SELENIUM IS INVOLVED IN REGULATION OF PERIPLASMIC HYDROGENASES GENE EXPRESSION IN *DESULFOVIBRIO VULGARIS* HILDENBOROUGH

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Abstract

Desulfovibrio vulgaris Hildenborough (*Dv*H) is a good model organism to study hydrogen metabolism in sulfate-reducing bacteria. Hydrogen is a key compound for these organisms since it is one of their major energy sources in natural habitats, but also an intermediate in the energy metabolism. The *Dv*H genome codes for six different hydrogenases, but only three of them, the periplasmic-facing [FeFe], $[FeNi]_1$ and [FeNiSe] hydrogenases, are usually detected. In this work we studied the synthesis of each of these enzymes in response to different electron donors and acceptors for growth, and the availability of Ni and Se. The formation of the three hydrogenases was not very strongly affected by the electron donors or acceptors used, but highest levels were observed after growth in hydrogen as electron donor and lowest with thiosulfate as electron acceptor. The major effect observed was with inclusion of Se in the growth media, which led to a strong repression of the [FeFe] and $[NiFe]_1$ hydrogenases, and a strong increase in the [NiFeSe] hydrogenase that is not detected in the absence of Se. Ni also led to increased formation of the $[NiFe]_1$ hydrogenase, except for growth with H_2 where its synthesis is very high even without Ni added to the medium. Growth with H_2 results in a strong increase in the soluble forms of the $[NiFe]_1$ and [NiFeSe] hydrogenases. This study is an important contribution to understanding why *Dv*H has three periplasmic hydrogenases. It supports their similar physiological role in H_2 oxidation and reveals that element availability has a strong influence in their relative expression.

Hydrogen plays a central role in the energy metabolism of sulfate reducing bacteria (17). H_2 is one of the major energy sources for these organisms in their natural habitats, but it may also be a product of their fermentative metabolism. Furthermore, a chemiosmotic mechanism involving production and oxidation of H_2 on opposite sides of the membrane has been proposed to explain energy transduction during sulfate respiration with lactate (21). In agreement with the important metabolic role of H_2, hydrogenases (Hases) are particularly abundant proteins in sulfate reducing bacteria, and many species have several different Hases (39). However, it is still not clear what are the exact physiological roles of each of these enzymes, or what is the reason for this apparent redundancy. *Desulfovibrio vulgaris* Hildenborough (*Dv*H), whose genome was recently sequenced (13), is a good model organism to study hydrogen metabolism in sulfate reducing bacteria. The *Dv*H genome encodes for six different Hases, four of which are periplasmically oriented (two [NiFe] isoenzymes, one [NiFeSe] and one [FeFe] Hase) and two that are facing the cytoplasm (two multi-subunit membrane [NiFe] Hases). The [FeFe] Hase (14, 24), $[NiFe]_1$ Hase (29) and [NiFeSe] Hase (36) of *Dv*H have been characterized, and are the dominant Hases detected in extracts of cells grown in standard lactate/sulfate conditions (10, 36).

The [FeFe] Hase is a soluble periplasmic protein with both high affinity for H_2 (37) and a very high H_2-uptake activity (9). It is noteworthy that *Desulfovibrio* spp. are the only known microorganisms with a periplasmic [FeFe] Hase, since these enzymes are most commonly cytoplasmic. A *Dv*H deletion mutant for the [FeFe] Hase showed that its physiological role is hydrogen uptake (26). The $[NiFe]_1$ and [NiFeSe] both belong to the family of uptake Hases (38). Contrary to most Hases in this family they both lack the membrane-bound heme *b* subunit responsible for electron transfer to the membrane quinone pool. However, both $[NiFe]_1$ and [NiFeSe] Hases are membrane-associated proteins (29, 36), but a soluble form of the [NiFeSe] Hase was also detected. The [NiFeSe] Hase is structurally very similar to the [NiFe] Hases, with the major differences being that it has a selenocysteine as one of

the ligands to the active-site Ni, and the small subunit has a medial $[4Fe4S]^{2+/+}$ rather than a $[3Fe4S]^{+/0}$ cluster (11, 36). In standard assays the [NiFeSe] Hase displays much higher activity values than the $[NiFe]_1$ Hase, and it is also resistant to inactivation by oxygen (36). A comparison of the catalytic activities of the three *Dv*H Hases was recently reported (36). As for the [FeFe] Hase, the physiological role of the $[NiFe]_1$ and [NiFeSe] Hases is apparently in H_2 oxidation, and all three enzymes share as a common electron acceptor a periplasmic cytochrome *c* (the type I cytochrome c_3) (25, 36). This raises the question of what distinguishes these three Hases in physiological terms, and one possibility could be differences in expression conditions. Interestingly, the genes coding for the $[NiFe]_1$ and [NiFeSe] Hases are adjacently located in the genomes of both *Dv*H and *D. desulfuricans* G20, suggesting the possibility of coordinated regulation. Regulation of bacterial Hase gene expression is exerted mainly at the transcriptional level and responds to four major types of signals: H_2, O_2, nickel ions and the electron donors and acceptors available (reviewed in (38)). In this work we investigated how different growth conditions and the presence of iron, nickel and selenium in the growth medium affect the expression of the *Dv*H [FeFe], $[FeNi]_1$ and [FeNiSe] Hases.

Gene regulation by both Fe and Ni in prokaryotes is well documented (2, 12, 19). In the particular case of Hases, Ni is required for complete maturation of [NiFe] Hases (32), and transcriptional regulation by Ni has been shown for example in *Bradyrhizobium japonicum* (22), and in *Methanothermobacter marburguensis*. In the latter organism growth in Ni-limited conditions leads to increased expression of the Ni-free Hase H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) and strongly decreased expression of the [NiFe] Hase F_{420}-reducing hydrogenase (Frh) (1). Both these Hases are regulated at transcriptional level, whereas the decreased expression of the [NiFe] methyl viologen reducing hydrogenase (Mvh) in these conditions is due to regulation at a post-transcriptional level (34).

Selenium is an essential trace element for higher animals where selenoproteins are involved in immune function and cellular defence against oxidative stress (5). In most selenoproteins selenium is found as selenocysteine, the 21st amino acid that is co-translationally incorporated into the polypeptide chain (6). Prokaryal selenoproteins have no counterpart in eukarya and are generally involved in energy metabolism. They are found in only a small fraction of bacterial and archaeal genomes, and their number within a given prokaryotic genome is in the order of one to ten (15). Some selenoprotein-containing organisms are absolutely dependent on the availability of selenium, whereas others are able to express cysteine-containing homologues of their selenoproteins. The activity of selenoproteins is usually higher than their cysteine-containing homologues (3, 16) due to the lower pKa and higher nucleophilicity of the selenol group compared to that of the thiol group. The involvement of selenium in transcriptional regulation of selenoproteins has been investigated in detail for two types of Hases in *Methanococcus voltae* (33). This organism has genes encoding for two pairs of homologous [NiFe] Hases, the F_{420}-reducing Hases (Frc and Fru) and the F_{420}-nonreducing Hases (Vhc and Vhu). In each pair one of the enzymes contains selenocysteine as a ligand to the Ni (Fru and Vhu), and the other a cysteine in the corresponding position (Frc and Vhc). Selenium is involved in negative regulation of the two selenium-free Hases, whose genes are linked by a common intergenic region and are only expressed in the absence of Se (4). This intergenic region includes a common negative regulatory element to which a LysR-type regulator binds, as well as two activator elements that are specific for each of the two transcription units (20, 35). *Methanococcus jannaschi* that only has genes encoding for seleno-containing Hases is completely dependent on the presence of selenium in the medium (31). In this work we report that Ni and Se are also involved in the regulation of the periplasmic *Dv*H Hases and that the [NiFeSe] Hase is the main Hase present when the cells are grown in the presence of Se.

MATERIALS AND METHODS

Cell growth and preparation of crude, soluble and membrane extracts

Desulfovibrio vulgaris Hildenborough (DSM 644) was grown in medium C (27) with an iron concentration of 25µM. Medium C contains yeast extract which is a potential source of Ni and Se. The levels of these elements in medium C without additions were below the respective detection limits as determined by atomic absorption spectrometry (< 0.01 mg/l for Ni and < 0.04 mg/l for Se). Medium C was supplemented with different electron donors and electron acceptors, all at a 40 mM concentration. The growth conditions tested were: lactate/sulfate, lactate/thiosulfate, hydrogen/acetate/sulfate (20mM acetate), pyruvate/sulfate, and only pyruvate (fermentative conditions). For each condition three different cultures were performed in parallel using the same inoculum (grown in medium C): one in medium C with no further additions, one in medium C supplemented with 1 µM nickel chloride, and one in medium C supplemented with nickel chloride plus sodium selenide, both at a concentration of 1µM. All cultures were performed in closed 1l glass flasks containing half their volume of culture and N_2 as gas headspace, with the exception of H_2 grown cells that were cultured in a 2.2L bioreactor, with a flow of H_2/CO_2 (80:20) of 900ml/min. and stirring at 250 rpm. Cells were harvested by centrifugation at late exponential phase and resuspended in 20mM Tris-HCl buffer pH 7.6. After addition of DNase the cells were disrupted by passing twice in a French-press cell. The extract was then centrifuged at 10.000g, 15 min, 4ºC to remove cell debris yielding the crude extract, a part of which was centrifuged again at 100.000g, 15min., at 4ºC in order to obtain the soluble, and the membrane fractions. The membranes were resuspended in 20mM Tris-HCl buffer pH 7.6.

H_2 production activity

Hydrogen production activity of crude, soluble and membrane extracts was assayed in 50mM Tris-HCl buffer pH7.6 with 1mM methyl viologen and 15mM of dithionite as electron donors. Quantification of hydrogen in the gaseous phase was carried out by Gas Chromatography as described in (23).

Gels stained for hydrogenase activity

For activity staining gels, samples were run aerobically in a 7.5% gel under native conditions, containing 0.1% Triton X-100 in a refrigerated chamber at 4ºC. The running buffer also contained 0.1% Triton X-100. The gel was then placed in a closed flask with degassed 50mM Tris-HCl buffer pH8, 0.5mM methyl viologen, and flushed with argon, followed by H_2 gas. After development the bands were fixed by adding a 1% 2,3,5-triphenyltetrazolium chloride solution.

Western blot analysis

The protein content of crude extracts was determined by the Bradford assay with gamma-globulin used as protein standard. The crude cell extracts were run in a 12% SDS gel with 0.1% TritonX-100. Proteins were then transferred to 0,45 µm PVDF membranes (Roche) for 1h at 100mV and 4ºC, in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were equilibrated with Tris-buffered saline solution with Tween 20 (TBST) (20mM Tris-HCl pH7.5, 150 mMNaCl, 0.05% Tween 20), and then treated with antiserum raised against the [FeFe], $[FeNi]_1$ and [NiFeSe] Hases of *D.vulgaris* H. The [FeFe] and $[FeNi]_1$ Hase antibodies were diluted 1:5000 whereas the [NiFeSe] Hase antibody was only diluted 1:500, in TBST. Unbound antibodies were removed by three 5 min washes with TBST. Immunodetection of bound antibodies was done by treatment with anti-mouse immunoglobulin G (H+L) alkaline phosphatase conjugate (from Promega), diluted 1:5000, followed by a solution of nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt

(NBT/BCIP solution from Roche). Quantification of immunodetected Hase bands was performed by densitometric analysis with the program Image Master Total Lab v.2.01, after scanning of the blots with the Image Scanner system of Amersham.

RT-PCR experiments

*Dv*H cells were grown anaerobically to the mid-exponential phase in lactate/sulfate medium with addition of Fe, Fe+Ni or Fe+Ni+Se as described before. Total RNA was extracted from the pellets using the hot acid phenol:chloroform method and treated with DNaseI, essentially as described in (8). Based on the gene sequences for the three Hases, retrieved from the *Dv*H genome (available at www.tigr.org), forward and reverse primers were designed for PCR amplification of the following DNA fragments: 584-bp for the [FeFe] Hase large subunit gene (*hydA*) (FH1: 5'-AGTACTGCCCCACCGCCGCCATCTTC-3' and FH2: 5'-CTTTGCGATGCAGGGCATGATGGAGAC-3'); 657-bp for the [NiFe] Hase large subunit gene (*hynA-1*) (FNH1: 5'-GACCCGGCCAAGGCTGCGAAGA-3' and FNH2: 5'-CAGGTCGGTGTACTTGGGTTCTGTCG -3'); 492 for the [NiFeSe] Hase large subunit gene (*hysA*) (FNSH1: 5'-GATACGGCTCCTTTCGTTCCG-3' and FNSH2: 5'-GTAATTGAGGCCGGTCGTCTC-3'). Prior to RT-PCR experiments optimisation of the PCR amplification conditions was established, for each pair of primers, using *Dv*H genomic DNA as template. For each RNA sample, control experiments in which the RT step was omitted were performed, and confirmed the absence of any residual DNA. The reactions were performed using a RT-PCR Master Mix (USB) and 100 ng of total RNA. As loading control the 16S rRNA was used. The results were analysed by Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

RESULTS

We recently reported the isolation of the [NiFeSe] Hase as a major Hase present in cells of *Dv*H grown in lactate/sulfate medium (36). Since the three periplasmic-facing Hases of *Dv*H have apparently a similar physiological function of H_2 uptake, we decided to study their expression in different growth conditions by varying electron donors and acceptors, and also the availability of the elements Ni and Se. The main electron donors used for growing *Desulfovibrio* spp. are lactate, pyruvate and hydrogen, which were tested with sulfate as electron acceptor. We also tested thiosulfate as an alternative electron acceptor with lactate as electron donor, and fermentative conditions with pyruvate as electron donor in the absence of sulfate. Growth curves were obtained for all these conditions so that cells could be collected at the same point of the growth curve for the different conditions. The addition of Ni or Se to the standard Fe-containing medium C did not affect the growth rate or growth yield for the lactate/sulfate (L/S), lactate/thiosulfate (L/T) or hydrogen/sulfate (H_2/S) conditions. For pyruvate/sulfate (P/S) or pyruvate (P) growth in the presence of Se led to a slightly faster growth rate, but similar yield, as compared to Fe or Fe+Ni conditions (data not shown).

Analysis of periplasmic DvH hydrogenases by activity-stained gels

The periplasmic [FeFe], $[NiFe]_1$ and [NiFeSe] Hases of *Dv*H are easily detected in crude cell extracts by PAGE gels stained for H_2-uptake activity (10, 36). The gels have to be run in native conditions in the presence of detergent, since the $[NiFe]_1$ and [NiFeSe] Hases are both membrane-associated proteins and absence of detergent leads to severe band streaking along the gel. Thus, the relative formation of the periplasmic *Dv*H Hases was initially probed by activity-stained gels using cells grown under different conditions. Besides the effect of the different electron donors/acceptors, we wanted also to test the effect of the elements Fe, Ni and Se, which are present in the metal centres of the periplasmic Hases, in the synthesis of these proteins. Thus, for each electron donor/acceptor pair, *Dv*H cells were

grown in the presence of only Fe (25μM), with both Fe (25μM) and Ni (1μM), and in the presence of the three elements Fe (25μM), Ni (1μM) and Se (1μM). The activity stained gel comparing cells grown with only Fe and with Fe+Ni are shown in Figure 1a. For cells grown in the presence of these metals only the [FeFe] and $[NiFe]_1$ Hases are observed, whereas the [NiFeSe] Hase is not detected. In Fe-only media the [FeFe] Hase is predominant, except for growth with H_2/S in which the $[NiFe]_1$ Hase displays a similar band intensity. The [FeFe] Hase band intensity is highest in P/S and H_2/S, and lowest in L/T.

The inclusion of Ni in the medium leads to a strong reduction in the band intensity of the [FeFe] Hase in L/S medium, and a smaller effect is also observed in P/S and pyruvate fermentative conditions. The presence of Ni increases the intensity of the $[NiFe]_1$ Hase for all conditions tested except in H_2/S in which the [FeFe] and $[NiFe]_1$ Hases show similar band intensities in Fe-only or Fe+Ni media. For Fe+Ni media the $[NiFe]_1$ Hase shows highest band intensity in H_2/S followed by L/S, whereas it is almost not detected in L/T.

The activity stained gel comparing cells grown with Fe+Ni and with Fe+Ni+Se are shown in Figure 1b. The Fe+Ni conditions are repeated from Figure 1a, as controls for the Fe+Ni+Se conditions, since band intensity comparisons should only be performed for samples in the same gel. It is readily apparent that inclusion of Se in the growth media has a profound effect on the pattern of the *Dv*H periplasmic Hases, leading to the appearance of a strong band of the [NiFeSe] Hase, which becomes the major Hase detected, and a concomitant decrease in the band intensity of the other two Hases. This effect is observed irrespective of the electron donor/acceptor present. For Fe+Ni+Se media the [NiFeSe] Hase shows highest band intensity in H_2/S and lowest in L/T. For two of the growth conditions (L/S and P) we also tested whether the effect of the three elements on the Hase synthesis was constant along the growth curve. Activity-stained gel analysis of cells collected at initial, middle and late exponential phase and at stationary phase showed that the effect of Ni and Se was constant along the growth curve (data not shown).

Previous work has shown that in *Dv*H grown in the standard L/S conditions the [FeFe] Hase is a soluble periplasmic protein, whereas the $[NiFe]_1$ and [NiFeSe] are both membrane-associated proteins (29, 36). However, a soluble form of the [NiFeSe] Hase was also detected. In order to check whether the Hases are soluble or membrane-bound in the growth conditions tested here, we separated the soluble and membrane extracts and analysed these with the activity-stained gels. Only the Ni+Fe *versus* Ni+Fe+Se conditions were tested since the difference in Hase composition between the Fe and the Fe+Ni crude extracts was less marked. For the L/S, L/T, P/S and P the same pattern was observed where the [FeFe] Hase is only present in the soluble extract, and the $[NiFe]_1$ and [NiFeSe] Hases are found mainly in the membrane fraction, but are also detected with much smaller intensity in the soluble fraction (Figure 2a, only L/S is shown). Interestingly, the cells grown in H_2/S displayed a different pattern where the band intensity of both the $[NiFe]_1$ and [NiFeSe] Hases in the soluble fraction is strongly increased relative to the other conditions. An [FeFe] Hase band in the membrane fraction of Ni+Fe grown cells is also apparent (Figure 2b). This indicates that the increased synthesis of the three Hases in the H_2/S conditions is accompanied by changes in the cellular localization of the three Hases.

Analysis of H_2-producing activity in DvH cell extracts

In the activity-stained gels the band intensity is related to the H_2-uptake activity of the three Hases. To complement these results we tested also the H_2-production activity of the soluble and membrane extracts of *Dv*H cells grown in the different conditions, by GC chromatography (Figure 3). These results cannot be directly compared to the previous ones because it is not possible to discriminate between the three different Hases. Also, the relative ratio of the H_2-uptake activities for the three Hases is different from the ratio of their H_2-production activities. Nevertheless, it is also of interest to analyse the effect of the different growth conditions in the H_2-producing activity. A fairly rough analysis of each Hase contribution can be attempted based on the fact that the soluble extract contains most of the

[FeFe] Hase and only a small amount of the other two Hases, whereas the membrane extract contains the $[NiFe]_1$ and [NiFeSe] Hases (except in the H_2/S conditions). Overall, for both the soluble and membrane extracts, the H_2-production activity is highest in H_2/S and P/S conditions, and lowest in L/T. The soluble extracts of P/S and P grown cells display similar activities in the three element conditions (Figure 3a). The soluble extracts of L/S and L/T display higher activities in Fe-only medium probably due to the increased expression of the [FeFe] Hase in these conditions. The soluble extracts of H_2/S grown cells have twice the activity level in Ni+Fe+Se medium relative to the other two metal conditions, which is likely due to the high activity of the [NiFeSe] Hase that under H_2/S conditions is almost equally distributed between the soluble and membrane extracts, as shown above. The H_2-production activity of membrane extracts for all growth conditions tested displays a sharp increase in the Ni+Fe+Se media (Figure 3b), reflecting the increase in the membrane-bound form of the [NiFeSe] Hase under these growth conditions, which has a much higher H_2-production activity than the soluble form (36).

Analysis of periplasmic DvH hydrogenases by Western blot

The results presented above show striking differences in the Hase pattern of *Dv*H under different growth conditions, particularly in response to Ni and Se. However, they may be fallible for assessing the quantity of these proteins since they are based on the enzymatic activity. The activity of each of the three proteins is different, and is particularly sensitive to their activation state, which in turn is affected by any possible contact with oxygen. Furthermore, the effects observed could be due to an increase/decrease in activity and not to an actual change in protein content of the extracts. In order to have a more clear picture of the Hase pattern in *Dv*H we analysed these proteins in cell extracts by Western blots. For this we obtained polyclonal antibodies against the [FeFe], $[NiFe]_1$ and [NiFeSe]

Hases purified from *Dv*H. These antibodies proved to be very selective, detecting only the respective Hase, and showing no cross-reactivity with the other two Hases when tested with the pure proteins.

Crude cell extracts of *Dv*H cells grown in the conditions mentioned were analysed by Western blot (Figure 4). The pattern of the three Hases under the different growth conditions was broadly in agreement to the results observed by activity-stained gel (Figure 1). Densitometric analysis of the immunoblot bands permits a more quantitative view of the results that is easier to analyse (Figure 5). In Fe-only media the [FeFe] Hase shows highest synthesis with P/S, followed by H_2/S, L/S, P and is lowest in L/T (Figure 5A). As observed in the activity-stained gels, the addition of Ni to the media induces no pronounced effect on the [FeFe] Hase for the H_2/S and L/T media, whereas its formation is reduced in P and P/S conditions and even more markedly in L/S. In all Fe+Ni+Se media the synthesis of the [FeFe] Hase decreases relative to Fe+Ni conditions, but is then constant irrespective of the electron/donor acceptor.

The synthesis of the $[NiFe]_1$ Hase (figure 5B) increases upon addition of Ni to the growth media, except in H_2/S conditions where its synthesis is surprisingly high even in Fe-only medium, and is not affected by the further inclusion of Ni, as observed before. In Fe+Ni media the formation of the $[NiFe]_1$ Hase is highest with H_2/S, followed by P/S, L/S and P and is lowest in L/T. As observed in the activity-stained gels, the addition of Se to the media causes a sharp decrease in the $[NiFe]_1$ Hase amount for all conditions tested.

The [NiFeSe] Hase is only detected in Fe+Ni+Se media (Figure 5C), and its synthesis is not markedly affected by the electron donor/acceptor, showing a small increase in H_2/S and a small decrease in L/T. Thus, the Western blot analyses confirms the effect of the elements Ni and Se on the levels of the *Dv*H Hases, particularly on the $[NiFe]_1$ and [NiFeSe] Hases.

Detection of [FeFe], [NiFe]$_1$ and [NiFeSe] Hase transcripts by RT-PCR

The results described above indicate that Ni, and even more strongly Se, are involved in regulation of the periplasmic *Dv*H Hase expression. To address the question of whether this regulation occurs at transcriptional or post-transcriptional level we analysed the mRNA levels of the three Hases in L/S medium. For this, primers were designed for RT-PCR amplification of DNA fragments from *hydA* (coding for the [FeFe] Hase large subunit), *hynA-1* (coding for the [NiFe] Hase large subunit) and *hysA* (coding for the [NiFeSe] Hase large subunit).

The results shown in Figure 6 indicate that addition of Ni to the L/S medium does result in a decrease of the [FeFe] Hase large subunit transcript, and a similar level is observed in the presence of Fe+Ni+Se, in agreement with the immunoblot analysis. The mRNA levels of the [NiFe]$_1$ Hase large subunit show a very weak increase in Ni+Fe L/S medium compared to Fe-only medium. Further addition of Se results in a decrease of the [NiFe]$_1$ Hase large subunit transcript, confirming the previous observations. The level of the [NiFeSe] Hase large subunit transcript is very low in Fe-only L/S medium as predicted, but this level is quite high in Fe+Ni conditions and only slightly lower than the level observed in Fe+Ni+Se conditions, which contrasts to the results obtained by activity-stained gel and Western blot for which the [NiFeSe] Hase was only detected in cells grown in medium containing selenium.

DISCUSSION

In this work we investigated the expression pattern of the *Dv*H [FeFe], [NiFe]$_1$ and [NiFeSe] Hases in response to different electron donors and acceptors, and also to the elements Ni and Se that are constituents of the active site of the two latter Hases. The [FeFe], [NiFe]$_1$ and [NiFeSe] are the major Hases detected in *Dv*H and previous work has indicated a similar physiological role of H_2 oxidation for

the three enzymes, suggesting that the difference between them could lie in different expression conditions.

An analysis of each Hase individually shows that the synthesis of the [FeFe] Hase in Fe-only medium was highest in P/S, followed by H_2/S. Inclusion of Ni in the medium led to a significant reduction of this Hase only in L/S medium, but Se further reduced its amount in all conditions tested. The $[NiFe]_1$ Hase showed highest formation upon growth with H_2/S, even if Ni was not added to the growth medium. For the other conditions, its synthesis increased in Fe+Ni media compared to Fe-only media. Inclusion of Se in the growth media led to a significant decrease of this Hase for all conditions tested. The [NiFeSe] Hase was only detected when Se was included in the media, and in these conditions it did not show strong variations in response to the electron donors/acceptors tested, albeit showing increased synthesis upon growth with hydrogen and lower when thiosulfate was used as electron acceptor.

The most striking result obtained in this study was that Se is involved in the negative regulation of the [FeFe] and $[NiFe]_1$ Hases and the positive regulation of the [NiFeSe] Hase, irrespective of the growth medium used. This Hase is not detected in cells grown in the absence of selenium, as would be expected since selenocysteine is part of its polypeptide chain. However, when cells are grown in the presence of Se the [FeFe] and $[NiFe]_1$ Hases are strongly (but not completely) downregulated and the [NiFeSe] Hase becomes the dominant Hase present. Downregulation of the $[NiFe]_1$ Hase in the presence of Se was somewhat expected. This Hase and the [NiFeSe] one are homologous, and structurally very similar, and their genes are adjacently located hinting at coordinated regulation, in a situation analogous to that found for the Hases of *M. voltae*. In contrast, the downregulation of the [FeFe] Hase in response to Se is unexpected as this Hase belongs to a completely different family from the [NiFe] Hases. These results are in agreement with the [NiFeSe] Hase having a similar physiological role to the [FeFe] and $[NiFe]_1$ Hases and so being capable of replacing them. Its preferred synthesis is

probably linked to its higher catalytic efficiency and possibly also to its resistance to oxygen inactivation (36). The repression of the [FeFe] and [NiFe]$_1$ Hases in the presence of Se occurs at a transcriptional level, as revealed by RT-PCR.

The situation found in *Dv*H is similar, but not identical, to that in *M. voltae* and *M. maripaludis*, since in these organisms the selenium-free Hases are completely repressed when Se is present in the growth medium and are only detected in its absence (4, 18, 30), whereas in *Dv*H the [NiFe]$_1$ and [FeFe] Hases are still present, albeit at a lower level, in Se-grown cells. In *M. maripaludis* a mutant of the *selB* gene encoding an archaeal translation factor specialised for selenocysteine insertion did not show repression of the Se-independent enzymes in the presence of Se, revealing that it is not selenium itself that is involved in regulation, but a molecule derived from it such as a selenoprotein or a selenocysteyl-tRNA (30).

Ni also affects synthesis of the [FeFe] and [NiFe]$_1$ Hases, although in a less clear cut way than Se. Growth in the presence of Ni led to a significantly reduced amount of the [FeFe] Hase only for L/S and P/S media, whereas for other media the effect was not significant. The regulation of the [FeFe] Hase by Ni in L/S medium occurs at the level of transcription. A nickel-dependent regulator, NikR, has been identified in the genomes of *Dv*H and *Desulfovibrio desulfuricans* G20, and putative binding sites for this regulator were identified upstream of genes encoding Ni-transport systems (28). Interestingly, in *D. desulfuricans* (but not *Dv*H) a similar binding site is present upstream of the [FeFe] Hase genes supporting regulation of these genes by Ni (28).

Formation of the [NiFe]$_1$ Hase is increased in the presence of Ni for all growth conditions, except in growth with hydrogen/sulfate. For this growth medium the amount of the [NiFe]$_1$ Hase is very high even in the Fe-only conditions, when Ni is not added. This indicates that H_2 acts as a very strong activating signal for expression of this Hase, and that there is enough Ni in the basal medium C to allow for maturation of a large amount of this Hase, even though the Ni concentration in this

medium was below the detection limit (0.01 mg/l) as determined by atomic absorption spectrometry. With lactate as electron donor (L/S and L/T) there is a strong increase in $[NiFe]_1$ Hase synthesis when 1 μM Ni is included in the medium, whereas with pyruvate as electron donor (P/S and P) there is a less pronounced increase. The RT-PCR studies of the Hase transcripts in L/S medium indicate that Ni does not regulate transcription of the $[NiFe]_1$ Hase, since similar mRNA levels are observed in growth in Fe and Fe+Ni conditions. In these conditions it is likely that the regulation by Ni is due to modulation of the post-translational processing, since insertion of Ni is one of the important steps in maturation of Hases (7, 38). The different behavior of the $[NiFe]_1$ Hase synthesis between the H_2/S medium and the other conditions suggests that hydrogen may induce a high-affinity uptake system for Ni, which is able to scavenge the low levels of Ni in the Fe-only medium in order to support a high expression level of this Hase, and that this system is not present in the other conditions. In *E. coli*, it was recently shown that transcription of the nickel transport system NikABCDE is regulated in order to match the Hase expression level of the cell (32). Regarding the effect of Ni on the [NiFeSe] Hase, this protein is not detected, by activity stained gel or Western blot, in cells grown in media containing Fe+Ni. However, its large subunit transcript is present in these conditions at an almost similar level to that found after growth in Fe+Ni+Se, indicating that the gene is transcribed even in the absence of Se, but this absence obviously interferes with translation as selenocysteine is part of the C-terminus of the polypeptide chain.

In terms of the effect of the electron donors/acceptors available for growth on the synthesis of the Hases, these had less impact than the two elements tested, with the three Hases being formed in all the conditions tested. Growth with thiosulfate as electron acceptor led to the lowest levels of the three Hases. On the other hand, hydrogen was undoubtedly the strongest activator for synthesis of the three Hases, which agrees with a function of H_2 uptake for all three proteins. Nevertheless, this effect was more pronounced for the $[NiFe]_1$ Hase than for the other two. A striking observation is that upon

growth with H_2, the [NiFeSe] and the $[NiFe]_1$ Hases, which are mainly membrane-associated in all other growth conditions, are almost equally distributed between the soluble and the membrane fraction. This is also reflected in the fact that the soluble fraction of cells grown in H_2/S in the presence of Se have a very high level of H_2-production activity when compared, for example, to the soluble fraction of P/S grown cells with Se, which have an almost similar level of the [NiFeSe] Hase that remains membrane-associated in this case. The mode of association of the [NiFeSe] and $[NiFe]_1$ Hases to the membrane has not been completely established but is presumably due to a lipidic group attached to the N-terminal (36). An operon containing Hase maturation genes, just downstream of the [NiFeSe] and $[NiFe]_1$ Hase genes in *Dv*H and *D. desulfuricans* genomes, includes a lipase, which is unprecedented as a Hase maturation protein. This lipase may be involved in the release of the lipid anchor from the two Hases resulting in soluble forms of the proteins, as observed after growth in hydrogen. A soluble form of the [NiFeSe] Hase, apparently lacking the lipidic group, has been detected in *Dv*H (36). It can be speculated that H_2 uptake might be more efficient by having a higher amount of the $[NiFe]_1$ and [NiFeSe] Hases present in both soluble and membrane-bound forms.

In conclusion, this work revealed that inclusion of Se in the growth medium of *Dv*H leads to a strong downregulation of both [FeFe] and $[NiFe]_1$ Hases and a strong increase in the level of the [NiFeSe] Hase. This Hase is not detected when Se is not present during growth, but when Se is added it becomes the major Hase present replacing the other two. Ni has a less clear effect, possibly causing repression of the [FeFe] Hase in L/S medium, but not in other conditions. Ni leads to increase of the $[NiFe]_1$ Hase in all media, except with H_2 as electron donor where its amount is highest even without Ni added. Three observations made in this work support the proposal that the [FeFe], $[NiFe]_1$ and [NiFeSe] Hases have a similar physiological role of H_2 oxidation in *Dv*H metabolism: i) all three hydrogenases are synthesised to highest levels upon growth with hydrogen; ii) the [NiFeSe] Hase mostly replaces the other two when Se is added; iii) synthesis of the three *Dv*H Hases responds in a

similar way to the different electron donors and acceptors. However, the fact that the [FeFe] and [NiFe]$_1$ Hases are still present even after growth with Se may indicate that not all functions of these Hases are shared. The flexibility of Hase expression in response to the elements available surely contributes to *Dv*H adaptation and survival in changing environmental conditions. Further work should be carried out to clarify the mechanisms of Hase regulation by Se and Ni in *Dv*H.

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Abbreviations: *Dv*H: *Desulfovibrio vulgaris* Hildenborough; Hase: Hydrogenase; L/S: lactate/sulfate; L/T: lactate/thiosulfate; H_2/S: hydrogen/sulfate; P/S: pyruvate/sulfate; P: Pyruvate.

Figure 1

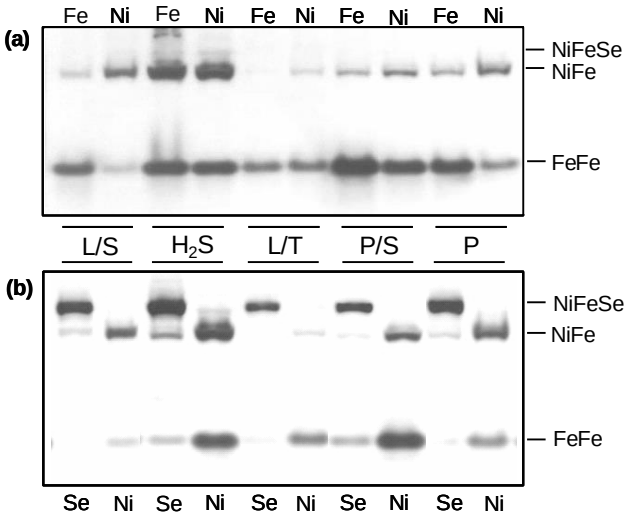
Fe Ni Fe Ni Fe Ni Fe Ni Fe Ni
(a)
NiFeSe
NiFe
FeFe
L/S
H2S
L/T
P/S
P
(b)
NiFeSe
NiFe
FeFe
Se Ni Se Ni Se Ni Se Ni Se Ni

Figure 2

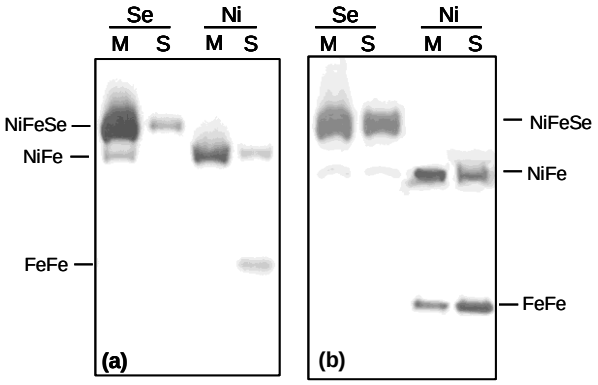

Figure 3

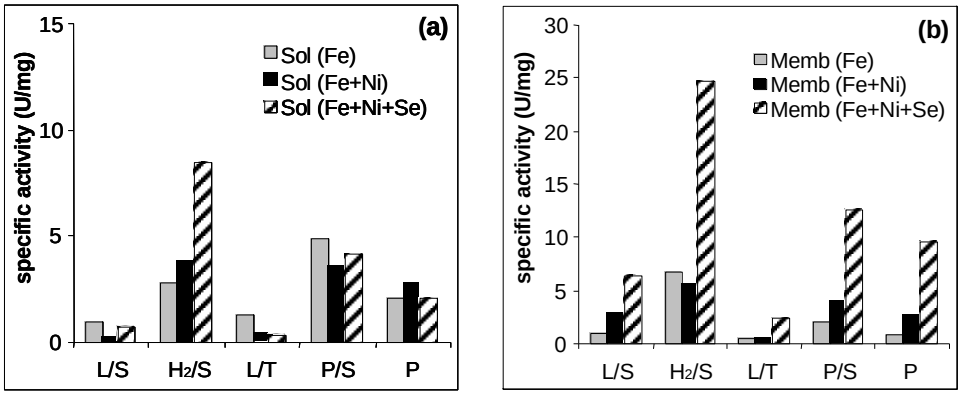
(a)
specific activity (U/mg)
15
10
5
0
Sol (Fe)
Sol (Fe+Ni)
Sol (Fe+Ni+Se)
L/S
H2/S
L/T
P/S
P
(b)
specific activity (U/mg)
30
25
20
15
10
5
0
Memb (Fe)
Memb (Fe+Ni)
Memb (Fe+Ni+Se)
L/S
H2/S
L/T
P/S
P

Figure 4

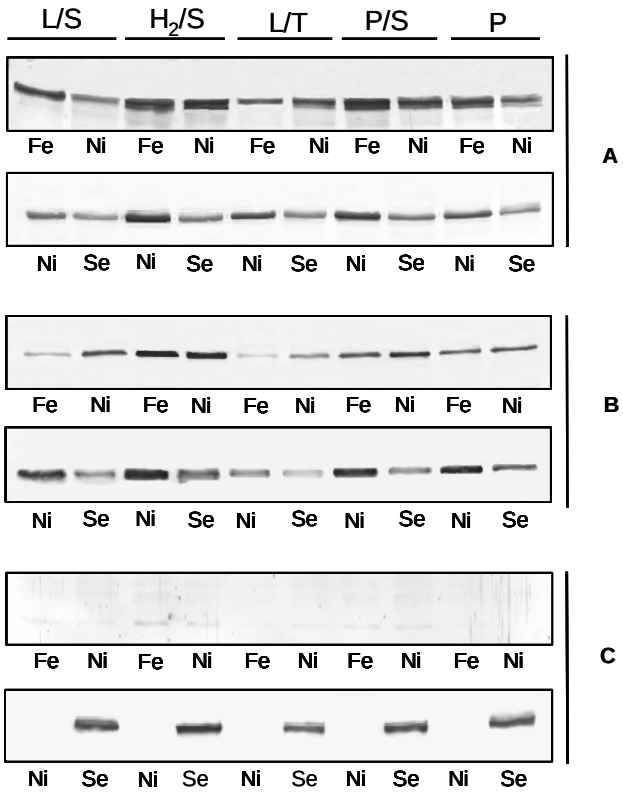
L/S
H2/S
L/T
P/S
P
Fe Ni Fe Ni Fe Ni Fe Ni Fe Ni
A
Ni Se Ni Se Ni Se Ni Se Ni Se
Fe Ni Fe Ni Fe Ni Fe Ni Fe Ni
B
Ni Se Ni Se Ni Se Ni Se Ni Se
Fe Ni Fe Ni Fe Ni Fe Ni Fe Ni
C
Ni Se Ni Se Ni Se Ni Se Ni Se

Figure 5

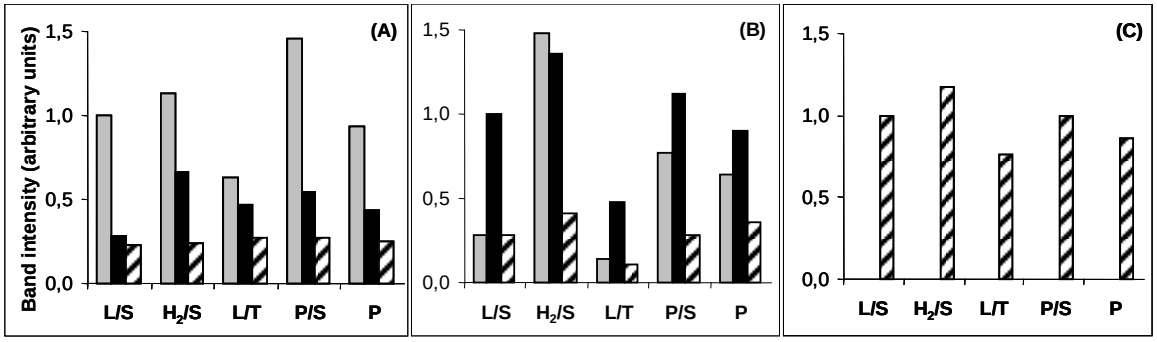

Figure 6

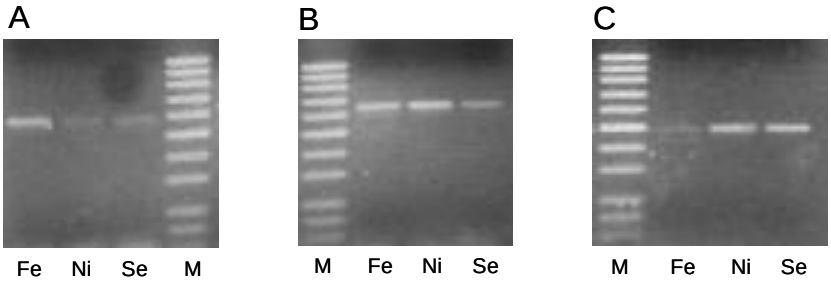

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Figure legends

Figure 1 – Activity-stained gels of crude cell extracts (50µg) from *Dv*H grown cells in: lactate/sulfate (L/S), hydrogen/sulfate (H_2/S), lactate/thiosulfate (L/T) pyruvate/sulfate (P/S), and pyruvate (P). Fe – medium containing only Fe; Ni – medium containing Fe+Ni; Se - medium containing Fe+Ni+Se. Results were reproduced in triplicate experiments. FeFe- [FeFe Hase; NiFe- $[NiFe]_1$ Hase; NiFeSe- [NiFeSe] Hase. a) gel comparing crude cell extracts of cells grown in Fe and Fe+Ni; b) gel comparing crude cell extracts of cells grown in Fe+Ni and Fe+Ni+Se. The Fe+Ni extracts are repeated in both gels because comparison of band intensities should only be performed within the same gel.

Figure 2 – Activity-stained gels of membrane (M) and soluble (S) extracts (50µg) from *Dv*H cells grown in lactate/sulfate (a) and hydrogen/sulfate (b). Se - medium containing Fe+Ni+Se; Ni –medium containing Fe+Ni.

Figure 3 –Hydrogen production activity (U/mg) of soluble (a) and membrane (b) extracts of *Dv*H cells grown in lactate/sulfate (L/S); hydrogen/sulfate (H_2/S); lactate/thiosulfate (L/T); pyruvate/sulfate (P/S) and pyruvate (P), in the presence of Fe (grey bars), Fe+Ni (black bars) or Fe+Ni+Se (stripe bars).

Figure 4 - Western blots of *Dv*H crude extracts from cells grown in lactate/sulfate (L/S), hydrogen/sulfate (H_2/S), lactate/thiosulfate (L/T), pyruvate/sulfate (P/S), pyruvate (P), in medium containing only iron (Fe), iron and nickel (Ni), or iron and nickel and selenium (Se), using antibodies against the *Dv*H [FeFe] (panel A), $[NiFe]_1$ (panel B) and [NiFeSe] Hases (panel C). The amounts used for imunnodetection were 20 µg of crude extracts for [FeFe] Hase antibodies, and 5µg for $[NiFe]_1$ and [NiFeSe] Hase antibodies.

Figure 5 – Densitometric analysis of imunoblots in Figure 4 using antibodies against [FeFe] Hase (A), $[NiFe]_1$ Hase (B) and [NiFeSe] Hase (C). Media containing only iron (grey bars), iron plus nickel (black bars) or iron plus nickel plus selenium (stripe bars). Values are normalized to the L/S medium in Fe-only conditions in (A), Fe+Ni conditions in (B) and Fe+Ni+Se in (C), and are averages of two experiments.

Figure 6 – RT-PCR analysis of the transcription of the genes encoding the *Dv*H [FeFe], $[NiFe]_1$ and [NiFeSe] Hases on L/S media: Fe – medium containing Fe; Ni – medium containing Fe+Ni; Se – medium containing Fe+Ni+Se; M – 100bp DNA ladder. Panel A – *hydA* ([FeFe] Hase large subunit) transcription; Panel B – *hynA-1* ([NiFe] Hase large subunit) transcription; Panel C - *hysA* ([NiFeSe] Hase large subunit) transcription. RT-PCR reactions were done with 100ng of total RNA.