Hydrogen sulfide increases thermotolerance and lifespan in *Caenorhabditis elegans*

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Hydrogen sulfide (H$_2$S) is naturally produced in animal cells. Exogenous H$_2$S has been shown to effect physiological changes that improve the capacity of mammals to survive in otherwise lethal conditions. However, the mechanisms required for such alterations are unknown. We investigated the physiological response of *Caenorhabditis elegans* to H$_2$S to elucidate the molecular mechanisms of H$_2$S action. Here we show that nematodes exposed to H$_2$S are apparently healthy and do not exhibit phenotypes consistent with metabolic inhibition. Instead, animals exposed to H$_2$S are thermotolerant and long-lived. These phenotypes require SIR-2.1 activity but are genetically independent of the insulin signaling pathway, mitochondrial dysfunction, and caloric restriction. These studies suggest that SIR-2.1 activity may translate environmental change into physiological alterations that improve survival. It is interesting to consider the possibility that the mechanisms by which H$_2$S increases thermotolerance and lifespan in nematodes are conserved and that studies using *C. elegans* may help explain the beneficial effects observed in mammals exposed to H$_2$S.

Early life arose in highly reducing conditions where hydrogen sulfide (H$_2$S) was abundant and molecular oxygen (O$_2$) was scarce (1, 2). Early organisms were likely to have extracted energy from the environment initially by anoxygenic photosynthesis using chemicals, including sulfide, as electron donors, followed by anaerobic respiration using sulfate as an electron acceptor (resulting in the production of sulfide), and, finally, by aerobic respiration (1). The success of primordial eukaryotes may have resulted from their ability to take advantage of chemically unstable mixtures of H$_2$S and O$_2$. In modern marine sediments, eukaryotic microbes accumulate at locations where O$_2$ and H$_2$S coexist, allowing for maximal energy production by redox chemistry (1, 3).

The importance of O$_2$ in biology is widely recognized; however, the ancient nature of H$_2$S suggests that it also might impact fundamental aspects of biological processes. H$_2$S is naturally produced by animal cells, and endogenous H$_2$S affects various aspects of physiology (4, 5). In addition, recent evidence suggests that H$_2$S can have dramatic effects on mammalian physiology (6) but is not apparently toxic to the worms. Animals raised in H$_2$S are visually indistinguishable from untreated controls and produce statistically identical numbers of progeny (221 ± 35 in H$_2$S, compared with 234 ± 15 in control conditions; $P > 0.05$; $n = 5–10$ in each group). Neither embryonic nor postembryonic development is delayed by H$_2$S (Table 1). In addition, the rate of egg-laying is not significantly different in H$_2$S (Table 2). The rate of egg-laying is tightly correlated with oocyte production (14), an energetically expensive activity that is a sensitive readout of metabolic capacity. Consistent with this, we observe a 2-fold decrease in the rate of egg-laying when ambient O$_2$ tension is reduced to 2% (from 21% O$_2$ in room air), a perturbation that was previously shown to decrease the metabolic rate of worms by ~50% (15). H$_2$S does not further alter the rate of egg-laying in environments with reduced ambient O$_2$ (Table 2). These data contrast with hypometabolic phenotypes commonly observed in nematodes with defective, including *clk-1(qm30)* mitochondrial function, including *clk-1(qm30)* animals (16–19). Our experiments demonstrate that apparent metabolic output is not appreciably changed when animals are raised in H$_2$S. However, we cannot definitively conclude that mitochondrial energy production has not been affected in these conditions. In addition, we observe that H$_2$S exposure does not induce expression of several transgenes driven by heat-shock promoters, including *hsp-16.2::GFP* and *hsp-4::GFP* (SI Fig. 6) (20–23). Together these data indicate that animals grown in H$_2$S are as healthy as untreated controls, and that in our conditions this concentration of H$_2$S does not affect apparent metabolic rate.

Results and Discussion

*C. elegans* is not adversely affected when grown in atmospheres containing 50-ppm H$_2$S (0.005%) in room air (hereafter referred to simply as H$_2$S) [supporting information (SI) Fig. 5]. We chose 50-ppm H$_2$S because this concentration has been shown to affect mammalian physiology (6) but is not apparently toxic to the worms. Animals raised in H$_2$S are visually indistinguishable from untreated controls and produce statistically identical numbers of progeny (221 ± 35 in H$_2$S, compared with 234 ± 15 in control conditions; $P > 0.05$; $n = 5–10$ in each group). Neither embryonic nor postembryonic development is delayed by H$_2$S (Table 1). In addition, the rate of egg-laying is not significantly different in H$_2$S (Table 2). The rate of egg-laying is tightly correlated with oocyte production (14), an energetically expensive activity that is a sensitive readout of metabolic capacity. Consistent with this, we observe a 2-fold decrease in the rate of egg-laying when ambient O$_2$ tension is reduced to 2% (from 21% O$_2$ in room air), a perturbation that was previously shown to decrease the metabolic rate of worms by ~50% (15). H$_2$S does not further alter the rate of egg-laying in environments with reduced ambient O$_2$ (Table 2). These data contrast with hypometabolic phenotypes commonly observed in nematodes with defective, including *clk-1(qm30)* mitochondrial function, including *clk-1(qm30)* animals (16–19). Our experiments demonstrate that apparent metabolic output is not appreciably changed when animals are raised in H$_2$S. However, we cannot definitively conclude that mitochondrial energy production has not been affected in these conditions. In addition, we observe that H$_2$S exposure does not induce expression of several transgenes driven by heat-shock promoters, including *hsp-16.2::GFP* and *hsp-4::GFP* (SI Fig. 6) (20–23). Together these data indicate that animals grown in H$_2$S are as healthy as untreated controls, and that in our conditions this concentration of H$_2$S does not affect apparent metabolic rate.

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Conflict of interest statement: The authors acknowledge a potential conflict of interest in that both authors are named as inventors on at least one patent that was licensed to a private company, founded by Mark Roth, to commercialize this technology.

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We observed that animals grown in H$_2$S are more thermotolerant than untreated controls (Fig. 1). At high temperature, animals grown in H$_2$S have a mean survival time up to 8-fold longer than untreated controls. Although the maximum extension of survival time observed varied between experiments, the effect was quite robust, with an average of 77% of H$_2$S-treated animals alive when all untreated animals had died (15 independent experiments) (SI Fig. 7). In this experiment, animals were raised in H$_2$S and challenged with high temperature in the presence of H$_2$S. In fact, we observe that animals grown in room air were more sensitive to thermal stress in H$_2$S (Fig. 1B). Thus, H$_2$S does not act directly to prevent damage associated with thermal stress. Moreover, unlike thermotolerance induced by prior stress such as heat shock (24) or azide (25), continuous exposure to H$_2$S is required for increased thermotolerance (Fig. 1C). These data suggest that H$_2$S exposure initiates physiological alterations, one manifestation of which is increased survival at high temperature.

In C. elegans, resistance to high temperature is often correlated with increased lifespan (24). Indeed, we observe that animals grown in H$_2$S are long-lived compared with controls (Fig. 2). The mean lifespan of animals grown in H$_2$S is 9.6 days greater than the untreated population, an increase of 70%. Maximum lifespan was similarly increased as H$_2$S-treated animals reached 75% mortality 10 days after the control population. Increased lifespan is not observed when animals are moved into H$_2$S at the beginning of the lifespan experiment as L4 larvae. In fact, the lifespan of these animals is slightly shorter than untreated controls (Fig. 2B). These data suggest that H$_2$S cannot act postdevelopmentally to slow the rate of aging (see also SI Fig. 7). However, these animals produce normal numbers of progeny, suggesting that overall physiological function is not impaired (227 ± 18 progeny when moved to H$_2$S, compared with 208 ± 16 in room air; P > 0.05, n = 5 in each group). Lifespan extension requires the continuous presence of H$_2$S in the atmosphere insofar as animals grown in H$_2$S but moved to room air have a normal lifespan (Fig. 2C). This finding indicates that H$_2$S exposure solely during development is insufficient for increased lifespan. We conclude that the increase in lifespan is another manifestation of the physiological alterations resulting from the exposure to H$_2$S.

Most genes that influence lifespan can be categorized into one of three genetically independent pathways in C. elegans (26–28). We considered the possibility that exposure to H$_2$S and associated physiological alterations may modulate one or more of these pathways. To evaluate this possibility, we tested whether exposure to H$_2$S caused increased thermotolerance in mutant animals with defects in these pathways.

In C. elegans, the insulin/IGF signaling (IIS) pathway regulates the decision to enter into an alternative third larval stage, the dauer, upon exposure to unfavorable conditions, such as high population density, low food, or high temperature (29). Mutations in the insulin-like receptor DAF-2 that reduce IIS increase the probability of entry into the dauer state and, in adults, increase thermotolerance and lengthen lifespan even without entry into dauer (30–32). All known phenotypes of daf-2 mutants can be suppressed by mutations in the DAF-16 FOXO transcription factor (26, 30, 31). Our data suggest that exposure to H$_2$S does not result in decreased IIS. First, H$_2$S exposure starting in adulthood does not increase lifespan (Fig. 2B), whereas knockdown of daf-2 by RNAi starting in adulthood is sufficient to decrease lifespan (32). Second, daf-2-mutant animals are more resistant to high temperature when grown in H$_2$S (Fig. 3A). Third, H$_2$S does not induce entry into the dauer state in wild-type nematodes because postembryonic development time is not extended (Table 1), nor does it affect entry into or exit

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**Table 1. Developmental rate in H$_2$S**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Embryogenesis</th>
<th>Postembryonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>12.6 ± 0.4 (43)</td>
<td>49.6 ± 0.2 (15)</td>
</tr>
<tr>
<td>H$_2$S</td>
<td>13.3 ± 0.4 (34)</td>
<td>49.3 ± 0.2 (15)</td>
</tr>
<tr>
<td>clk-1(qm30)</td>
<td>16.9 ± 2.0 (17)*</td>
<td>69.0 ± 0.3 (15)*</td>
</tr>
</tbody>
</table>

*Developmental time is significantly different (P < 0.05) from untreated controls by log-rank analysis.

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**Table 2. Rate of egg-laying in H$_2$S**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Room air</th>
<th>H$_2$S</th>
<th>2% O$_2$</th>
<th>2% O$_2$ plus H$_2$S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.8 ± 1.5</td>
<td>8.3 ± 3.0</td>
<td>4.2 ± 1.3</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td>H$_2$S</td>
<td>nd</td>
<td>7.8 ± 2.0</td>
<td>4.9 ± 1.4</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>clk-1(qm30)</td>
<td>4.2 ± 0.6*</td>
<td>3.2 ± 1.1*</td>
<td>2.4 ± 0.8*</td>
<td>2.5 ± 1.0*</td>
</tr>
</tbody>
</table>

*Rate of egg-laying is significantly different (P < 0.05) than untreated controls in same conditions by Student’s t test.

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![Fig. 1.](image-url) **Fig. 1.** H$_2$S increases thermotolerance in wild-type C. elegans. (A) Animals exposed to H$_2$S survive longer than untreated controls at high temperature. Nematodes were moved to 35°C in the same gaseous atmosphere in which they had been cultured (SI Fig. 5). The mean survival time of animals grown in H$_2$S was 65.5 h (solid line; n = 136), compared with 9.1 h (n = 96) for untreated controls (dashed line). (B) Prior exposure to H$_2$S is required to survive high temperature in H$_2$S. All animals were grown in room air without H$_2$S and then moved to 35°C in the presence or absence of 50-ppm H$_2$S. Animals first exposed to H$_2$S at high temperature had a mean survival time of 2.1 h (solid line; n = 20), whereas the control group exposed in room air survived for 7.3 h (dashed line; n = 20). (C) The continuous presence of H$_2$S in the atmosphere is required for increased survival at high temperature. Animals were exposed to 35°C in room air. Animals grown in H$_2$S before heat shock survived 7.3 h (solid line; n = 20), which is not significantly longer than untreated controls (dashed line; 7.0 h; n = 20). Indicated P values were determined by log-rank analysis.
from dauer in daf-2(e1370) mutants (data not shown). Finally, daf-16-mutant animals become thermotolerant upon exposure to H$_2$S (Fig. 3A). These data suggest that the mechanism by which H$_2$S increases lifespan and thermotolerance is independent of the IIS pathway.

Reduction of mitochondrial function is a well established mechanism for increasing the lifespan of C. elegans (27). In vitro, H$_2$S is an inhibitor of cytochrome c oxidase, the terminal enzyme in the electron transport chain (9). However, we do not observe hypometabolic phenotypes in animals grown in H$_2$S (Tables 1 and 2), suggesting that mitochondrial function is not grossly affected in these conditions. In addition, depletion of mitochondrial components by RNAi only during development increases lifespan (16), whereas animals grown in H$_2$S but moved to room air as adults, are not long-lived (Fig. 2C). These data suggest that H$_2$S exposure has characteristics distinct from mitochondrial dysfunction. In support of this premise, we observe that isp-1- and clk-1-mutant animals, which have defects in mitochondrial function and are long-lived (17, 18), become more resistant to high temperature when grown in H$_2$S (Fig. 3B). We conclude from these data that the effect of H$_2$S on lifespan is mediated by a genetic mechanism distinct from mitochondrial dysfunction.

Reduced caloric intake or dietary restriction (DR) extends lifespan in a wide range of organisms (33). C. elegans subjected to DR appear thin and pale, develop slowly, and have reduced fecundity, which are phenotypes not observed in animals grown in H$_2$S (Tables 1 and 2) (19, 34). Furthermore, DR can increase lifespan when initiated in adults (35-37), whereas H$_2$S exposure cannot (Fig. 2B). Therefore, we consider it unlikely that H$_2$S acts through the DR pathway. Consistent with this interpretation, eat-2-mutant animals, which are long-lived because of DR (38), become thermotolerant upon exposure to H$_2$S (Fig. 3C). We conclude that H$_2$S alters the physiology of worms in a manner distinct from DR, suggesting that it acts through a separate mechanism.

In addition to, but perhaps overlapping with, these genetically defined pathways, Sir2 homologues influence lifespan in many organisms, including C. elegans (39-42). Overexpression of the C. elegans Sir2 homologue, sir-2.1, increases lifespan by 18-50%
humans, with potentially wide-ranging implications in both basic insights into similar mechanisms in higher organisms, including increased SIR-2.1 expression. Indeed, does not require sir-2.1 increased thermotolerance and lifespan in a manner distinct from type) and mutant nematode strains were grown at room temperature on Growing Nematodes in H

Fig. 4. sir-2.1 is required for increased thermotolerance and lifespan upon exposure to H2S. (A) H2S does not increase thermotolerance of animals that have a deletion in sir-2.1. The mean survival time of sir-2.1(ok434) animals grown in H2S and exposed to high temperature in H2S (solid line) is 9.8 ± 0.3 h (n = 20), which is not significantly longer than untreated controls in room air (dashed line; mean survival 9.6 ± 0.3 h; n = 20). (B) H2S does not increase the lifespan of sir-2.1(ok434) animals. The lifespan of sir-2.1(ok434) animals raised in H2S is 20.0 ± 1.6 days (solid line; n = 47), which is statistically indistinguishable from control animals in room air (dashed line; 22.2 ± 1.2 days; n = 26). Indicated P values were determined by log-rank analysis.

(A) Our data indicate that sir-2.1 is required for increased thermotolerance and lifespan upon exposure to H2S. In contrast to wild type (Figs. 1A and 2A), the thermotolerance and lifespan of nematodes harboring a deletion in the sir-2.1 gene are unchanged when the animals are grown in H2S (Fig. 4). However, we consider it unlikely that H2S results in increased lifespan as a result of increased SIR-2.1 expression. H2S effects on lifespan are independent of daf-16 (Fig. 3A), whereas lifespan extension by overexpression of sir-2.1 requires DAF-16 activity (43). Indeed, sir-2.1 transcript levels in animals grown in H2S are indistinguishable from untreated controls as measured by quantitative RT-PCR, and animals overexpressing sir-2.1 become more thermotolerant when grown in H2S (SI Fig. 8). We conclude that H2S modulates SIR-2.1 activity to impart increased thermotolerance and lifespan in a manner distinct from sir-2.1 overexpression. The fact that these phenotypes require sir-2.1 supports the interpretation that the effects of H2S are distinct from DR because increased lifespan resulting from DR does not require sir-2.1 (44). Moreover, the finding that SIR-2.1 activity is required for increased thermotolerance and lifespan in H2S further suggests that these phenotypes do not result from nonspecific metabolic suppression.

Sir2 homologues are NAD+-dependent deacetylase enzymes that may have a variety of substrates (45). This finding raises the possibility that H2S shifts redox homeostasis, thereby increasing the available NAD+ (or the NAD+/NADH ratio) and resulting in increased SIR-2.1 activity (40, 46). Alternatively, H2S may directly modify SIR-2.1 to alter its activity (47). It also is possible that SIR-2.1 is indirectly activated by some other aspect of H2S-induced physiological alterations. Whatever the mechanism by which H2S-induced physiological alterations are translated into the phenotype of increased lifespan, our studies raise the possibility that endogenous H2S naturally regulates SIR-2.1 activity. It may be that Sir2 homologues are involved in mediating the physiological alterations observed in mammals exposed to exogenous H2S. Further investigations of genetic mechanisms that mediate H2S-induced phenotypes in nematodes may yield insights into similar mechanisms in higher organisms, including humans, with potentially wide-ranging implications in both basic research and clinical practice.

Materials and Methods
Growing Nematodes in H2S-Containing Atmospheres. Bristol strain N2 (wild-type) and mutant nematode strains were grown at room temperature on nematode growth medium (NGM) plates seeded with live Escherichia coli OP50 food (48). Mutant strains obtained from the C. elegans genetic stock center were as follows: CB130, daf-2(e1370); DR26, daf-16(m26); VC520, isp-1(gk267); MO130, clk-1(qm30); DA1116, eat-2(ad1116); and VC199, sir-2.1(ok434).

Plates were maintained in atmospheric chambers sealed with Dow Corning Vacuum Grease (Sigma–Aldrich). Care was taken to ensure that cultures did not starve. Chambers were continuously perfused with room air or 50-ppm H2S that was freshly mixed into room air (SI Fig. 5). Gasses were hydrated by using gas wash bottles (Fisher) and moved through a 28-inch outer diameter FEP tubing (Cole Parmer) with connections by snap connectors (Cole Parmer), stainless-steel quick-connect fittings, or compression fittings (Sealite Fluid Systems). The H2S-containing atmospheres were constructed by mixing 5,000-ppm H2S (balanced with N2) with room air by using mass flow controllers (model no. 810 and Smart-Track Series 100; Sierra Instruments). All compressed gas mixtures used in this study were obtained from Byrne Gas and were certified standard to within 2% of indicated concentration. Flow tubes (Aalborg) were used to distribute the gas mixture to different chambers. Gas flow rate was 100 cm3/min to small boxes (100–300 ml) and 800 cm3/min to the large boxes (1–3 liters) used to culture nematode strains at room temperature. At these flow rates, the gaseous environment of the atmospheric chambers is exchanged every 20–30 min. The concentration of H2S was monitored with a Surecell H2S detector ( sixth sense) containing a three-electrode electrochemical Surecell H2S detector (Sixth Sense). The detector was zeroed with room air and spanned with 100-ppm H2S before each use. Data were collected by using Chart software with a Powerlab data acquisition unit (ADInstruments) and analyzed with EXCEL. The concentration of H2S measured was consistently within 10 ppm of the reported value and was stable from day to day. The H2S-containing atmospheres did not alter the pH of the NGM plates.

Brood Size Measurement. To determine the number of viable progeny produced by nematodes, individual fourth-stage larvae (L4) were transferred to NGM plates with OP50 food at room temperature. Animals were moved daily until they quit laying fertilized eggs. Progeny were counted as L4 young adult.

Measuring Developmental Rates. The time required for embryonic development was determined by measuring the time required for two-cell embryos to hatch. Two-cell embryos were isolated from log-phase adults as previously described (49). Briefly, adults were chopped with a razor blade and ~20 two-cell embryos were moved to NGM plates without food by mouth pipette. The number of embryos that hatched was monitored every 45–60 min beginning 6–8 h after embryos were picked. Embryos that did not hatch after 36 h were considered dead and were not included in the analysis. Median time of hatching was determined by log-rank analysis in Sigmapstat (Systat). Data from one representative experiment for both embryonic and postembryonic development are shown in Table 1, although each experiment was repeated several times with similar results.

Postembryonic development was measured as the time required for starved first-stage larvae (L1) to become gravid, egg-laying adults. Starved L1 were isolated by picking 20–30 adults from each population into 10 µl of hypotonic solution (2.5 N KCl, 5% NaOCl) on a small (unseeded) NGM plate. After 5 min, 1 ml of M9 buffer (48) was added to the plate, and the embryos were returned to the atmospheric chambers. After 24–36 h, starved L1 were moved onto NGM plates with OP50, returned to the chamber, and allowed to develop at room temperature. After 30–48 h, individual larvae were moved to NGM plates with a 10-µl spot of OP50. Each worm was monitored every 6–12 h until it began laying eggs (intervals became closer as time progressed and the worm became gravid). If more than one embryo had been laid, the time that the first egg was laid was determined assuming that one egg was laid every 15 min for wild type and every 30 min for the clk-1(qm30) mutants. This value was determined empirically by counting the number of embryos laid by each worm for the 6-h period after it began egg-laying. Data were analyzed by using log-rank analysis in Sigmapstat (Systat).

The rate of egg-laying was determined for first-day gravid adults from populations cultured in each condition (room air with or without 50-ppm H2S). Animals were picked as L4 from mixed-stage populations and allowed to develop for 20–30 h in the same conditions at room temperature. Individual worms were then placed onto NGM plates with a 10-µl spot of OP50 food. The number of embryos laid in 3–5 h was counted to determine the rate of egg-laying. To create an atmosphere with 2% O2, N2 was mixed with 5% O2 balanced with N2 (Smart-Track Series 100 mass flow controllers (Sisra Instruments) were used to mix the gas and split it into two atmospheric chambers. Using a model 810 mass flow controller (Sisra Instruments), we then added H2S to the 2% O2 that flowed into one of the chambers. Student’s t test was
used to determine whether the rate of egg-laying varied significantly between conditions, assuming two-tailed distributions with unequal variance (EXCEL). In each experiment, 10–15 individuals were included in each group. The data shown in Table 2 are from one experiment that is representative of at least three independent assays.

**Thermotolerance Assay.** Cultures of nematodes were established in 50-ppm H$_2$S or room air control conditions and maintained for at least 1 week before thermotolerance measurement. Care was taken to prevent the population from starving. Nematodes were picked from these mixed-stage populations as S-containing chambers with the initial investigations of H$_2$S in nematodes. This work was supported by National Institutes of Health Grant R01 GM48435 (to M.B.R.), National Research Service Award Fellowship 1F32FM073369 (to D.L.M.), the Caenorhabditis Genetics Center, and the National Institutes of Health/National Research Resources.

**Lifespan Measurements.** On day 0, groups of 20–30 L4 animals from populations in each condition were picked from mixed-stage cultures onto NGM plates seeded with live OP50 and spotted with 25 μl of 50 μg/ml 5-fluoro-2'-deoxyuridine (Sigma–Aldrich) to prevent growth of progeny. Control experiments indicated that the lifespan of nematodes in H$_2$S was not affected by the 5-fluoro-2'-deoxyuridine. Plates were placed into atmospheric chambers at room temperature. Every few days, the plates were removed from the chambers to monitor the number of animals remaining alive. Nematodes were considered dead when they no longer responded to repeated prodding with a platinum wire. Data were analyzed with SigmaStat (Systat) by using Kaplan–Meier survival analysis. Each assay was repeated at least twice with similar results.

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