The Response of *Caenorhabditis elegans* to Hydrogen Sulfide and Hydrogen Cyanide

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ABSTRACT Hydrogen sulfide (H_2S), an endogenously produced small molecule, protects animals from various stresses. Recent studies demonstrate that animals exposed to H_2S are long lived, resistant to hypoxia, and resistant to ischemia–reperfusion injury. We performed a forward genetic screen to gain insights into the molecular mechanisms *Caenorhabditis elegans* uses to appropriately respond to H_2S. At least two distinct pathways appear to be important for this response, including the H_2S-oxidation pathway and the hydrogen cyanide (HCN)-assimilation pathway. The H_2S-oxidation pathway requires two distinct enzymes important for the oxidation of H_2S: the sulfide:quinone reductase *sqrd-1* and the dioxygenase *ethe-1*. The HCN-assimilation pathway requires the cysteine synthase homologs *cysl-1* and *cysl-2*. A low dose of either H_2S or HCN can activate hypoxia-inducible factor 1 (HIF-1), which is required for *C. elegans* to respond to either gas. *sqrd-1* and *cysl-2* represent the entry points in the H_2S-oxidation and HCN-assimilation pathways, respectively, and expression of both of these enzymes is highly induced by HIF-1 in response to both H_2S and HCN. In addition to their role in appropriately responding to H_2S and HCN, we found that *cysl-1* and *cysl-2* are both essential mediators of innate immunity against fast paralytic killing by *Pseudomonas*. Furthermore, in agreement with these data, we showed that growing worms in the presence of H_2S is sufficient to confer resistance to *Pseudomonas* fast paralytic killing. Our results suggest the hypoxia-independent *hif-1* response in *C. elegans* evolved to respond to the naturally occurring small molecules H_2S and HCN.

HYDROGEN sulfide (H_2S) is an endogenously produced molecule with profound physiological effects (Kabil and Banerjee 2010). While first noted for its potent toxicity, it is now clear that organisms and H_2S have long had an intimate relationship (Philippot *et al.* 2007). Prior to the appearance of oxygen as a component of the earth's atmosphere, H_2S was abundant at the earth's surface (Reinhard *et al.* 2009). Sulfur was likely important for redox biochemistry in early life, similar to the role of oxygen today (Philippot *et al.* 2007). Sulfur-containing redox reactions are still essential in modern organisms in the form of glutathione and thioredoxin (Holmgren *et al.* 2005) and in iron–sulfur clusters in the electron transport chain and aconitase in the tricarboxylic acid (TCA) cycle (Rouault and Tong 2008). Recent molecular phylogenetic data confirm that H_2S-interacting enzymes predated oxygen-binding enzymes (David and Alm 2011). H_2S is also essential for the existence of sulfur-containing amino acids because cysteine synthase uses H_2S as the sulfur source during *de novo* cysteine biosynthesis (Feldman-Salit *et al.* 2009).

In animals, H_2S is generated by dissimilating H_2S from cysteine (Kabil and Banerjee 2010). The numerous activities ascribed to H_2S in animals include its function as a neuromodulator in the brain and as a mediator of vascular tone and smooth muscle of the heart (Li *et al.* 2011). The ability of H_2S to affect mammalian tissue has recently been exploited for therapeutic uses (Mustafa *et al.* 2009). When rodents are exposed to H_2S, they enter a reversible state of decreased metabolic rate and core body temperature (Blackstone *et al.* 2005). Exogenous H_2S has shown benefit in a number of animal-disease models. For example, H_2S protects mice from hypoxic death (Blackstone and Roth 2007), helps rats survive severe hemorrhage (Morrison *et al.* 2008), and diminishes ischemia–reperfusion injury (Elrod *et al.* 2007).

Our understanding of the mechanism by which H_2S affects biology is incomplete. To fill these gaps, we have developed *C. elegans* as a genetically amenable model system for studying the response of animals to H_2S. Previous work has shown that *C. elegans* becomes longer lived and

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thermotolerant when grown in H_2S (Miller and Roth 2007). We showed that H_2S induces the accumulation of the hypoxia-inducible factor 1 (HIF-1) (Budde and Roth 2010). In turn, *hif-1* is required for survival in the presence of H_2S, and keeping HIF-1 levels constitutively high confers resistance to otherwise lethal H_2S concentrations. HIF-1 is a transcription factor and thus the observed protective effects are likely to be mediated by increased expression of genes regulated by HIF-1. To determine the molecular mechanism by which tolerance to H_2S is conferred, we performed a forward genetic screen for *C. elegans* strains sensitive to H_2S and report on two *hif-1* target genes that function in the first steps of two distinct pathways important for survival in H_2S.

Materials and Methods

Strains

C. elegans were grown at room temperature on nematode growth medium plates seeded with live *Escherichia coli* OP50 food (Brenner 1974). The following mutant strains were obtained from the *Caenorhabditis* Genetics Center: *cysl-1*(*ok762*), *cysl-2*(*ok3516*), *cysl-4*(*ok3359*), *cbs-2*(*ok666*), *hif-1* (*ia04*), *sqrd-2* (*ok3440*), and CB4856. The following strains were provided by Shohei Mitani of the National Bio-Resource Project: *sqrd-1*(*tm3378*) and *ethe-1*(*tm4104*). *Pseudomonas aeruginosa* strain PA01 was obtained from Colin Manoil (University of Washington, Seattle, WA).

Atmospheric chambers and viability tests

H_2S-containing atmospheric chambers were generated by mixing air with small amounts of defined compressed H_2S balanced with N_2 (Budde and Roth 2010). The resulting gas mixture is essentially room air containing hydrogen sulfide (RA/H_2S). Unless otherwise stated, RA/H_2S is room air containing 50 ppm of H_2S. All survival experiments were performed in 1000-ml chambers, with a gas flow of 1000 ml/min. HCN-containing atmospheres were created in the same way as the H_2S atmospheres except HCN balanced with N_2 was used, and they are referred to as RA/HCN (essentially room air containing hydrogen cyanide). Unless otherwise stated, RA/HCN is room air containing 5 ppm of HCN.

Viability tests were performed by picking L4 larval stage worms onto OP50-seeded NGM plates and placing them into the gaseous atmosphere for 24 hr (Budde and Roth 2010). For RA/H2S exposures, all animals observed after 24 hr were either healthy adults or appeared to have perished shortly after initiation of the experiment. For RA/HCN exposures, developmentally arrested L4 animals with very slow movement were occasionally observed. If maintained in RA/HCN, these animals invariably perished after several days as L4. If removed from RA/HCN after 24 hr, some of these animals eventually recovered to become fertile adults. For the data in this article, animals with this developmental arrest were scored as not surviving the RA/HCN exposure. All error values presented are standard deviation (SD).

Video acquisition and automated worm speed analysis

A Quickcam Communicate STX (Logitech) webcam was affixed to the eyepiece of a stereomicroscope. Images were acquired every 12 sec and analyzed with the Multi-Worm Tracker (MWT) (Swierczek *et al.* 2011). The video was analyzed three times on the MWT and the speed was averaged every 2.5 min.

Genetic screen for H_2S-sensitive mutants

Ethyl methanesulfonate (EMS) mutagenesis was performed as previously described (Brenner 1974) and was optimized to maximize the number of unique mutations observed (Shaham 2007). N2 animals at the L4 or young-adult stage were incubated with EMS for 4 hr. The worms were then washed once, aliquoted onto four OP50-seeded NGM plates and allowed to lay eggs overnight. The next day, the adults were washed off the plates and the F_1 animals were grown to adults. Each pool of 25,000 F_1 adults was bleached to generate synchronized F_2 animals. A total of 100,000 F_2 L4 animals were screened (25,000 from each pool).

The screen for nematodes that are sensitive to H_2S was performed as follows. When the F_2 worms entered the L4 larval stage, the plate of worms was exposed to RA/H_2S for 4 hr. The worms were moved to room air for 5 min and then all paralyzed worms were picked from the plate within 15 min of removal from RA/H_2S. From this screen, 791 F_2 animals were singled out to generate 544 viable strains. Of these viable strains, 124 strains exhibited a lethality phenotype during a 24-hr exposure to RA/H_2S. Of the 124 sensitive strains, 23 were 100% lethal when exposed to RA/H_2S for 24 hr as L4 and were annotated as strongly penetrant (Figure 2).

Mapping mutations to chromosomes and complementation tests

Prior to complementation testing, strongly penetrant strains were crossed to the Hawaiian strain CB4856 and SNP enrichment analysis was performed (Davis *et al.* 2005). Male CB4856 worms were mated into the 23 mutant strains. F_1 animals were isolated and synchronous F_2 populations were created by egg lays. When F_2 animals reached L4 larval stage, the plates were exposed to RA/H_2S for 4 hr. Thirty paralyzed animals were picked as phenotypic, and 30 motile animals were picked as aphenotypic. In most cases, ~25% of F_2 worms became paralyzed when exposed to RA/H_2S and were annotated as recessive. In four cases, ~75% of worms became paralyzed and these mutants were annotated as dominant.

After the restriction digests were separated by gel electrophoreses, the gel was visually examined for genomic regions where the DNA from phenotypic and aphenotypic animals did not cut equally well with *Dra*I. Ethidium bromide fluorescence was quantified with Quantity One 1-D analysis software (Bio-Rad) and a fluorescence ratio of cut/uncut DNA was generated for each SNP on the chromosome of interest. The SNPs on two neighboring chromosomes are presented as a background control. Strains that appeared to show similar

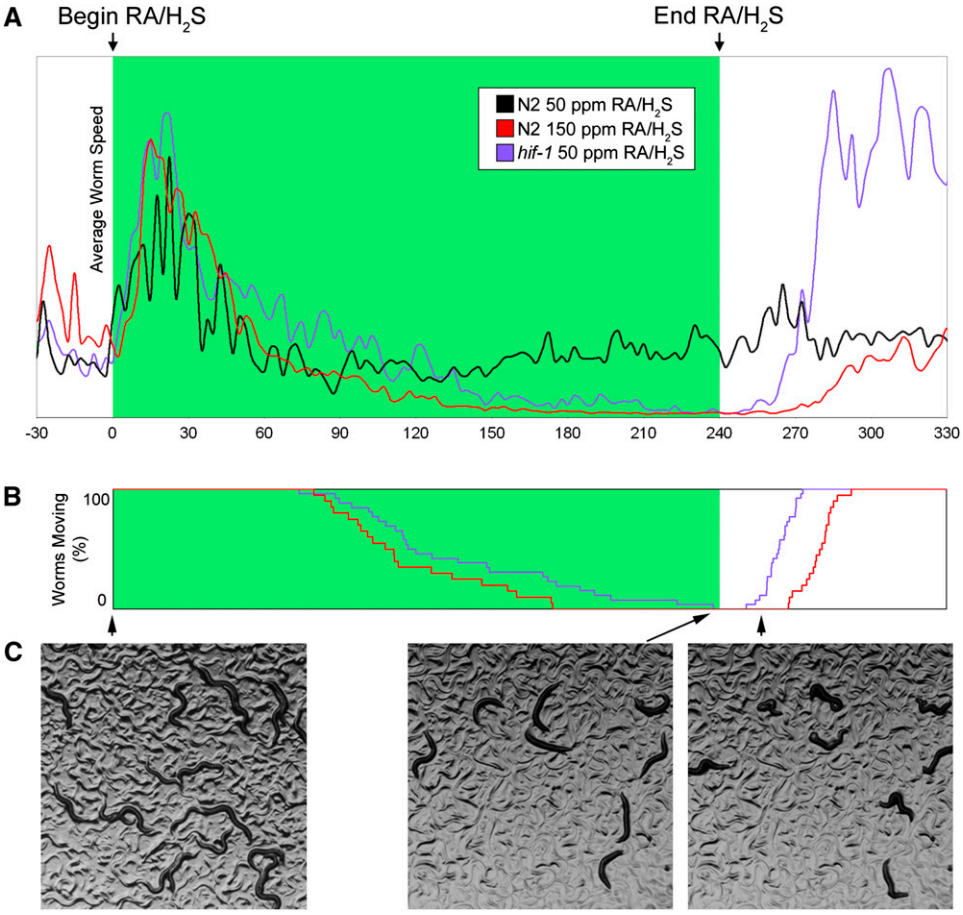

Figure 1 Wild-type nematodes exposed to 50 ppm RA/H2S exhibit a brief increase in movement initially and then continue to move (A, black line). When either wild-type or *hif-1* mutant animals are exposed to 150 ppm RA/H2S or 50 ppm RA/H2S, respectively, they stop moving and when the RA/H2S is removed, they resume movement (blue and red lines, A and B). Examples of *Hif-1* moving, stopped, and reanimated worms are shown in C.

enrichment patterns were grouped together as suspected complementation groups (Figure 3).

Complementation tests were carried out by crossing homozygous males of one strain into another strain and testing the F_1 progeny for survival in RA/H_2S. Sequencing of *cysl-1* and *sqrd-1* was accomplished by PCR amplification of genomic DNA and then using nested primers for the labeling reaction. After the point mutations were identified, SIFT-BLink (Ng and Henikoff 2001) (http://sift.bii.a-star.edu.sg/www/SIFT_BLink_submit.html) was used to determine whether the mutations are predicted to be tolerated or not tolerated. In parallel, all splice mutations were annotated as not tolerated.

Protein homology

Protein–protein Basic Local and Alignment and Search Tool (BLASTp) was used to locate homologous genes. Potential homologs were reciprocally BLAST searched to ensure specificity. Amino acid alignment was performed with ClustalX2 using the following National Center for Biotechnology Information reference sequence IDs: *Arabidopsis thaliana* CAS, NP_191703.1; *A. thaliana* CYS, NP_193224.1; *Solanum tuberosum* CAS, BAB18760.1; *S. tuberosum* CYS, AAC25635.1; *Zea mays* CAS, ADG60236.1; *Z. mays* CYS, NP_001105469.1; *Mus musculus* CBS, NP_659104.1; *Homo sapiens* CBS, NP_000062.1; *Ciona intestinalis* CBS, XP_002120247.1; and *Drosophila melanogaster* CBS, NP_608424.1.

Psuedomonas aeruginosa dependent paralysis

Pseudomonas experiments were performed with strain PA01 as previously described (Shao *et al.* 2010). Briefly, a full lawn of *P. aeruginosa* was grown on brain heart infusion agar (37 g Difco BHI, 20 g agar per liter) on 3.5-cm plates for 24 hr at 37°. The plates were cooled to room temperature and 10 L4 animals were placed onto the bacterial lawn and the plate was sealed with parafilm. The number of animals moving and not moving after 6 hr was recorded.

mRNA detection by QRT–PCR

Quantitative reverse transcriptase PCR was performed on a Bio-Rad C1000 Thermal Cycler with a CFX96 real-time system, with iQ SYBR green supermix (Bio-Rad) as previously described (Budde and Roth 2010). Experimental mRNA was compared to the average mRNA level of the housekeeping genes *tba-1* and *tbb-2*. The primer sequences are listed in Supporting Information, Table S1.

Western blotting

Antibody was generated and affinity purified using the amino acids 419–431 of SQRD-1 (wormbase.org WP: CE17629), amino acids AMETTPFDQSKPTY, by ProSci Incorporated (San Diego, CA). Western blotting was performed as previously described (Budde and Roth 2010)

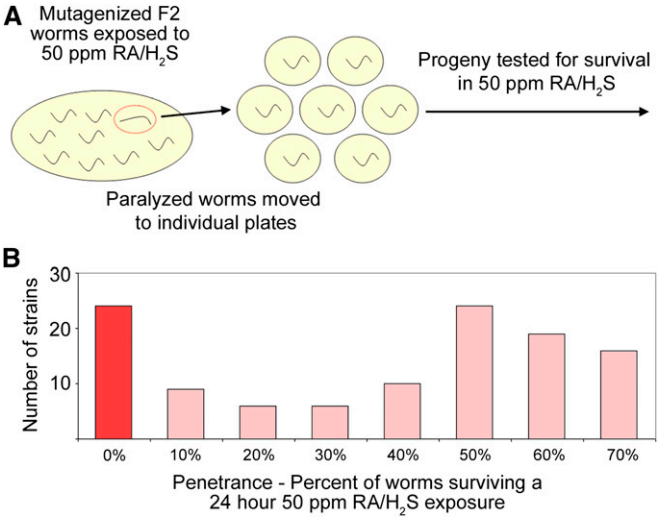

Figure 2 H_2S sensitivity screen method and results. (A) Schematic of initial paralysis screen and lethality rescreen. (B) Histogram of the number of strains isolated for each penetrance bin. The strains with zero survivors after sulfide exposure are highlighted in red. These strains were subsequently characterized.

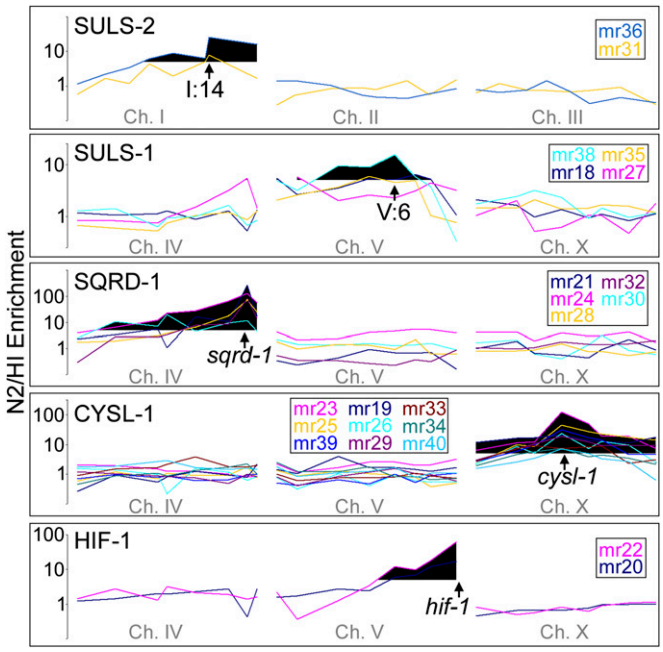

Figure 3 Enrichment of H_2S-sensitive phenotype after crossing mutant lines with the Hawaiian strain CB4856. SNP mapping data show five apparent complementation groups. For *suls-1* and *suls-2* the apparent location of mutation is indicated by an arrow. The known locations of *sqrd-1*, *cysl-1*, and *hif-1* are also indicated by arrows. Black indicates an N2/HI ratio >5.

with the following changes: primary antibody was diluted 1:500. Goat antirabbit IgG (H+L)-horseradish peroxidase (catalog no. 111-035-003; Jackson ImmunoReseach Laboratories, West Grove, PA) was used as the secondary antibody.

Fosmid recombineering

GFP was inserted into fosmid WRM066CH11 as previously described (Dolphin and Hope 2006). The oligonucleotides used to insert the RT cassette were:

RT cassette forward -gtttgattaaaggatactggaatggaccagctacac tcagaaattgtacaTCGCTGTCGAGATATGACGGTG
RT cassette reverse -acaaataaaaacgacaaagtgggaaatatctattta gactttaccaatcgGATGATAAGCTGTCAAACATGAG
GFP forward -acaaataaaaacgacaaagtgggaaatatctatttagacttta ccaatcgTTTGTATAGTTCATCCATGCCATGTG
GFP reverse -gtttgattaaaggatactggaatggaccagctacactcagaaat tgtacaATGAGTAAAGGAGAAGAACTTTTC

Uppercase letters indicate primers for the PCR reaction.

Generation of transgenic animals

Wild-type *C. elegans* were injected with 50 ng/μl fosmid together with 100 ng/μl pRF4, which confers a dominant roller [*rol-6*(*su1006*)] phenotype (Mello *et al.* 1991). Rolling F_1 animals were picked onto single plates to generate transgenic lines. Animals were exposed to 50 ppm RA/H_2S prior to confocal fluorescent microscopy, as previously described (Budde and Roth 2010).

RNAi experiments

RNAi was performed as previously described (Frazier and Roth 2009) using WBRNAi00013759 to target *egl-9*. L4 animals were moved onto the RNAi food and the progeny were moved onto NGM-OP50 or BHI-PA01 plates and immediately assayed.

Results

We showed previously that hypoxia-inducible factor, *hif-1*, is required for growth and survival in H_2S (Budde and Roth 2010). HIF-1 protein concentration and activity are increased when *C. elegans* are moved into RA/H_2S, and *egl-9* mutant animals, which have constitutively high HIF-1 expression and activity, are resistant to H_2S.

During our investigations with *hif-1* mutants, we noticed that although *hif-1* mutant animals are unable to survive prolonged exposure to RA/H_2S (24 hr), a brief exposure (4 hr) causes a reversible paralysis (Figure 1A). Roughly 2 hr after entering an environment containing RA/H_2S, *hif-1* mutant L4 animals enter a state of suspended animation, characterized by a complete cessation of movement and feeding (Figure 1B), and the animals lose their characteristic sinusoidal shape (Figure 1C). If removed to fresh air during this suspended state, the animals resume moving after ~20 min and proceed to become fertile adults. This phenotype is also present in wild-type animals exposed to 150 ppm RA/H_2S (Figure 1, A and B; File S1). We used this phenotype as a basis for screening mutant *C. elegans* that are sensitive to H_2S, in an attempt to find genes that confer resistance to H_2S (Figure 2A).

About 200,000 mutant haploid genomes were screened for H_2S sensitivity. Through screening, we obtained 124

Table 1 Alleles unable to survive in RA/H_2S

Gene name	Chromosome	Allele	Alive/total (% surviving) 32 ppm RA/H_2S	15 ppm RA/H_2S	7.5 ppm RA/H_2S	Dominance
N2	—	—	12/12 (100)	12/12 (100)	13/13 (100)	—
cysl-1	X	ok762	0/20 (0)	0/18 (0)	0/11 (0)	—
cysl-1	X	mr19	0/13 (0)	0/12 (0)	0/10 (0)	Recessive
cysl-1	X	mr23	0/16 (0)	0/10 (0)	0/10 (0)	Recessive
cysl-1	X	mr25	0/13 (0)	0/10 (0)	0/6 (0)	Recessive
cysl-1	X	mr26	0/12 (0)	0/10 (0)	0/11 (0)	Recessive
cysl-1	X	mr33	0/15 (0)	5/9 (56)	10/10 (100)	Recessive
cysl-1	X	mr34	0/10 (0)	0/11 (0)	1/10 (10)	Recessive
cysl-1	X	mr29	0/11 (0)	0/10 (0)	1/13 (8)	Recessive
cysl-1	X	mr39	0/10 (0)	0/10 (0)	13/13 (100)	Recessive
cysl-1	X	mr40	0/10 (0)	0/10 (0)	1/11 (9)	Recessive
sqrd-1	IV	tm3378	5/10 (50)	10/10 (100)	10/10 (100)	—
sqrd-1	IV	mr24	10/14 (71)	11/11 (100)	—	Recessive
sqrd-1	IV	mr30	2/13 (15)	9/9 (100)	—	Recessive
sqrd-1	IV	mr32	12/13 (92)	10/10 (100)	—	Recessive
sqrd-1	IV	mr21	6/15 (40)	10/10 (100)	—	Recessive
sqrd-1	IV	mr28	5/9 (56)	10/10 (100)	11/11 (100)	Recessive
hif-1	V	ia04	0/15 (0)	0/11 (0)	10/10 (100)	—
hif-1	V	mr20	0/14 (0)	0/10 (0)	10/13 (77)	Recessive
hif-1	V	mr22	0/15 (0)	1/11 (9)	8/10 (80)	Recessive
suls-1	V	mr18	6/12 (50)	11/12 (92)	10/10 (100)	Dominant
suls-1	V	mr27	2/9 (22)	10/10 (100)	—	Dominant
suls-1	V	mr35	0/5 (0)	0/10 (0)	0/10 (0)	Dominant
suls-1	V	mr38	0/9 (0)	8/8 (100)	—	Dominant
suls-2	I	mr31	7/8 (88)	8/8 (100)	—	Recessive
suls-2	I	mr36	8/9 (89)	11/11 (100)	—	Recessive
—	—	mr37	11/12 (92)	11/11 (100)	—	—

lines that were sensitive to H_2S (Figure 2B). Of these lines, 23 were completely penetrant, where penetrance is defined as the percentage of worms unable to survive a 24-hr RA/H_2S exposure. These mutant strains were chosen for further characterization.

The 23 strongly penetrant mutant lines were crossed with the Hawaiian CB4856 strain for mapping the sensitivity trait to chromosomal regions using enrichment of SNPs. The SNP analysis revealed that 22 mutants map to only five chromosomal regions (Figure 3, Table 1); only *mr37* showed no Mendelian inheritance of the trait. These mutants were then crossed to confirm that each chromosomal region contained a single complementation group (Figure 4). One group on chromosome V (*mr18*, *mr27*, *mr35*, and *mr38*), exhibits dominant genetics and thus could not be further tested for complementation. We named this gene *suls-1* (**sul**fide **s**ensitive) and *suls-1* (*mr35*) was mapped to the 4-cM region between SNP pkP5097 and pkP5068. The two alleles on chromosome I were named *suls-2*. The genes mutated in *suls-1* and *suls-2* strains remain to be identified. Two alleles on chromosome V (*mr20* and *mr22*), mapped near the hif-1 locus and failed to complement the *hif-1*(*ia04*) mutation, which confirms that we successfully screened for our phenotype of interest.

The 23 strongest mutant lines were next tested for survival in room air containing lower H_2S concentrations in an effort to phenotypically discriminate between the complementation groups. Table 1 shows that complementation groups have differences in H_2S sensitivity, with the group mapping to the X chromosome being the most sensitive and *suls-2* being the least sensitive. In contrast to the other complementation groups, *suls-1* alleles vary in sensitivity to H_2S.

A cysteine synthase homolog, cysl-1, is required for survival in H_2S

mr23 was used as a representative allele of the group of alleles on the X chromosome (*mr19*, *mr23*, *mr25*, *mr26*, *mr29*, *mr33*, *mr34*, *mr39*, and *mr40*) and was mapped to a 0.2-cM region between SNPs pas23398 and snp_F41E7 [3]. This region contains 70 predicted genes. Of the deletion mutants available for that region, *ok762* phenocopies and fails to complement these H_2S-sensitive mutations. The *ok762* allele deletes five of the six exons of the gene *C17G1.7*. Sequencing a portion of *C17G1.7* of our isolated strains revealed adenine-to-guanine point mutations, which are characteristic of EMS mutagenesis (Figure S1). All identified mutations were either missense or splice mutations.

C17G1.7 is a previously uncharacterized gene encoding a member of the **c**ystathionine **β**-**s**ynthase (CBS)-like subgroup, which is made up of enzymes important in cysteine production, CBS and **cy**steine **s**ynthase (CYS) (OAS-TL and CysK) (Feldman-Salit *et al.* 2009). Animals generally have CBS homologs, and bacteria and plants tend to have CYS homologs (Hell *et al.* 2008). CBS is critical for generating

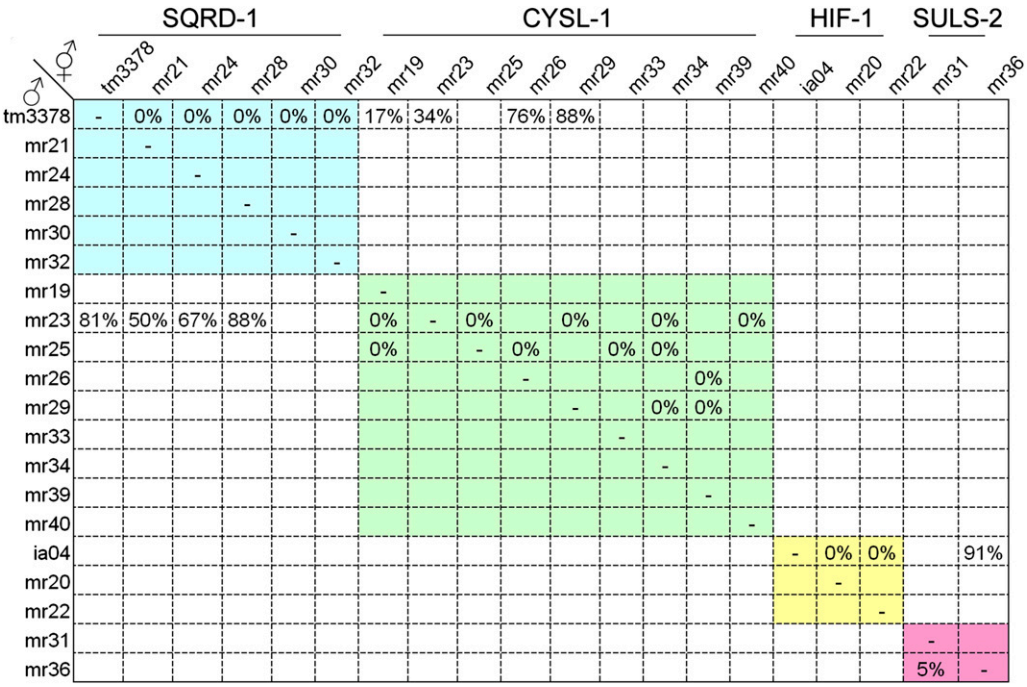

Figure 4 Complementation data showing that each genetic locus consists of only one complementation group (blue, green, yellow, and pink shades). *suls-1* is not shown because it is dominant. *sqrd-1*, *cysl-1* double heterozygotes are slightly sensitive to RA/H_2S.

cysteine in animals, which make cysteine from dietary methionine (Finkelstein *et al.* 1988). CBS is also one of the only H_2S-generating enzymes in mammals (Kabil and Banerjee 2010). In plants and bacteria, which are cysteine prototrophs, CYS combines H_2S with O-acetylserine (OAS) to generate cysteine (Figure 5A) (Wirtz *et al.* 2010). Humans and mice each have one CBS-like family member. In contrast, *C. elegans* has six: *C17G1.7*, *K10H10.2*, *R08E5.2*, *F59A7.9*, , and *F54A3.4* (Table 2).

C17G1.7 (*cysl-1*), *K10H10.2* (*cysl-2*), *R08E5.2* (*cysl-3*), and *F59A7.9* (*cysl-4*) are more similar to CYS from plants and bacteria, whereas *ZC373.1* (*cbs-1*) and *F54A3.4* (*cbs-2*) are more similar to CBS from animals (Figure 5C). A BLAST search reveals that the genus *Caenorhabditis* contains the only known animal CYS homologs. We have renamed these genes *cysl-1* (**cy**steine **s**ynthase-**l**ike), *cysl-2*, *cysl-3*, *cysl-4*, *cbs-1*, and *cbs-2* to reflect this homology. Depletion of H_2S levels, as it is used as a substrate by CYS, may explain why *cysl-1* mutant animals are sensitive to H_2S. Nematodes appear to have acquired the ability to assimilate H_2S, and *cysl-1* is likely using this reaction to drive down internal H_2S levels.

cysl-2 mediates immunity to fast paralytic killing by Pseudomonas

The existence of three additional Cysl homologs in *C. elegans* raises the possibility that these other Cysl enzymes are also important in H_2S detoxification. This possibility is especially intriguing, because we have shown a *hif-1*–dependent increase in *cysl-2* transcript levels during RA/H_2S exposure (Budde and Roth 2010). We obtained the available knockout strains of *cysl-2*, *cysl-4*, and *cbs-2* and tested them for RA/H_2S sensitivity, but none showed a RA/H_2S sensitivity phenotype (Table 2).

In plants, CYS and cyanoalanine synthase (CAS) are closely related and have overlapping catalytic activities (Figure 5, A and B). The CAS reaction combines cysteine and HCN to produce β-cyanoalanine and H_2S, lowering cellular HCN levels (Figure 5B). Furthermore, *cysl-2* mRNA was previously shown to be upregulated by HIF-1 (Shen *et al.* 2005; Shao *et al.* 2010; Budde and Roth 2010), and high HIF-1 activity has been shown to create resistance to HCN-mediated fast paralytic killing by *P. aeruginosa* (Darby *et al.* 1999; Gallagher and Manoil 2001; Shao *et al.* 2010). Therefore, *cysl-2* may be induced not to effect H_2S levels, but instead may be the mediator of resistance to HCN-dependent fast paralytic killing. To test this hypothesis, we exposed *cysl-1*, *cysl-2*, and *cysl-4* mutant animals to RA/HCN and found that both *cysl-1* and *cysl-2* mutant animals are sensitive to RA/HCN (Table 2). The specificity of *cysl-2* mutant sensitivity to cyanide suggests that this enzyme is directly acting to diminish HCN levels rather than to affect H_2S levels. The sensitivity of *cysl-1* mutants to both RA/H_2S and RA/HCN suggest that *cysl-1* acts downstream of *cysl*-2, and *cysl-1* mutants are being overwhelmed by the H_2S produced by *cysl-2* during HCN detoxification (Figure 8).

Next, we tested whether these enzymes are responsible for HIF-1–mediated innate immunity to *P. aeruginosa*. We showed that, similar to previous studies (Gallagher and Manoil 2001; Shao *et al.* 2010), *egl-9*–deficient animals have increased innate immunity to *P. aeruginosa* (Table 3). Next, we demonstrated that *cysl-1* and *cysl-2* are both required for the observed innate immunity to *P. aeruginosa*, demonstrating a mechanism of innate immunity to *Pseudomonas*-dependent fast paralytic killing. Since we previously showed that animals grown in RA/H_2S have increased *cysl-2* mRNA expression, we assayed whether growth in H_2S confers

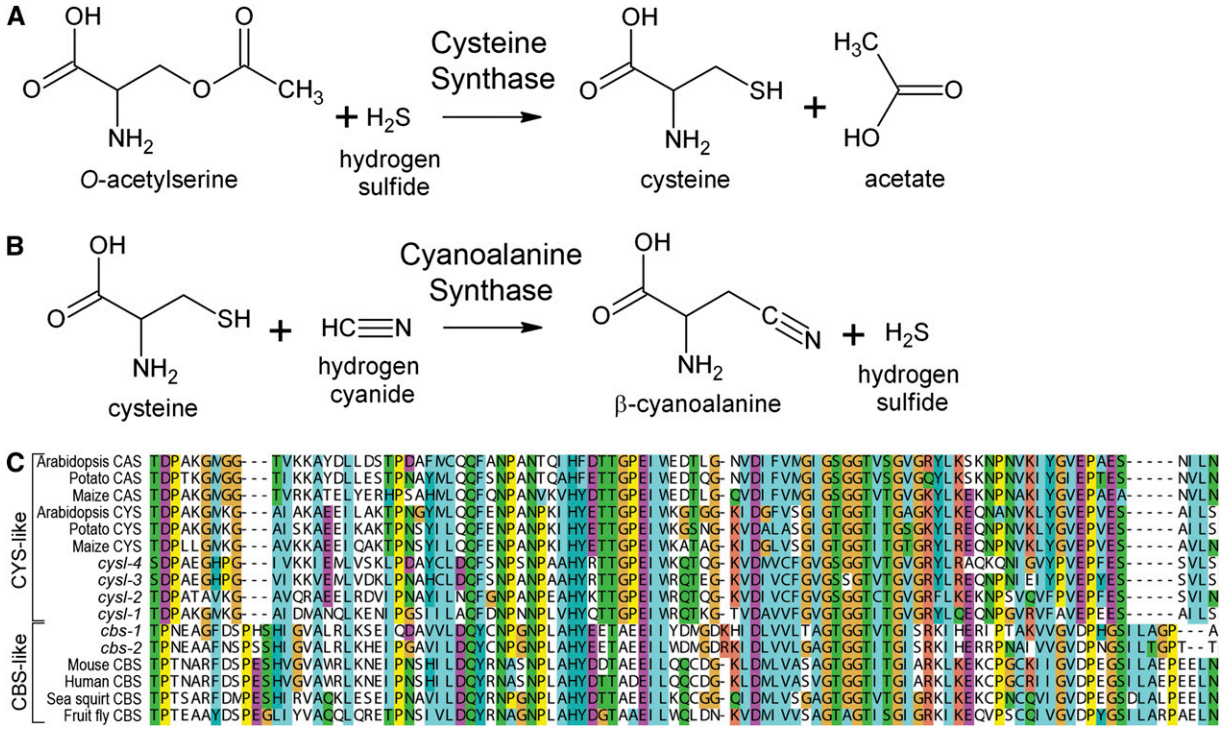

Figure 5 (A) Cysteine synthase (CYS) catalyzes the formation of cysteine and acetate from OAS and H_2S. (B) Cyanoalanine synthase (CAS) catalyzes the formation of β-cyanoalanine and H_2S from cysteine and HCN. (C) Amino acid alignment of a selected region of CYS and CBS homologs. *C. elegans* Cysl genes are homologous to CYS, whereas *C. elegans* Cbs genes are homologous to cystathionine β-synthase (CBS).

resistance to *P. aeruginosa*. Indeed, our results show that growth in H_2S increases the innate immunity of *C. elegans* to *P. aeruginosa* (Table 3).

sqrd-1 is required for survival in H_2S

The group of alleles on chromosome IV, *mr21*, *mr24*, *mr28*, *mr30*, and *mr32* were mapped near the SNP pkP4095 located at the genetic location IV:12. Analysis of the region revealed a gene, *F02H6.5*, encoding an apparent homolog of the protein **s**ulfide:**q**uinone **r**eductase **d**ehydrogenase (SQRD), which we have named *sqrd-1* to reflect this homology. SQRD was initially described in prokaryotes as oxidizing sulfide and transferring the electrons to ubiquinone for generation of ATP during oxidative phosphorylation (Vande Weghe and Ow 1999). The available deletion allele of *sqrd-1* (*tm3378*) phenocopies and fails to complement these mutants. Sequence analysis revealed A-to-G transitions in our isolated mutants (Figure S1B).

The *C. elegans* gene *sqrd-1* encodes a member of the group II SQRD enzymes, which also includes human, mouse, and *Drosophila* homologs. The *C. elegans* genome is predicted to encode another SQRD homolog, *Y9C9A.16* (*sqrd-2*), which was initially classified as a pseudogene. We obtained the deletion allele of *sqrd-2* and found that it is not sensitive to RA/H_2S.

Transcriptional regulation of identified genes

We undertook this screen to determine the mechanism by which HIF-1 confers resistance to H_2S. Therefore, we were interested to learn whether either *cysl-1* or *sqrd-1* is transcriptionally regulated by HIF-1 in response to RA/H_2S exposure. Figure 6A shows that *sqrd-1* and *cysl-2* mRNA levels

Table 2 Genes named in this article

Gene name	Gene ID	Allele tested	50 ppm RA/H_2S survival (%)	5 ppm RA/HCN survival (%)
cysl-1	C17G1.7	*ok762*	0 ± 0*	0 ± 0*
cysl-2	K10H10.2	*ok3516*	100 ± 0	0 ± 0*
cysl-3	R08E5.2	—	—	—
cysl-4	F59A7.9	*ok3359*	92 ± 11	100 ± 0
cbs-1	ZC373.1	—	—	—
cbs-2	F54A3.4	*ok666*	100 ± 0	100 ± 0
sqrd-1	F02H6.5	*tm3378*	0 ± 0*	97 ± 5
ethe-1	C33A12.7	*tm4101*	0 ± 0*	100 ± 0

* $P < 0.05$ as compared to N2 wild-type animals.

Table 3 Innate immunity to *P. aeroginosa* requires *hif-1*, *cysl-1*, and *cysl-2*

Strain	Pretreatment	Allele	Survival on *P. aeruginosa* (%)
N2	Control RNAi	—	0 ± 0
N2	Egl-9 RNAi	—	89 ± 19
hif-1	Egl-9 RNAi	*ia04*	10 ± 16*
egl-9	Egl-9 RNAi	*sa307*	100 ± 0
cysl-1	Egl-9 RNAi	*ok762*	4 ± 7*
cysl-2	Egl-9 RNAi	*ok3516*	0 ± 0*
sqrd-1	Egl-9 RNAi	*tm3378*	100 ± 0
ethe-1	Egl-9 RNAi	*tm4101*	100 ± 0
N2	H2S grown	—	83 ± 29†

* $P < 0.05$ as compared to egl-9 knockdown in N2 animals. †$P < 0.05$ as compared to untreated control RNAi N2 animals.

increase during RA/H_2S and RA/HCN exposure, but *cysl-1* mRNA levels remain unchanged. Furthermore, *hif-1(ia04)* mutant animals fail to increase *sqrd-1* and *cysl-2* mRNA levels during RA/H_2S exposure (Figure 6B), suggesting that upregulation of *sqrd-1* by HIF-1 is a mechanism for surviving in the presence of H_2S.

To determine whether or not SQRD-1 protein levels increase during RA/H_2S exposure, we performed a Western blot to determine relative protein concentrations. Figure 6C shows that SQRD-1 protein levels increase during RA/H_2S exposure in the parental N2 Bristol strain. No SQRD-1 protein accumulation is observed in the *sqrd-1(tm3378)* or in the *hif-1(ia04)* mutant animals. *egl-9(sa307)* mutant animals have constitutively high levels of SQRD-1 protein, which may explain their ability to survive in 100 times higher RA/H_2S concentration than *hif-1(ia04)* mutant animals.

SQRD-1::GFP protein is expressed during H_2S exposure

Next, we analyzed the expression pattern of SQRD-1 in *C. elegans*. We created a SQRD-1::GFP fusion construct because the antibody we created was unable to stain worms by immunofluorescence. The presence of a predicted SL2 alternative splice site upstream of *sqrd-1* indicated that a standard promoter fusion might not accurately recapitulate the endogenous expression pattern. Therefore, we used the recombineering approach for GFP fusion construction, which includes a 35-kb region surrounding the 4.5-kb *SQRD-1* gene. RA/H_2S exposure causes a fluorescence increase in recombinant animals that is most apparent in the anterior of the worm (Figure 7). Expression appears limited to the hypodermis, the body wall muscle, and the anterior bulb of the pharynx (Figure 7C). A cross section through the worm confirms that expression is limited to the most exposed parts of the worm (Figure 7D). It is possible that this SQRD-1 expression pattern reflects a function of SQRD-1 to prevent H_2S from reaching the interior tissue.

We conclude from these experiments that HIF-1 acts to initiate the response of *C. elegans* to H_2S by upregulating the SQRD-1 protein levels. SQRD-1 is an oxidoreductase, oxidizing H_2S to polysulfide and reducing ubiquinone (Theissen *et al.* 2003). The oxidation of sulfide generates polysulfide (Marcia *et al.* 2009), which can then be further oxidized via the H_2S oxidation pathway to yield sulfate and thiosulfate (Kabil and Banerjee 2010). The decrease in H_2S caused by SQRD-1 is likely to reduce internal H_2S levels to a tolerated range.

A second enzyme required for the H_2S oxidation pathway is ETHE1 (Tiranti *et al.* 2009). ETHE1 is a sulfur dioxygenase required in mammals for oxidation of sulfur to sulfate (Kabil and Banerjee 2010). *C. elegans* has a single homolog of Ethe1, *C33A12.7*, which we named *ethe-1*. We obtained the available *ethe-1* (*tm4101*) deletion allele and tested for H_2S sensitivity (Table 2). Similar to *sqrd-1* (*tm3378*), *ethe-1* (*tm4101*) is sensitive to RA/H_2S, which suggests that *sqrd-1* confers resistance to H_2S through the sulfur–oxidation pathway.

Epistasis with egl-9

Egl-9–deficient animals have been shown to be resistant to both H_2S and HCN and our data suggest that resistance is mediated by HIF-1 upregulating *sqrd-1* and *cysl-2*, respectively. To test this hypothesis, we used RNAi to knockdown *egl-9* in mutant animals, which we have described as H_2S and HCN sensitive and tested them for resistance to H_2S and HCN. Table 4 shows that *egl-9* knockdown does not mediate resistance to HCN in *cysl-1* and *cysl-2* mutant backgrounds,

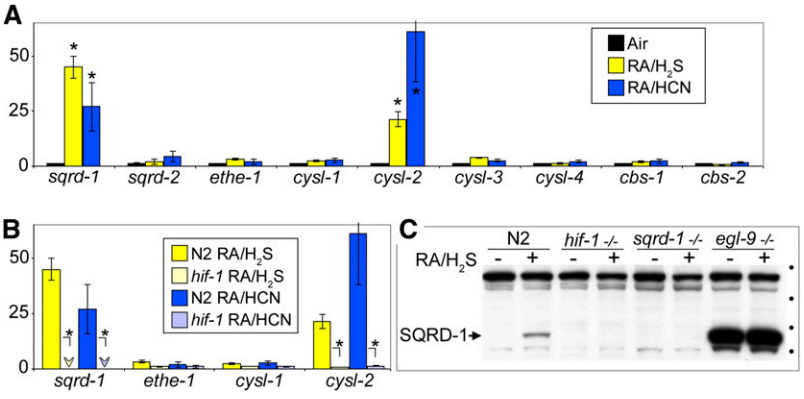

Figure 6 (A) Both *sqrd-1*and *cysl-2* mRNA levels increase relative to untreated animals as measured by quantitative reverse transciptase PCR. *Y* axis is relative mRNA concentration compared to room air-treated control animals. *$P < 0.05$ as compared to animals grown in room air. (B) *hif-1* is required for *sqrd-1* and *cysl-2* mRNA induction when exposed to RA/H_2S or RA/HCN. Arrowheads indicate that mRNA levels were observed at very low levels. *$P < 0.05$ when comparing wild-type and *hif-1* mutant animals. (C) Western blot shows SQRD-1 protein induction in RA/H_2S. This induction requires *hif-1*. No immunoreactivity is observed in *sqrd-1* mutant animals. SQRD-1 protein is abundant in *egl-9* mutant animals. Circles indicate protein size markers of 116, 82, 62, and 49 kDa from top to bottom. The calculated molecular weight of 53 kDa corresponds well with the observed relative migration.

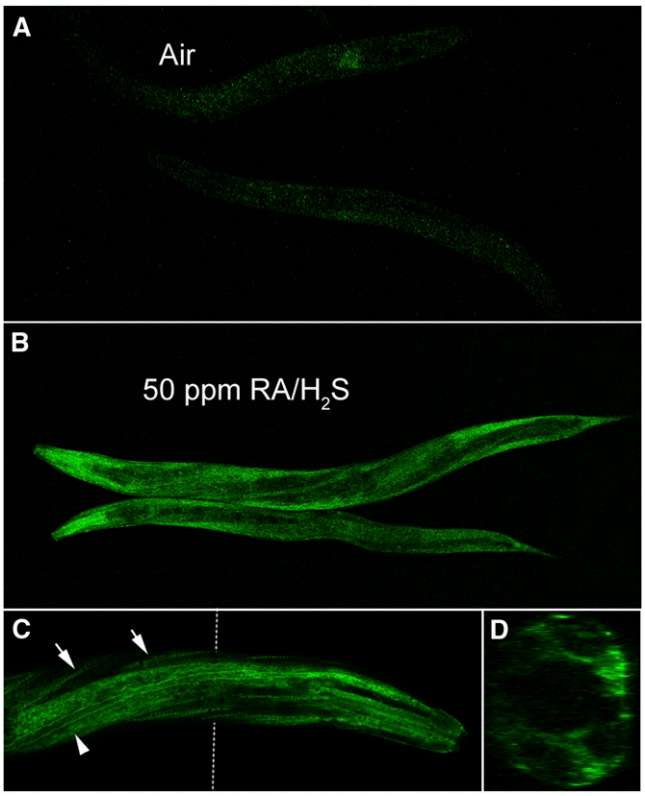

Figure 7 SQRD-1::GFP expression is induced by exposure to RA/H_2S. (A) SQRD-1::GFP is not expressed in room air. (B) RA/H_2S at 50 ppm induces expression of SQRD-1::GFP. (C) Confocal microscopy at ×40 magnification shows that the expression is brightest in the muscle dense bodies (tailed arrows) and the hypodermis (tail-less arrow). (D) Cross-section (*Z*-stack) shows expression is most intense in the hypodermis.

and therefore *cysl-1* and *cysl-2* are epistatic to *egl-9*. *Sqrd-1* and *ethe-1* mutant animals with knocked down *egl-9* are partially resistant to H_2S.

Discussion

C. elegans likely encounters H_2S in the soil as a result of breakdown of organic sulfur (Morra and Dick 1991) and HCN produced by both bacteria (Gallagher and Manoil 2001) and plants (García *et al.* 2010). This study has examined the mechanisms that *C. elegans* uses to respond to high levels of H_2S and HCN. RA/H_2S and RA/HCN both cause an increase in HIF-1 activity, which increases the expression of *sqrd-1* and *cysl-2* to catalyze the first steps of the metabolism of H_2S and HCN, respectively. *sqrd-1* acts to oxidize H_2S, ultimately preventing internal H_2S levels from exceeding the maximum tolerated concentration. *cysl-2* acts in parallel to keep HCN levels low; however, equimolar amounts of H_2S are generated as a consequence of *cysl-2* activity. *cysl-1* is required to reduce H_2S levels that are produced by *cysl-2* during RA/HCN exposure (Figure 8).

Here, we provide direct evidence that H_2S acts through *hif-1* to increase both SQRD-1 mRNA and protein expression. This initiates the sulfide–oxidation pathway, which lowers internal H_2S levels (Figure 8). SQRD-1 expression appears to be localized to the cells most readily exposed to exogenous H_2S in the worm. In mammals, colon epithelial cells that express high levels of SQRDL (Pontén *et al.* 2008) are likely to do so because a prominent enteric bacteria, *Desulfovibrio piger*, produces high levels of H_2S in the lower intestine (Loubinoux *et al.* 2002). The requirement for *ethe-1* during RA/H_2S exposure confirms that the sulfide–oxidation pathway is the essential function of *sqrd-1*. The sulfide–oxidation pathway requires oxygen, both to maintain a functional electron transport chain (to generate oxidized ubiquinone) and to directly oxidize sulfur via *ethe-1*. Exposure of mammals to exogenous H_2S results in many alterations in physiology, including the induction of a suspended-animation–like state and improved outcome in ischemia reperfusion injury. It will be interesting to learn whether SQRDL expression is induced upon exposure to H_2S in mammals.

The question remains as to why SQRD-1 is inducible at all. Surrounded by decaying matter, *C. elegans* must encounter areas of high H_2S in its natural environment. Having constitutively high SQRD-1 levels, as in *egl-9* mutant animals, allows survival when wild-type animals with lower HIF-1 activity would perish. However, low levels of H_2S are important for biological function, so perhaps nematodes are using SQRD-1 to deliberately titrate the cellular concentration of H_2S by controlling its rate of destruction. In mammals, the H_2S-generating enzymes CBS, CSE, and 3MP control sulfide levels (Kabil and Banerjee 2010). However, our study suggests that mammals may also modulate cellular H_2S levels by regulating SQRDL-dependent destruction.

In addition to furthering our understanding of the sulfide oxidation pathway, this study also provides insight into two

Table 4 Epistasis relationships with *egl-9*

	15 ppm RA/HCN		250 ppm RA/H_2S	
	Empty vector (%)	egl-9 RNAi (%)	Empty vector (%)	egl-9 RNAi (%)
N2	0 ± 0	92 ± 10	0 ± 0	98 ± 4
hif-1 (*ia04*)	0 ± 0	0 ± 0*	0 ± 0	0 ± 0*
egl-9 (*sa307*)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
sqrd-1 (*tm3378*)	0 ± 0	87 ± 16	0 ± 0	47 ± 15*
ethe-1 (*tm4101*)	0 ± 0	89 ± 14	0 ± 0	73 ± 10*
cysl-1 (*ok762*)	0 ± 0	27 ± 35*	0 ± 0	68 ± 30
cysl-2 (*ok3516*)	0 ± 0	0 ± 0*	0 ± 0	94 ± 6

* $P < 0.05$ as compared to egl-9 RNAi knockdown in wild-type animals. Data are presented as percentage of survival.

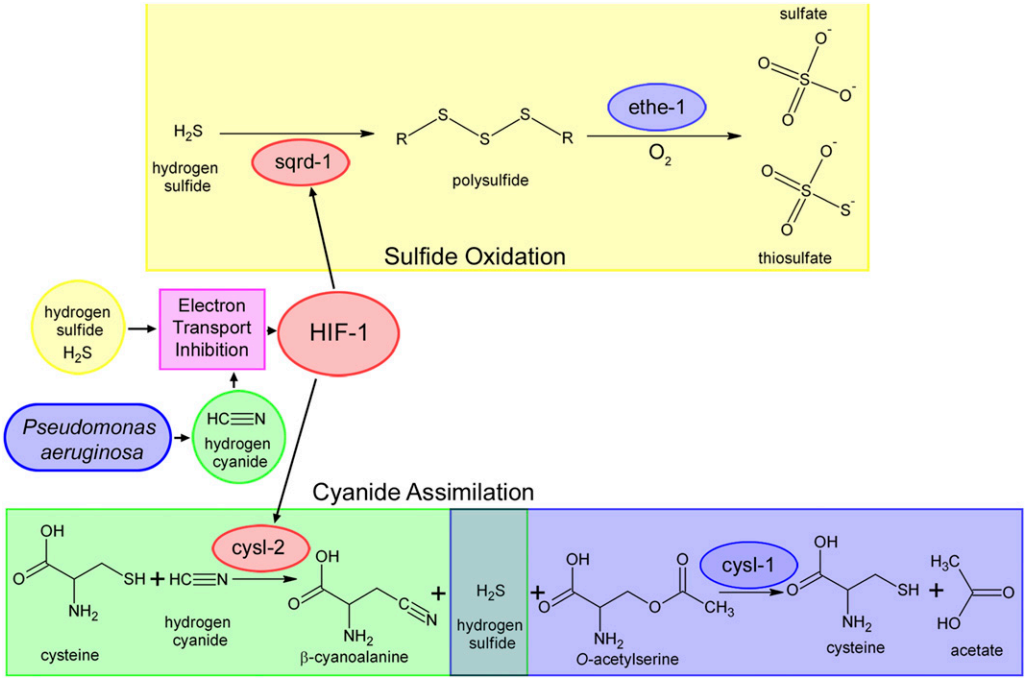

Figure 8 Proposed mechanism. Both H_2S and HCN can inhibit electron transport. Electron transport inhibition causes induction of HIF-1 activity resulting in high expression of SQRD-1 and CYSL-2. SQRD-1 catalyzes the first step in the H_2S-oxidation pathway, ultimately resulting in production of sulfate and thiosulfate. CYSL-2 catalyzes the first step in the HCN assimilation pathway, producing H_2S. The resultant H_2S is detoxified by CYSL-1.

new metabolic activities previously unknown in animals, cysteine synthase and cyanoalanine synthase. *cysl-1* mutant animals are sensitive to both RA/H_2S and RA/HCN, while *cysl-2* mutant animals are only sensitive to RA/HCN. This suggests that *cysl-1* is a cysteine synthase acting downstream of *cysl-2*, a cyanoalanine synthase (Figure 8). We hypothesize that *cysl-1* mutant animals could be succumbing to H_2S produced by CYSL-2 as a result of cyanide assimilation. In this way each molecule of HCN assimilated by CYSL-2 would produce one molecule of H_2S. In this reaction mechanism CYSL-1 might regenerate cysteine used in cyanide assimilation. Alternatively, it is possible that CYSL-1 is catalyzing the assimilation of both H_2S and HCN, a hypothesis supported by *in vitro* plant studies (Hell *et al.* 2002). The function of the two remaining cysteine synthase homologs, *cysl-3* and *cysl-4*, remains to be elucidated, but they may also have important CYS/CAS activity during development or in specific tissues.

To our knowledge, *cysl-1* is the first reported functional CYS homolog in animals. Indeed, while no orthologs appear to be present in animals outside of the genus *Caenorhabditis*, in plants and bacteria CYS uses OAS as the H_2S-accepting substrate (Feldman-Salit *et al.* 2009). Plants and bacteria generate OAS by acetylating serine with the enzyme serine acetyltransferase (SAT) (Wirtz *et al.* 2010), which has no homolog in *C. elegans*. How might *C. elegans* be obtaining OAS? As with many other amino acids, *C. elegans* is likely obtaining OAS from its bacterial food source. Alternatively, *C. elegans* might have evolved a mechanism of generating OAS using alternative enzymes. CYS activity is essential for cysteine biosynthesis in prototrophs (Feldman-Salit *et al.* 2009). Because the critical aspect of CYS appears to be the elimination of H_2S instead of the generation of cysteine, perhaps *C. elegans* is freed from using OAS as the H_2S acceptor and is using an alternative H_2S acceptor that does not generate cysteine. In mammals the closest mammalian homolog to CYS is CBS. Consistent with the idea that mammals have CYS activity, there is evidence that radiolabeled H_2S can be incorporated into cysteine in germ-free rats (Huovinen and Gustafsson 1967).

The protective effect of *cysl-2* to HCN explains the previous observation that *egl-9* mutant animals are resistant to the pathogenic *Pseudomonas* fast paralytic killing. *P. aeruginosa* is a clinically relevant pathogen and can produce toxic cyanide levels in the sputum of cystic fibrosis patients (Anderson *et al.* 2010). *P. fluorescens* is a cyanide-producing soil bacteria that has also been shown to exhibit cyanide-dependent fast paralytic killing effect on *C. elegans* (Romanowski *et al.* 2011). HIF-1 activates resistance to hypoxia, and this has been proposed to also confer resistance to the cytochrome *c* oxidase inhibiting effects of cyanide (Gallagher and Manoil 2001; Shao *et al.* 2010; Romanowski *et al.* 2011). While this certainly may play some role, we provide evidence here that the resistance of egl-9 mutant animals to *P. aeruginosa* requires cysl-2, likely by directly detoxifying cyanide to β-cyanoalanine (Figure 8). This reaction produces H_2S, which can be further detoxified by *cysl-1* and *sqrd-1*.

While we have demonstrated critical components of the RA/H_2S-induced *hif-1* response, the mechanism of *hif-1* induction remains unclear. The overlapping gene expression patterns between both H_2S and HCN suggests that *C. elegans* has a shared response to these two molecules. In fact, our studies suggest that *C. elegans* is unable to discriminate between H_2S and HCN, leading them to activate the degradation pathways of both molecules when either is presented. Thus, *C. elegans* treated with RA/H_2S or RA/HCN increases

both H_2S-detoxification enzymes and cyanide-detoxification enzymes (Figure 6A). A recent study showed that inhibition of electron transport through reduction of expression of electron transport proteins is sufficient to induce HIF-1 activity (Lee *et al.* 2010). H_2S and HCN are natural effectors of electron transport and cause increased HIF-1 activity; therefore, H_2S and HCN might be natural regulators of electron transport.

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GENETICS

Supporting Information
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The Response of *Caenorhabditis elegans* to Hydrogen Sulfide and Hydrogen Cyanide

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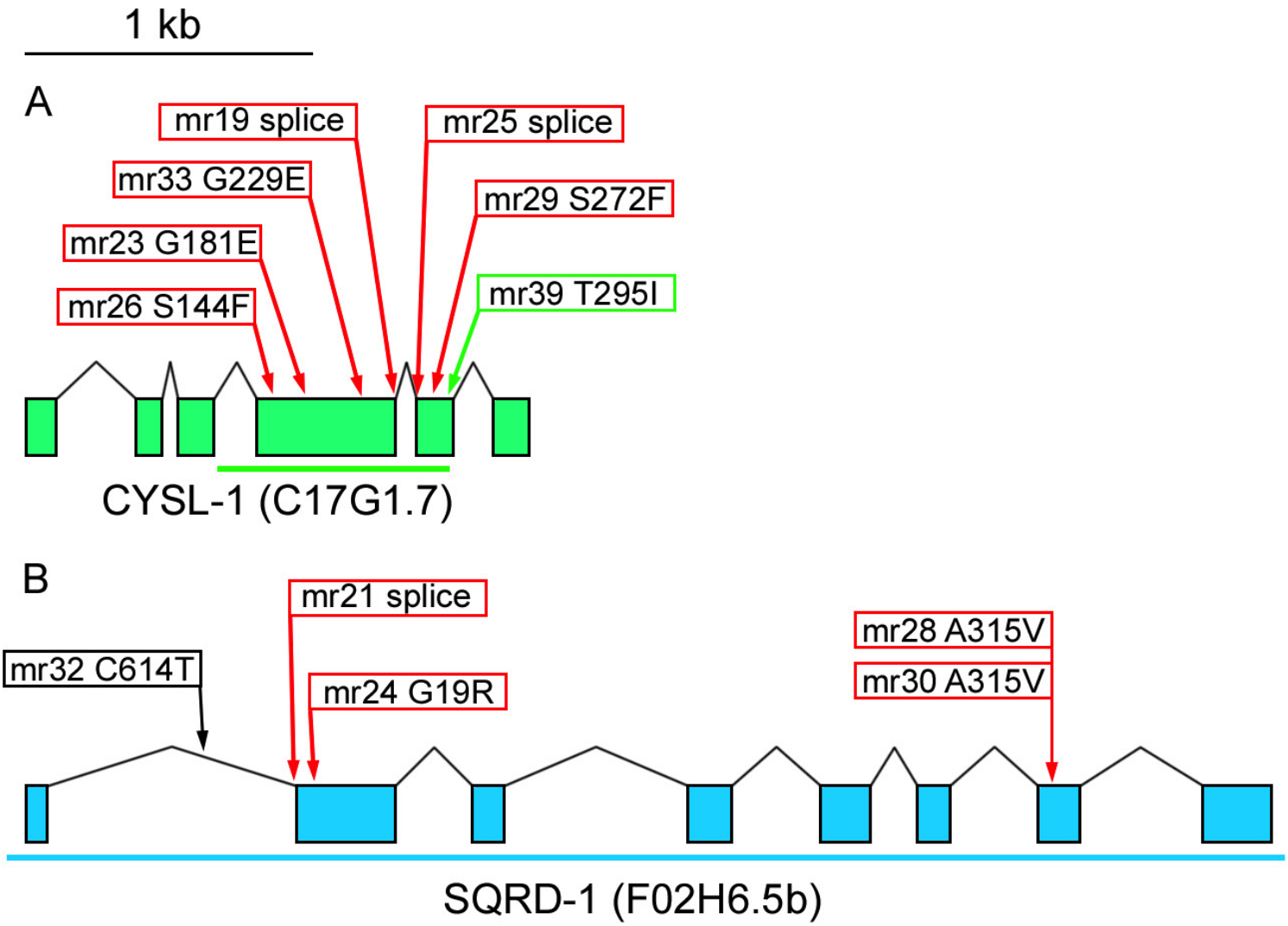

Figure S1 Identification of genetic mutations in isolated mutants. The region sequenced is indicated as a colored bar below the gene diagram. Mutations predicted not to be tolerated are highlighted in red. Mutation predicted be tolerated are highlighted in green. **A** Sequencing an 807 bp region of cysl-1 revealed seven point mutations, all of which except *mr39* are predicted not to be tolerated. **B** Sequence data was obtained from the entire *sqrd-1* gene and four novel point mutations were identified.

File S1
Supporting Movie

Wild-type *C. elegans* treated with four hours of 150 ppm RA/H2S enter a reversible state of suspended animation. This 60 second time-lapse video corresponds to 10 hours of recording.

File S1 is available for download as a compressed .avi file at http://www.genetics.org/cgi/content/full/genetics.111.129841/DC1.

Table S1 Primers used for quantitative PCR

Gene	Direction	Sequence
cysl-1	forward	gaaaggagcgattgacatgg
cysl-1	reverse	tgtccctttggtttgtctcc
sqrd-1	forward	gtgatcctcgcagaatttgg
sqrd-1	reverse	gctggtccattccagtatcc
sqrd-2	forward	ttgtccactggtcgtcagc
sqrd-2	reverse	ccagtgccctttttatcaatcc
ethe-1	forward	tcagtgctcagttcaaaatcg
ethe-1	reverse	tgcagatctcaatgaatgttcc
cysl-2	forward	tgctgaggtgattcttaccg
cysl-2	reverse	taatgggcttctggatttgc
cysl-4	forward	aaatcgagatgctcgtcagc
cysl-4	reverse	ccactttcccttgagtttgc
cysl-3	forward	agcttccaaatgctcattgc
cysl-3	reverse	tcctgtcacagttccactcg
cbs-1	forward	tggcttctcttggatcaacc
cbs-1	reverse	taacggatttcctggattgc
cbs-2	forward	ctgagaaaaccggcaaacc
cbs-2	reverse	cgtgatcaggcatttgtagc
tba-1	forward	cagccaccaactgttgttcc
tba-1	reverse	cattccttctccgacgtacc
tbb-2	forward	agaaggaatggacgagatgg
tbb-2	reverse	ctccagcttctccctcagc