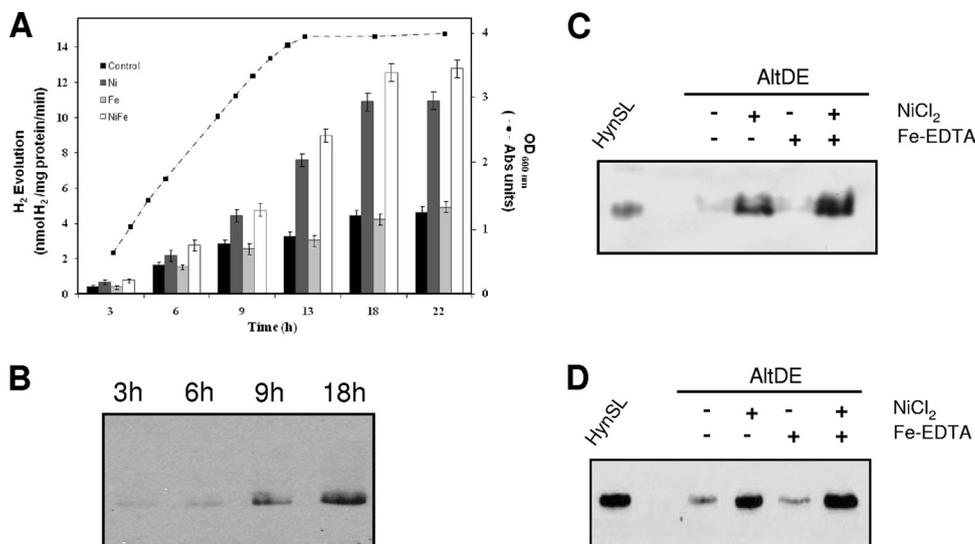
FIG. 3. Effect of nickel and iron on the activity and expression of *A. macleodii* HynSL. (A) Examination of *A. macleodii* hydrogenase activity in AltDE cells aerobically grown in marine broth medium (control), and in the medium supplemented with NiCl$_2$ (Ni-only), Fe-EDTA (Fe-only), or both NiCl$_2$ and Fe-EDTA (Ni+Fe). Growth curve of the AltDE cells in the marine broth medium (control) is presented. H$_2$ evolution activity was assayed using AltDE cells collected at time points as indicated. The bars represent average values ± the SD of three independent experiments. (B) Western blotting to detect expression of HynSL at various time points. Crude extracts from AltDE cells collected at different time points were used for native PAGE. Protein blots were probed with anti-TrHynL antibodies. Lane 1, 3 h (optical density at 600 nm [OD$_{600}$] = 0.5); lane 2, 6 h (OD$_{600}$ = 1.12); lane 3, 9 h (OD$_{600}$ = 2.15); lane 4, 18 h (OD$_{600}$ = 3.87). (C) H$_2$ uptake activity staining after native PAGE. Protein samples used for the staining were collected from the cells at 18 h. (D) Western blotting of protein samples at 18 h after native PAGE. Anti-TrHynL antibodies were used to detect HynL. Partially purified *A. macleodii* HynSL was loaded as a control for in-gel H$_2$ uptake activity staining and Western blotting. Portions (40 µg) of total protein were loaded into each lane.

RNA samples with a pair of primers specific for *hynS* generated a 700-bp specific product (see Fig. S4A in the supplemental material), whereas no products were generated in the no-RT control (see Fig. S4A in the supplemental material), indicating *hynS* was transcribed. Second, RT-PCR amplification with a pair of primers encompassing the junction region of *hynS* and *hynL* generated a 650-bp specific product (see Fig. S4A in the supplemental material), indicating that the *hynS* and *hynL* genes were cotranscribed together.

The effect of the Ni and Fe treatment on accumulation of the *hynL* transcript was examined by RT-PCR in aerobically grown AltDE cells after Ni-only, Fe-only, Ni+Fe, or neither metal (control) treatments. For exponential amplification of *hynL* transcripts, we determined optimal concentrations of RNA templates in RT-PCRs (see Fig. S4B in the supplemental material). Total RNA was isolated from the cells collected at 3, 6, and 9 h after inoculation. In this experiment the samples obtained at 13, 18, and 22 h were excluded from RNA analysis since the cells had reached the stationary phase, and these samples yielded RNA of poor quality that gave inconsistent results. No variation in the RT-PCR band intensity was observed among the metal-treated samples collected at a single time point (see Fig. S4C in the supplemental material). rRNA visualized in a formalin-denatured agarose gel confirms that similar amounts of RNA sample were added to the RT-PCRs (see Fig. S4C in the supplemental material). Thus, the increase in activity from NiCl$_2$ addition is not likely to be regulated by increased transcription of the hydrogenase genes.

Determination of subcellular localization of *A. macleodii* HynSL. Hydrogenases in microorganisms are targeted to different subcellular locations (47). To determine the subcellular

localization of *A. macleodii* HynSL, we prepared periplasmic, cytoplasmic, and membrane fractions from AltDE cells. To evaluate the purity of these fractions, we determined the activities of their corresponding marker enzymes: alkaline phosphatase was used as the marker for the periplasm fraction, malate dehydrogenase for the cytoplasmic fraction, and NADH oxidase for the membrane fraction. In each case, ~90% of the total marker enzyme activity was found in its corresponding fraction. The distribution of the *A. macleodii* hydrogenase activity among these three fractions was examined. The result (Fig. 4A) shows that the activity was present in cytoplasmic (70%) and membrane (30%) fractions but not in the periplasm. Therefore, the bipartite localization of *A. macleodii* HynSL between the cytoplasm and the membranes was in a ratio of 7:3. The presence of HynSL in the cytoplasmic and membrane fractions was further confirmed by immunodetection of HynL after native PAGE (Fig. 4B). It was observed that the ratio of the hydrogenase activity between the membrane and cytoplasmic fractions (roughly 1:2) (Fig. 4A) was significantly higher than that of the protein levels between these two fractions (roughly 1:10) (Fig. 4B). This observation suggests that the specific hydrogenase activity in the membrane fraction was significantly higher than in the cytoplasmic fraction.

Examination of thermostability of *A. macleodii* HynSL. The *T. roseopersicina* [NiFe] hydrogenase HynSL has extraordinary stability at high temperatures (20). We determined whether *A. macleodii* hydrogenase HynSL is also thermostable. Aliquots of partially purified *A. macleodii* HynSL were heated at 70°C or 80°C for 2 h before they were subjected to H$_2$ evolution activity assays. Parallel experiments were also performed on *T. roseopersicina* HynSL that was partially purified through the DEAE
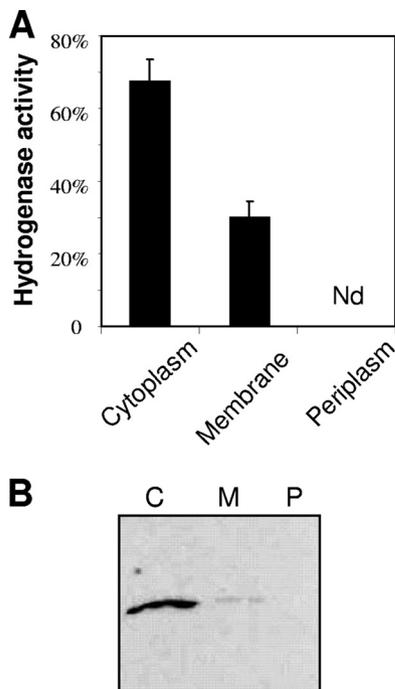
FIG. 4. Determination of *A. macleodii* HynSL localization. (A) Detection of *A. macleodii* HynSL activity in the cytoplasm, membrane, and periplasm fractions. Bars depict relative activity with respect to the total activity prior to cellular fractioning (100% activity). The data displayed represent average values ± the SD of three independent experiments. Nd, none detected. (B) Immunodetection of *A. macleodii* HynSL in the cytoplasm, membrane, and periplasm fractions. Three cellular fractions prepared from equal amounts of cells were separated on a native PAGE, and the protein blot was probed with anti-TrHynL antibodies. Lanes: C, the cytoplasmic fraction; P, the periplasmic fraction; M, the membrane fraction. A total of 40 μg of total protein was loaded into each lane for Western blotting.

52 cellulose column. Figure 5 shows the results of thermostability assays, where the activities of the heat-treated hydrogenase samples were compared to the untreated hydrogenase samples. The activity of the untreated samples was used as a standard and normalized to 100%. *A. macleodii* partially purified HynSL retained 94% of the original activity after the heat treatment at 70°C, whereas, after the 80°C treatment, the enzyme retained 84% of its activity (Fig. 5A). For *T. roseopersicina* partially purified HynSL 75% of the original activity remained after the treatment at 70°C and less than half (43%) remained following the 80°C treatment (Fig. 5B).

Examination of O$_2$ stability and catalytic activity of *A. macleodii* HynSL. To examine the O$_2$ stability of *A. macleodii* hydrogenase HynSL, we exposed the partially purified enzyme to air (21% O$_2$) at 4°C over a period of 45 days. The H$_2$ evolution activity of the enzyme was assessed at day 1 (on the day of purification), day 14, and day 45 by performing *in vitro* H$_2$ evolution activity assays under the anaerobic condition with reduced BV as an artificial electron donor. Our results show that at day 14 and day 45 the *A. macleodii* hydrogenase retained 100.1% ± 1% and 98.5% ± 0.9% of its activity at day 1, respectively. This result demonstrates that the partially purified HynSL has a remarkably high stability in O$_2$ and that its
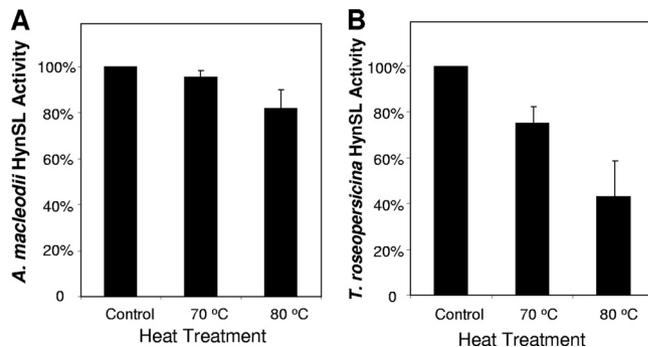

FIG. 5. Examination of thermostability of *A. macleodii* HynSL and *T. roseopersicina* HynSL. (A) Thermostability assay of *A. macleodii* HynSL. (B) Thermostability assay of *T. roseopersicina* HynSL. Partially purified HynSL from each organism was incubated under aerobic conditions for 2 h on ice (control), at 70°C, or at 80°C. Aliquots of the three treated samples were used for H$_2$ evolution assays. The activities of the untreated samples (49.30 nmol of H$_2$ mg^{-1} min^{-1} for *A. macleodii* HynSL and 97.85 nmol of H$_2$ mg^{-1} min^{-1} for *T. roseopersicina* HynSL) were used as standards (100% activity). The activities of heat-treated samples were then normalized to control values. The values represent averages ± the SD of three independent experiments.

activity can be fully restored under anaerobic conditions after being exposed to air for such a long period.

We further determined whether *A. macleodii* HynSL has any catalytic activity in the presence of O$_2$ by using the H-D exchange assay. This assay is based on the intrinsic property of a hydrogenase to catalyze an isotopic exchange reaction between the protons in H$_2$ and the deuterium ions in deuterium oxide (D$_2$O) to yield H-D. Since this reaction does not require any additional electron mediator, it can be used to assess the catalytic properties of the enzyme in O$_2$. The partially purified enzyme was used for H-D exchange activity assays in absence or in the presence of 1%, 3% or 5% O$_2$. Like most bidirectional hydrogenases, *A. macleodii* HynSL was able to catalyze the H-D exchange reaction when resuspended in D$_2$O in a H$_2$ atmosphere without any O$_2$. When the reaction mixture was exposed to 1% O$_2$ with continuous stirring, the enzyme retained 20.4% ± 1.9% H-D exchange activity, indicating *A. macleodii* HynSL had catalytic activity in 1% O$_2$. The activity dropped to 5.2% ± 1.4% when the reaction was exposed to 3% O$_2$, and no activity was detected in 5% O$_2$. We performed a parallel study on the *T. roseopersicina* hydrogenase HynSL, and no H-D exchange activity was detected in three O$_2$ concentrations tested, a finding that is consistent with the previous report (20).

DISCUSSION

This study demonstrates that the [NiFe] hydrogenase HynSL in the marine bacterium *A. macleodii* AltDE, a homolog of the *T. roseopersicina* stable [NiFe] hydrogenase HynSL, is naturally expressed and functionally active. *A. macleodii* HynSL was found in AltDE but not in another *A. macleodii* strain Alt107, suggesting the enzyme is not conserved among various *A. macleodii* strains. This lack of conservation is consistent with studies on the genomic sequences in *A. macleodii* (21). The 4.4-Mb genome of AltDE has 4,102 annotated genes. However, 1,242 of these genes, including all genes in the HynSL gene cluster,

were missing in the genome of Alt107. The HynSL gene cluster was discovered in a genomic island of ~95 kb in the AltDE genome (21), suggesting that the genes of the *A. macleodii* hydrogenase were likely introduced to *A. macleodii* through a horizontal gene transfer event. The fact that 65 transposable elements (including one near the gene locus of the *A. macleodii* hydrogenase) were identified in the AltDE genome (21) further supports this hypothesis.

In addition to the *T. roseopersicina* hydrogenase HynSL, the *A. macleodii* hydrogenase HynSL shares strong homology (60 to 69% identity at the protein level) with [NiFe] hydrogenases from *Thiobacillus denitrificans* ATCC 25259 (Tbd-1375 and -1378), *Thioalkalivibrio* sp. strain HL-EbGR7 (Tqr7-1266 and -1269), and *Allochromatium vinosum* DSM 180 (Alividraft-0802 and 08085) (8, 16, 26). The strong homology suggests common origins for these [NiFe] hydrogenases. Among *A. macleodii* HynSL and its four homologs, *Thiobacillus* and *Thioalkalivibrio* hydrogenases are most closely related to each other. Like *A. macleodii* HynSL, their structural and accessory genes are clustered together. Their gene clusters (*hynL*/*isp2*/*isp1*/*hynS*/*hupH*/*hynD*/*hypC*/*hypA*/*hypB*/*hypD*/*hypF*/*hypE*) share similar gene components and arrangement as the HynSL gene cluster, except that their structural genes are arranged differently. The structural genes *hynS* and *hynL* of *A. macleodii* HynSL are adjacent to each other, while the structural genes *hynS* and *hynL* for the *Thiobacillus* and *Thioalkalivibrio* hydrogenases are separated by *isp1* and *isp2* to form the cluster *hynL-isp2-isp1-hynS*. The protein products of *isp1* and *isp2* are involved in the *in vivo* function of the hydrogenase (34); however, no counterparts of *isp1* and *isp2* are found in AltDE. The other two homologs (16, 35) found in *T. roseopersicina* and *A. vinosum* also have the unusual arrangement (*hynL-isp2-isp1-hynS*) and, unlike *A. macleodii* HynSL, their structural and accessory genes are scattered throughout the genomes. The 10 proteins encoded by the *A. macleodii* gene cluster have the overall best matches with their *Thiobacillus* and *Thioalkalivibrio* counterparts. These findings suggest that *A. macleodii* hydrogenase HynSL is more closely related to the *Thiobacillus* and *Thioalkalivibrio* hydrogenases than to *T. roseopersicina* and *A. vinosum* hydrogenases. The *A. macleodii* HynSL gene cluster could have been acquired from *Thiobacillus denitrificans* and *Thioalkalivibrio* sp. by horizontal gene transfer. However, the G+C content of the HynSL gene locus (46%), which is similar to the overall G+C content of the AltDE genome (44.9%), is substantially lower than those of its homolog hydrogenases (63 to 68%) that are also in the same range as the overall G+C contents of their corresponding bacterial genomes. Therefore, they could have gone through substantial evolution since their genes were first introduced into *A. macleodii*.

Nickel is an essential component of the catalytic site of [NiFe] hydrogenases (4). Previous reports described the effects of nickel on the hydrogenase expression and activity (5, 18, 31, 37, 45). Our analysis at the functional, protein, and mRNA levels of HynSL in AltDE cells further reveals the mechanisms that govern the effects of nickel. The significantly higher amount of HynSL produced at the posttranscriptional level is likely due to improved translation or more efficient assembly/maturation of the hydrogenase in the presence of nickel. It is also possible that sufficient nickel increases enzyme stability.

Iron is also an essential component of the [NiFe]-catalytic site. However, in contrast to the strong stimulatory effect of nickel on hydrogenase activity, little effect of iron was observed. This could be the result of already high levels of total iron contained in the marine broth media (>400 μM). In this case, iron would not be limiting, and further addition would have no effect on iron metabolism in the cells.

Oxygen is important in the regulation of hydrogenase expression and activity (13, 49). Previous studies reported that the [NiFe] hydrogenases from organisms such as *Escherichia coli*, *Rhizobium japonicum*, *Bradyrhizobium japonicum*, *Alcaligenes eutrophus*, and *T. roseopersicina* are upregulated under microaerobic or anaerobic conditions (15, 24, 25, 28, 33). For instance, in *E. coli* the anaerobic global regulator Fnr (fumarate nitrate reduction) senses low concentrations of O_2 and activates the expression of the *hypABCDE* operon for the biosynthesis of hydrogenases (33). The AltDE genome encodes a homolog of the Fnr protein in *E. coli*, suggesting AltDE might have a similar regulatory mechanism in response to oxygen limitation. However, we found that both the protein abundance and enzymatic activity of *A. macleodii* HynSL are downregulated in the absence of oxygen, suggesting that AltDE may possess different cellular machinery for regulating hydrogenase expression.

Protein sequence alignment shows that *A. macleodii* HynSL belongs to the group 1 hydrogenases, a group of membrane-associated hydrogenases classified by Vignais et al. (48). In conjunction with the sequence alignment, our localization analysis demonstrates that HynSL is loosely membrane-bound. Similar to the cellular localization of the *T. roseopersicina* stable hydrogenase HynSL (6), *A. macleodii* HynSL was detected in both soluble and membrane fractions, and the specific activity in the membrane fraction was higher. We speculate that HynSL in the soluble fraction was released from the membrane because the hydrogenase was loosely bound. The finding that *A. macleodii* HynSL is membrane targeted is consistent with the fact that the small subunit (HynS) of HynSL carries a twin-arginine signal peptide at the N terminus. This signal peptide can target proteins to the membranes and its presence in HynS strongly suggests that *A. macleodii* HynSL could be targeted to the periplasmic face of the cytoplasmic membrane (9). A gene cluster similar to the Tat operon of *E. coli* (22, 39) was identified in the genomic sequence of AltDE, supporting that HynSL could be targeted to the membranes through the Tat pathway.

The *T. roseopersicina* [NiFe] hydrogenase HynSL is remarkably stable at high temperatures and in the presence of O_2 and its activities were even higher at 70°C in comparison to lower temperatures (20). The *A. macleodii* hydrogenase HynSL demonstrated similar enzyme characteristics to *T. roseopersicina* HynSL, consistent with the existence of strong similarity between these two hydrogenases. Like its homolog, the *T. roseopersicina* hydrogenase HynSL, the *A. macleodii* hydrogenase HynSL showed both H_2 uptake and H_2 evolution activity and the enzyme is also more active at higher temperatures. In the present study, *A. macleodii* HynSL appeared more thermostable and O_2 tolerant than the *T. roseopersicina* hydrogenase HynSL. However, since hydrogenases used for thermostability and O_2 tolerance assays were partially purified, coexisting cellular proteins may possibly provide a stabilizing effect to the

hydrogenases. This possible effect makes comparison of the stability between *A. macleodii* HynSL and *T. roseopersicina* HynSL complicated. Although the same DEAE column and protocol were used for purification, copurified cellular proteins between two hydrogenase preparations were likely different. Consequently, they could contribute differently to hydrogenase stabilities, if such a stabilizing effect did exist. Therefore, we cannot rule out the possibility that higher stability observed in the *A. macleodii* hydrogenase preparation was caused by other cellular components in AltDE.

Nevertheless, considering that partially purified *A. macleodii* HynSL has demonstrated unusual stability at high temperatures and in the presence of O_2, this hydrogenase is potentially a good candidate for biotechnological applications. The mechanism behind the stability remains to be determined. Small sequence differences between *A. macleodii* and *T. roseopersicina* hydrogenases may cause differences in their structural features associated with stability. Further structure-function analysis should reveal the structural determinants related to stability, which will facilitate designing more stable enzymes that can potentially be used for biotechnological purposes.

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