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Bacterial Nitric Oxide Extends the Lifespan of *C. elegans*

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SUMMARY

Nitric oxide (NO) is an important signaling molecule in multicellular organisms. Most animals produce NO from L-arginine via a family of dedicated enzymes known as NO synthases (NOSes). A rare exception is the roundworm *Caenorhabditis elegans*, which lacks its own NOS. However, in its natural environment, *C. elegans* feeds on *Bacilli* that possess functional NOS. Here, we demonstrate that bacterially derived NO enhances *C. elegans* longevity and stress resistance via a defined group of genes that function under the dual control of HSF-1 and DAF-16 transcription factors. Our work provides an example of interspecies signaling by a small molecule and illustrates the lifelong value of commensal bacteria to their host.

INTRODUCTION

Nitric oxide (NO) performs many important functions in living organisms (Kerwin et al., 1995; Lowenstein et al., 1994; Moncada et al., 1991). It modulates the activities of diverse proteins either directly by binding to their active centers or indirectly by posttranslationally modifying specific redox active thiols and other amino acid residues (Hanafy et al., 2001; Lima et al., 2010; Marshall et al., 2000). NO also protects bacteria against oxidative stress and antibiotics (Gusarov et al., 2009; Shatalin et al., 2008). The functions of this tiny molecule in cardiovascular, immune, and neuronal systems constitute the best-studied examples of NO-mediated signaling (Ignarro, 1999; MacMicking et al., 1997; Moncada and Higgs, 1991). Almost all eukaryotes are capable of generating NO by dedicated enzymes known as NO synthases (NOSes) (Stuehr, 1999). Surprisingly, however, the complete genome of *Caenorhabditis elegans* contains no genes that encode NOS (Table S1 available online). Because all eukaryotic and bacterial NOSes are homologous (Table S1) (Crane et al., 2010; Gusarov et al., 2008; Stuehr, 1999), it is evident that *C. elegans* is unable to produce NO by NOS-dependent synthesis. Considering the ubiquitous function of NO in animal physiology (Hanafy et al., 2001; Kerwin et al., 1995; Lima et al., 2010; Lowenstein et al., 1994; Moncada and Higgs, 1991), we hypothesized that *C. elegans* compensates for its own deficit of NO production by "hijacking" NO generated by its natural food, bacteria.

C. elegans is a soil-dwelling animal that feeds on live bacteria. In the laboratory, this nematode is maintained almost exclusively on *Escherichia coli*. However, in nature, it lives predominantly on decomposing plant material (Félix and Duveau, 2012; Kiontke et al., 2011) where *Bacillus subtilis* and related bacteria thrive (Siala et al., 1974). Thus, *C. elegans* normally consumes *Bacilli*, which, along with some other soil bacteria, including *Staphylococci* and *Streptomycies*, synthesize NO via bacterial NOS (bNOS) (Crane et al., 2010; Gusarov and Nudler, 2005; Gusarov et al., 2008, 2009; Johnson et al., 2008; Reece et al., 2009). The unique chemical properties of NO define its ability to function as a signaling molecule (Thomas et al., 2008). It is a small, short-lived, and pervasive free radical that diffuses freely across cellular compartments and membranes. For example, in the mammalian vasculature, NO is synthesized by endothelial cells and immediately diffuses into neighboring smooth muscle, where it activates soluble guanylate cyclase (sGS), leading to muscle relaxation and vasodilation (Hanafy et al., 2001; Kerwin et al., 1995; Lowenstein et al., 1994; Moncada and Higgs, 1991). By analogy, bacteria-derived NO could in principle diffuse into the worm and influence its physiology.

RESULTS

NO Produced by Bacteria Extends *C. elegans* Lifespan

To test our hypothesis, we examined the effect of bacterial NO on *C. elegans* physiology. The lifespan of worms feeding on NOS-deficient *B. subtilis* was reduced by 14.74% compared with that of worms consuming wild-type (WT) bacteria (Figure 1A; Tables 1 and S2), whereas other physiological parameters, such as egg-laying, postembryonic development, and size, were not affected (Figure S1). This result indicates that bacterial NO is generally beneficial to worms. Deletion of the *B. subtilis nos* gene abolished NO production (Figure S2A) but did not affect bacterial growth (Figure S2B) (Gusarov and Nudler, 2005; Gusarov et al., 2008). This rules out potential variations in food availability as a factor in the reduction of lifespan. We noticed that *B. subtilis* quickly formed spores on regular nematode growth medium (NGM) agar (Figure S2C). Such spores are metabolically dormant and do not make NO, which explains why *nos* deletion has no effect on lifespan of worms grown on NGM agar

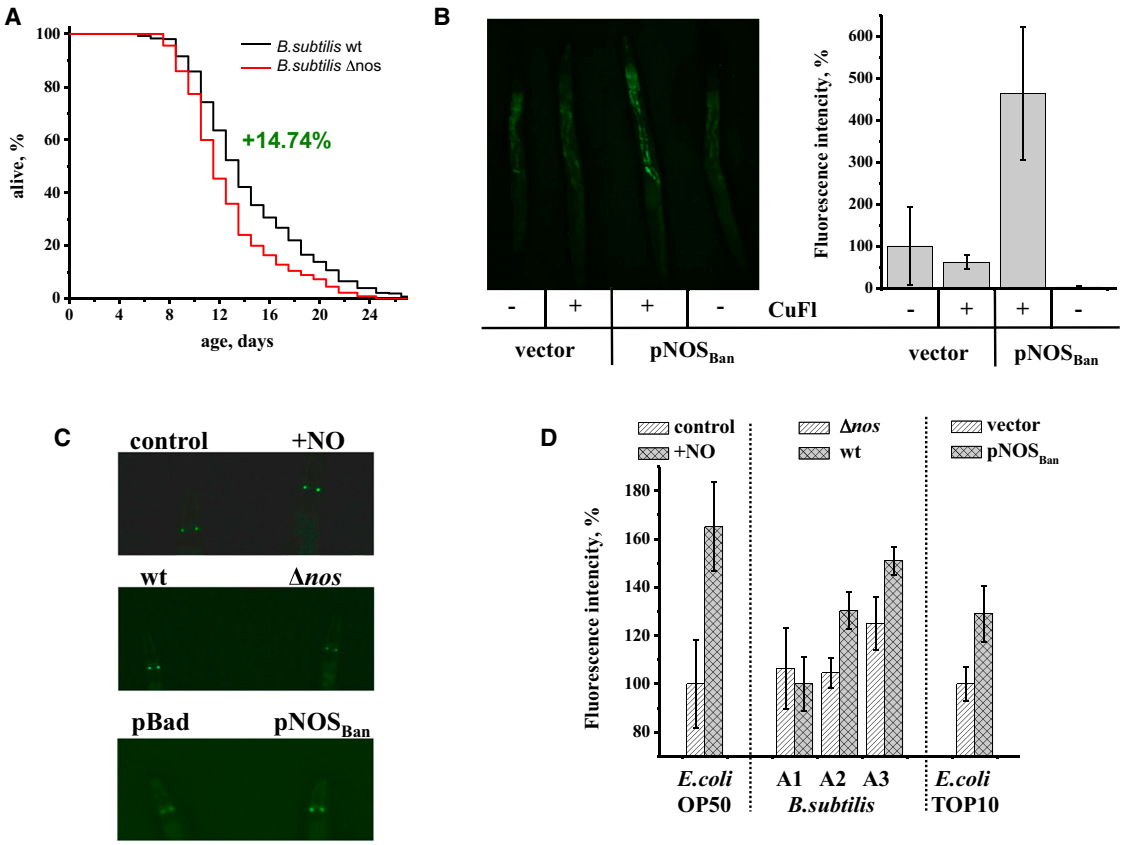

Figure 1. Bacteria-Derived NO Signals inside *C. elegans*

(A) Bacterial NO extends *C. elegans* lifespan. N2 worms were fed either WT or Δ*nos B. subtilis* on NGMga agar plates (black and red curves, respectively). Average values from three independent experiments are plotted (see also Tables 1 and S2). NGMga media (NGM plus 2% glucose and 0.5 mM arginine) was designed to inhibit sporulation and optimize NO production by *B. subtilis*. See also Figures S1, S2, and S3.

(B) Bacterial NO production in *C. elegans*. Representative fluorescent image (left) and quantification (right) of *C. elegans* treated with a Cu(II)-based NO-detecting probe (CuFL). WT worms were fed *E. coli* harboring either empty vector or pNOS$_{Ban}$ at 20°C. Twenty worms were used for each experimental condition. See also Table S1.

(C and D) sGS activation by NO in *C. elegans*. Representative fluorescent images (C) and fluorescence intensity quantification (D) of CX3553 (*str-1*::GFP) worms (n = 20) treated with NO donor (top panel and first two bars). Worms were fed with WT or Δ*nos B. subtilis* (middle panel and middle bars) or *E. coli* strains harboring either empty vector or pNOS$_{Ban}$ plasmid (lower panel and last two bars).

Error bars show means ±SD from at least three independent experiments. See also Figures S1, S2, and S3 and Tables S1 and S2.

(Figure S3A). To inhibit sporulation (Figure S2C) and achieve optimal NO production, we added glucose and arginine to the NGM media. As discussed below, NO-mediated life extension occurs irrespective of glucose and its negative effect on the worms' lifespan (Figure 2A; Tables 1 and S2).

Bacterial NO Is Produced and Acts inside the Worm

To trace bacterial NO inside *C. elegans* directly, we took advantage of the NO-specific fluorescent sensor CuFl (Lim et al., 2006). To circumvent the high level of autofluorescence of *B. subtilis*, we utilized *E. coli* expressing plasmid-borne bNOS (Gusarov et al., 2008). Only worms fed on bNOS-expressing bacteria became fluorescent after treatment with CuFl (Figure 1B). Fluorescence was detected in the gut, indicating that bacteria generated NO inside the worm (Figure 1B). Because addition of the NO sensor to worms feeding on control bacteria carrying an empty vector did not result in any fluorescent change above background (no probe control) (Figure 1B), we conclude that *C. elegans* does not make NO on its own (Table S1).

To confirm that bacterial NO reaches *C. elegans* tissues, we monitored the level of cellular cyclic guanosine monophosphate (cGMP). Soluble guanylyl cyclase (sGC) is the bona fide target for NO; the latter binds directly to the heme-iron active center of sGC to activate cGMP production (Hanafy et al., 2001; Kerwin et al., 1995). *C. elegans* has several heme-containing sGCs that could potentially be activated by NO (Fitzpatrick et al., 2006). To investigate this issue, we utilized CX3553 worms carrying green fluorescent protein (GFP) fused to the *str-1* promoter. GFP expression in AWB neurons of CX3553 depends on cGMP (van der Linden et al., 2008). We found that exposing *str-1*::GFP worms to the NO donor (MAHMA) resulted in robust fluorescence induction in AWB neurons (Figures 1C, top, and 1D), indicating that NO activates sGC in *C. elegans* as it does in other animals. We then fed CX3553 worms with WT and

Table 1. NO Increases *C. elegans* Lifespan

C. elegans Strain	Bacterial Strain	Media	50% Survival ±SE	NO-Dependent Increase, %
N2	*B. subtilis* 168	NGM	18.37 ± 0.18	0.97 ± 0.76
N2	*B. subtilis* Δnos	NGM	18.19 ± 0.31	
N2	*B. subtilis* 168	NGMga	13.75 ± 0.21	14.74 ± 2.0
N2	*B. subtilis* Δnos	NGMga	11.98 ± 0.14	
N2	*E. coli* OP50	NGM	17.18 ± 0.21	
N2	*E. coli* OP50	NGM+NO (MAHMA)	19.62 ± 0.62	14.13 ± 2.27
N2	*E. coli* OP50	NGM+NO (DETA)	18.55 ± 0.18	8.9 ± 0.45
N2	*E. coli* OP50	NGM+Glu	12.1 ± 0.2	
N2	*E. coli* OP50	NGM+Glu+NO (MAHMA)	14.33 ± 0.23	18.46 ± 0.03
hsf-1	*B. subtilis* 168	NGMga	12.5 ± 0.27	−7.2 ± 2.69
hsf-1	*B. subtilis* Δnos	NGMga	13.51 ± 0.68	
hsf-1	*E. coli* OP50	NGM	7.99 ± 0.085	
hsf-1	*E. coli* OP50	NGM+NO (MAHMA)	7.98 ± 0.005	−0.11 ± 1.12
hsf-1	*E. coli* OP50	NGM+Glu	7.56 ± 0.16	
hsf-1	*E. coli* OP50	NGM+Glu+NO (MAHMA)	7.37 ± 0.22	−2.5 ± 1.27
daf-16	*B. subtilis* 168	NGMga	12.5 ± 0.87	−3.21 ± 5.01
daf-16	*B. subtilis* Δnos	NGMga	12.89 ± 0.24	
daf-16	*E. coli* OP50	NGM	10.56 ± 0.26	
daf-16	*E. coli* OP50	NGM+NO (MAHMA)	10.29 ± 0.09	−2.43 ± 3.21
clk-1	*E. coli* OP50	NGM	8.85 ± 0.05	
clk-1	*E. coli* OP50	NGM+NO (MAHMA)	10.05 ± 0.2	13.55 ± 1.62

Summary of lifespan assays. Experimental conditions are as described in Experimental Procedures. Glu = 2% glucose, NGMga = NGM + 2% glucose + 0.5 mM arginine. NO-dependent lifespan increase was calculated for each group, $p < 0.05$. Change in lifespan is indicated in relation to the control lacking a source of NO. Data were analyzed and mean survival time was calculated using the OriginPro software package. See also Table S2.

Δ*nos B. subtilis* under the conditions of the "aging" experiment described above (Figure 1A). The fluorescence of AWB neurons decreased by 30% in the case of *nos*-deficient bacteria (Figures 1C, middle, and 1D), demonstrating that NO from *B. subtilis* penetrated *C. elegans* tissues and activated sGC. The same result was obtained with an *E. coli* strain expressing bNOS (Figures 1C, bottom, and 1D). Taken together, these experiments demonstrate that NO produced by bacteria in the *C. elegans* gut is capable of signaling in animal cells.

Exogenous NO Extends *C. elegans* Lifespan

In principle, the effect of bacterial NO on longevity could be indirect, e.g., via alterations in microbial metabolism. To address this issue and to determine whether NO itself affects aging of *C. elegans*, we supplied two different NO donors (MAHMA and DETA) to worms fed on *E. coli*. Gram-negative *E. coli* is evolutionarily distant from Gram-positive *B. subtilis* and cannot generate NO aerobically (Gusarov et al., 2008) unless it expresses bNOS from *Bacilli*, as described above. MAHMA and DETA increased the worms' lifespan by 14.1% and 8.9%, respectively (Figure 2A; Tables 1 and S2). The NO donors extended the lifespan of worms fed on modified or regular NGM equally, demonstrating that the antiaging effect of NO did not depend on modification of the media (Figure 2A). Moreover, we observed a similar increase in lifespan (12.7%) when we applied the NO donor to worms fed on *B. subtilis* spores (Figure S3B; Table S2). In all cases, the exogenous NO-mediated lifespan extension was comparable to that of bacteria-derived NO (Figures 1A and 2A; Tables 1 and S2), demonstrating that the antiaging effect of bacterial NO was direct, i.e., it was not mediated by variations in bacterial metabolism.

NO-Mediated Lifespan Extension Requires *daf-16* and *hsf-1* Activity

The lifespan of *C. elegans* is subject to regulation by several signaling pathways and transcription factors that sense stress and nutrient availability (Kenyon, 2010; Vijg and Campisi, 2008). Inhibition of insulin-like signaling (ILS) and perturbation of mitochondrial metabolism are among the most studied factors that affect worm lifespan (Kenyon, 2010; Lakowski and Hekimi, 1996; Mukhopadhyay et al., 2006). To determine whether NO affects either of these pathways, we exposed *daf-16* and *clk-1* mutant worms to NO. DAF-16 is a FOXO transcription factor that is necessary for life extension by ILS (Kenyon, 2010; Mukhopadhyay et al., 2006). *Clk-1* mutations reduce ubiquinone biosynthesis and content in mitochondria, and as a result, increase lifespan, slow development, and alter the cell cycle and rhythmic adult behaviors (Lakowski and Hekimi, 1996). Although the NO donor extended the lifespan of *clk-1* mutant worms (Figure 2B; Tables 1 and S2), it had no effect on *daf-16* mutant worms (Figure 2C; Tables 1 and S2). Moreover, *daf-16* mutant worms fed on WT *B. subtilis* had the same lifespan as those

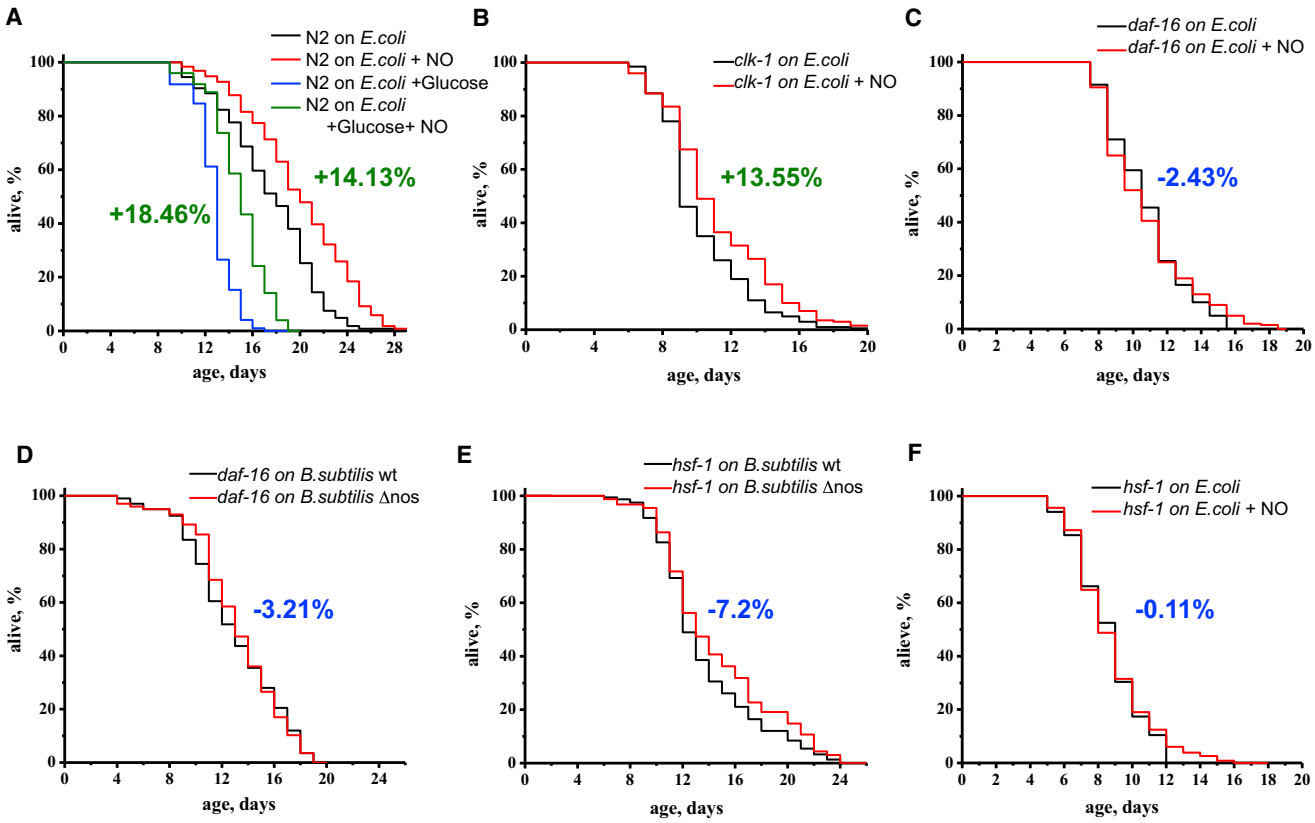

Figure 2. NO-Dependent Life Extension Depends on *daf-16* and *hsf-1*

In each case, the average values from at least three independent experiments are plotted (see also Tables 1 and S2).

(A) NO donor extends *C. elegans* lifespan. N2 worms were grown on *E. coli* until L4 and transferred to freshly prepared *E. coli* agar plates with (red and green) or without (black and blue) 1 mM MAHMA NONOate. NO treatment was repeated 2 and 4 days later. The experiment was performed on NGM (black and red) and NGMga (blue and green) agar plates. See also Figure S3B.

(B) NO donor extends *clk-1* lifespan. The graph demonstrates the adult lifespan without an extended developmental period. The experiment was performed on NGM media as described in (A). The mean from three experiments is presented.

(C and D) Exogenous or bacterially derived NO does not extend the lifespan of *daf-16* worms. The experimental conditions in (C) and (D) are as described in Figures 2A and 1A, respectively.

(E and F) Bacterially derived or exogenous NO does not extend the lifespan of *hsf-1* worms. The experimental conditions in (E) and (F) are as described in Figures 1A and 2A, respectively. Experiments with a NO donor were also performed on NGMga agar to demonstrate that glucose and arginine do not alter the effect of NO on aging (Tables 1 and S2).

See also Figure S3 and Table S2.

fed on *nos*-deficient bacteria (Figure 2D; Tables 1 and S2). Together, these results indicate that NO-mediated lifespan extension depends on *daf-16* activity.

A subset of genes that are vital for longevity is regulated by the dual control of the *daf-16* and *hsf-1* transcription factors (Hsu et al., 2003). HSF-1 is a master activator of heat shock (HS) genes that encode molecular chaperones and other cytoprotective molecules (Anckar and Sistonen, 2011; Morimoto, 2008). Inhibition of *C. elegans* HSF-1 leads to heat sensitivity, age-onset proteotoxicity (Cohen et al., 2006), and accelerated aging (Garigan et al., 2002; Morley and Morimoto, 2004). Increased *hsf-1* gene dosage prolongs lifespan and delays toxic protein aggregation (Hsu et al., 2003). Moreover, mild HS and elevated expression of several HS proteins (HSPs) delay aging of *C. elegans* and *Drosophila* (Kurapati et al., 2000; Tatar et al., 1997; Yokoyama et al., 2002). Because NO can induce HSPs via HSF-1 in cultured human cells (Xu et al., 1997), we examined whether NO-mediated lifespan extension involves activation of the *C. elegans* HS response (HSR). In contrast to the lifespan of WT worms, the lifespan of *hsf-1* worms (SY441 strain) was unaffected by bacterial NO production (Figure 2E; Tables 1 and S2). Consistent with this result, the NO donor failed to extend the lifespan of HSF-deficient animals (Figure 2F; Tables 1 and S2). We conclude that the antiaging effect of NO requires HSF-1 activity.

Exogenous or Bacterial NO Induces HSPs in *C. elegans*

To determine whether NO activates the HSR in nematodes, we used two reporter strains, TJ375 and AM446, in which GFP is expressed under the *hsp-16* and *hsp-70* promoters, respectively (Morley and Morimoto, 2004; Rea et al., 2005). Four hours after NO exposure, the *hsp-16::GFP* worms displayed strong

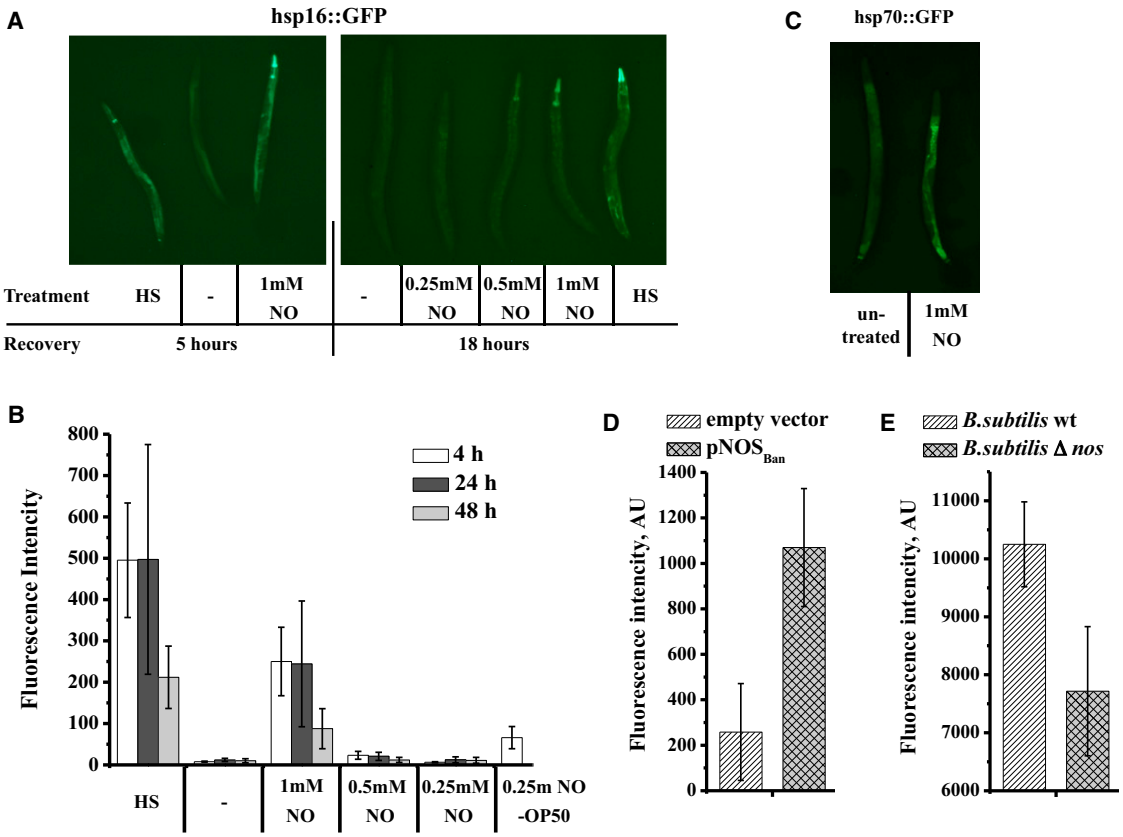

Figure 3. NO Induces the Expression of HSPs in *C. elegans*

(A) Representative images of TJ375 worms (*hsp16::GFP*) treated with NO. *C. elegans* grown on *E. coli* were exposed to the indicated amounts of MAHMA NONOate as described in Figure 2A. Worms were picked and photographed 5 and 18 hr later. HS: 30 min of HS at 30°C. See also Figure S4.

(B) Fluorescence quantification of TJ375 worms (*hsp16::GFP*) treated with NO. Twenty worms were randomly picked for quantification 4, 24, and 48 hr after NO exposure (see Experimental Procedures for details).

(C) Representative image of AM446 (*hsp70::GFP*) worms 18 hr after NO treatment. The experimental conditions were as described in (A).

(D) Fluorescence quantification of 20 1-day-old TJ375 (*hsp16::GFP*) adults fed *E. coli* that harbored either empty vector or $pNOS_{Ban}$ at 25°C. See also Figure S5A.

(E) Fluorescence quantification of TJ375 worms fed WT or Δ*nos B. subtilis*. Two-day-old worms were shifted to 34°C for 40 min and then allowed to recover for 4 hr at 20°C. See also Figures S5B and S6.

Error bars show means ±SD from at least three independent experiments. See also Figures S4, S5, and S6.

fluorescence that persisted for 2 days (Figures 3A and 3B). We also detected an increase in fluorescence in the *hsp70::GFP* strain 18 hr following NO treatment (Figure 3C). Together, these results demonstrate HSR induction by NO. Interestingly, the *hsp-16*-driven signal decreased on the second day after NO treatment but could be restored by additional NO treatment (Figure 3B and S4A). Thus, the pattern of NO application in our aging experiments described above ensured that small HSPs were continuously expressed to delay aging, similarly to repeated nonlethal HS (Olsen et al., 2006). The minimum concentration of bolus NO donor sufficient to induce HSR was 0.5 mM (Figures 3A, 3B, and S4B). However, it could be greatly reduced in the absence of live bacteria (Figures 3B and S4B), indicating that bacteria absorbed most of the NO. Notably, the NO^+-releasing agent S-nitroso-N-acetylpenicillamine (SNAP) failed to induce HSR (Figure S4C). In contrast to NO, NO^+ does not pass through cellular membranes, supporting the notion that bacterial NO must diffuse into the worms' cells to delay aging.

Feeding TJ375 worms with *E. coli* expressing plasmid-borne bNOS induced *hsp-16*-driven fluorescence as compared with the empty vector control (Figures 3D and S5A). In a separate experiment, we fed TJ375 with WT or Δ*nos B. subtilis* followed by a brief HS (35°C for 40 min). The fluorescence intensity of worms fed on WT bacteria was ~33% higher than that of worms fed on Δ*nos* bacteria (Figures 3E and S5B). Together, these experiments show that either exogenous or bacterial NO induces HSP expression in *C. elegans*.

In contrast, the expression of *sod-3::GFP* (CF1553), one of many genes that are regulated by *daf-16* (Libina et al., 2003), was not affected by NO (Figures S6A and S6B). This result indicates that although *daf-16* is necessary for NO-mediated life extension, the ILS pathway may not be a direct target of NO.

NO Promotes Thermotolerance in *C. elegans*

Because NO activates the HSR, we expected it to augment worm thermotolerance. Indeed, bacterially derived NO

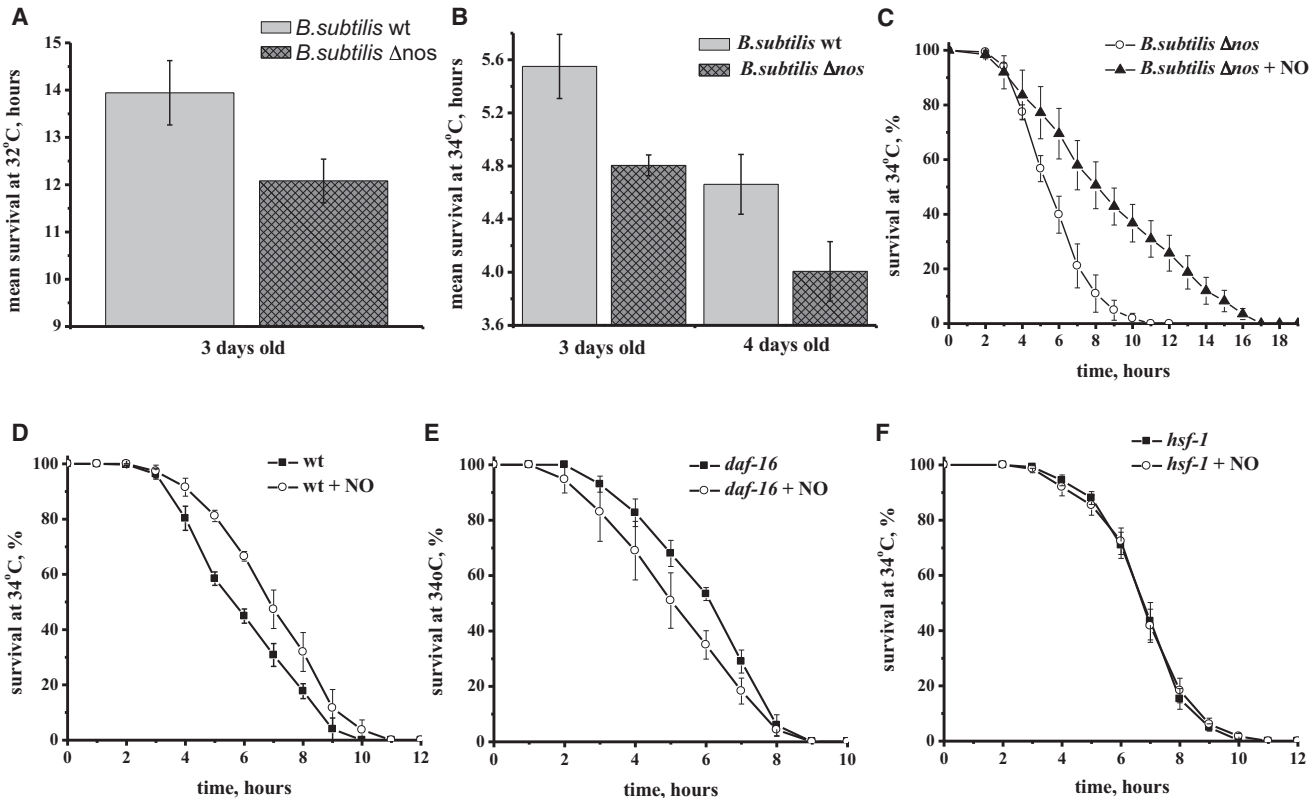

Figure 4. NO Increases *C. elegans* Thermotolerance

In each case, the graph shows the average survival ±SE of more than three independent experiments. See Table S3 for details on individual experiments.

(A and B) Bacterial NO increases thermotolerance. L4 *C. elegans* fed either WT or Δ*nos B. subtilis* were transferred to NGMga agar plates and incubated for 3 or 4 days at 20°C. To avoid mixing with the progeny, worms were transferred to fresh agar plates at days 2 and 3. The plates were then shifted to 32°C (A) or 34°C (B) and the median survival time was calculated as described in Experimental Procedures.

(C) Exogenous NO complements the lack of bacterial NO in restoring the thermotolerance of worms grown on Δ*nos B. subtilis*. Worms were treated twice with 1 mM MAHMA NONOate at L4 and A2. At A3, animals were shifted to 34°C and scored every hour for dead worms. The mean survival time was calculated as described in Experimental Procedures.

(D) Exogenous NO protects worms from lethal HS. WT worms were fed *E. coli* until they reached L4, treated with 1 mM MAHMA NONOate as described in Figure 2A, and then allowed to recover for 4 hr. They were then shifted to 34°C and scored every hour for dead worms, and the average of three independent experiments was plotted. See also Figure S7A.

(E) The initial NO-mediated increase in thermotolerance requires *daf-16*. Experimental conditions are the same as in (D). Note that, if *daf-16* worms were left to recover for 15 hr past NO treatment, they acquired partial thermotolerance (Figure S7A and Table S3).

(F) The NO-mediated increase of thermotolerance requires HSF-1. SY440 (*hsf-1*$^{-}$) worms were fed *E. coli* on agar plates until they reached stage L4. They were then treated with 1 mM MAHMA NONOate as described in Figure 2A, followed by 15 hr of recovery. The animals were then shifted to 34°C, the plates were scored every hour for dead worms, and the average of eight independent experiments was plotted. See also Figure S7B.

See also Figure S7 and Table S3.

protected *C. elegans* against lethal HS. Animals were fed WT or *nos*-deficient *B. subtilis* at 20°C prior to exposure to elevated temperatures. When shifted to 32°C or 34°C, 3-day-old worms fed on WT bacteria lived 16.18% or 15.4% longer, respectively, than those fed on NOS-deficient bacteria (Figures 4A and 4B; Table S3). Notably, the difference in survival time increased as the age of the animal increased (4-day-old worms fed on WT bacteria lived 19% longer than worms fed on NOS-deficient bacteria), probably due to their longer exposure to NO (Figure 4B). To further demonstrate that NO induces thermotolerance, we treated worms with an NO donor while feeding them with *nos*-deficient *B. subtilis*. Two consecutive exposures increased the mean survival time by 58% at 34°C (Figure 4C; Table S3), indicating that NO is responsible for elevated thermotolerance.

To investigate the role of DAF-16 and HSF-1 in NO-mediated thermotolerance, we grew WT and mutant worms on *E. coli* until stage L4, followed by treatment with a NO donor. A single NO exposure increased the mean survival of WT worms by 22.1% and 14.5% at 34°C after 4 and 16 hr of recovery, respectively (Figures 4D and S7A; Table S3). Thus, 4 hr after NO challenge, the worms were able to mount a robust response that rendered them more resistant to a subsequent lethal HS. In contrast, the thermotolerance of *daf-16* worms was not increased but was slightly decreased after NO treatment (Figure 4E), demonstrating the critical role of *daf-16* in the NO-mediated response. After

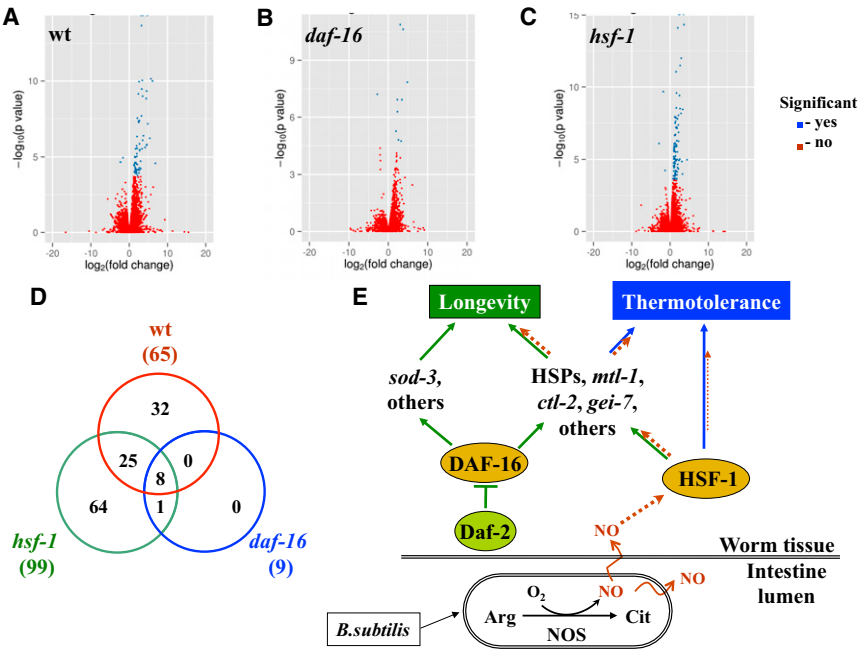

Figure 5. Response of *C. elegans* to NO

(A–C) Volcano plots demonstrating the results of differential expression analyses of NO-mediated response in (A) WT, (B) *daf-16*, and (C) *hsf-1 C. elegans*.

(D) Venn diagram demonstrating the key role of *daf-16* and *hsf-1* in response to NO. NO treatment deregulates 65 genes in WT (red circle), 99 genes in *hsf-1* (green circle), and 10 genes in *daf-16* (blue circle) strains. Expression of 57 and 32 genes was lost in the *daf-16* and *hsf-1* strains, respectively. Note that the induction of 14 out of 25 genes shared between WT and *hsf-1* strains was more than 2-fold smaller in the case of *hsf-1*. See also Tables S4, S5, and S6.

(E) Model for bacterial NO signaling in *C. elegans*. While in the worm's intestine, vegetative *B. subtilis* produces NO. This small lipophilic molecule diffuses freely into *C. elegans* cells. There it induces a specific response that depends strictly on DAF-16 and HSF-1 activity and results in the induction of 65 genes, including *hsps* and several other genes (mtl-1, ctl-2, gei-7, and T19B10.2) (Table 2) that have been implicated in longevity and stress resistance (Halaschek-Wiener et al., 2005; Murphy et al., 2003; Samuelson et al., 2007). A proposed NO activated pathway is indicated in red.

See also Tables S4, S5, and S6.

15 hr of recovery from NO treatment, however, a partial increase in the thermotolerance of *daf-16* worms was detected (Figure S7A), indicating that some effects of NO on thermotolerance were *daf-16* independent. As expected, NO failed to increase the thermotolerance of *hsf-1* (SY441) worms (Figure 4F). To further confirm the role of HSF-1 in NO-mediated thermotolerance, we knocked HSF-1 down by means of RNA interference (RNAi). This experiment showed that indeed HSF1-depleted worms became more thermosensitive and ceased to be protected by NO (Figure S7B; Table S3).

A signal from thermosensory neurons in *C. elegans* has been reported to activate HSF-1 in somatic cells (Prahlad et al., 2008). However, we observed NO-mediated thermotolerance in both OH8 (*ttx-3*) and IK597 (*gcy-23*, *gcy-8*, and *gcy-18*) animals, indicating that NO activates HSR in somatic cells directly (Figure S7A).

The NO-Dependent Regulon in *C. elegans* Depends on the Combined Activity of HSF-1 and DAF-16

To determine NO-mediated transcriptomic changes in *C. elegans*, we performed next-generation RNA sequencing (RNA-seq). RNA-seq revealed 65 genes induced by NO in WT worms (Figure 5A; Tables 2 and S4), indicating that the response was highly specific. In agreement with the above results, *hsps* were among those genes. Also, *mtl-1*, *ctl-2*, *gei-7*, *nlp-29*, and T19B10.2 genes previously associated with longevity (Halaschek-Wiener et al., 2005; Murphy et al., 2003; Samuelson et al., 2007) were upregulated by NO (Table 2). These five genes along with *hsps* are likely to be responsible for NO-mediated life extension and stress resistance. Eight genes from Table 2 (*hsp-16.1*, *hsp-16.11*, *cnc-4*, ZK970.7, F20G2.5, *fip-6*, *nlp-29*, and *Cyp-14A5*) have been shown to respond to various pathogens (Troemel et al., 2006; http://www.wormbase.org). Interestingly, there is also an overlap between NO and Cd^{2+} responses (Cui et al., 2007). Seven genes in Table 2 caused Cd^{2+} hypersensitivity when they were suppressed by RNAi or were induced by Cd^{2+} treatment (Cui et al., 2007; http://www.wormbase.org). Thus, NO signaling bridges the components of at least three known stress pathways: HS, Cd^{2+} toxicity, and the pathogenic response. The function of most of these genes has not been elucidated, hence the significance of their induction by NO remains unknown.

Because *daf-16* and *hsf-1* mutants do not exhibit NO-mediated lifespan extension (Figure 2), we analyzed their transcriptomic responses to NO and compared them with those of WT worms (Tables S5 and S6). In *daf-16*, only nine genes were affected by NO (Figure 5B; Table 2), and eight of these were induced in the WT as well, meaning that the induction of 57 genes in the WT animals was *daf-16* dependent (Figure 5D). This result explains the key role of *daf-16* in NO-mediated longevity as well as in HS resistance after NO preconditioning (Figure 4E). The induction of 32 out of 65 genes by NO was lost in the *hsf-1* mutant, and the expression of another 14 genes decreased more than 2-fold (Figures 5C and 5D; Table 2). Thus, full activation of 46 out of 65 genes by NO requires HSF-1. Eleven genes that were previously shown to affect aging, including six *hsps*, *mtl-1*, *ctl-2*, *gei-7*, *nlp-29*, and T19B10.2, are among those controlled by HSF-1 (Table 2). Finally, the induction by NO of only four genes was not decreased in either *daf-16* or *hsf-1* animals (Table 2). Together, these results demonstrate that NO imposes a narrow and highly specific response in *C. elegans* that is orchestrated by both DAF-16 and HSF-1 (Figure 5).

DISCUSSION

C. elegans has become a favorite model system for study by gerontologists because of its relatively short lifespan and genetic tractability. Genetic and chemical manipulations of this animal have provided important insights into the mechanisms of aging during the past two decades. However, these studies largely ignored the fact that *C. elegans* feeds on actively metabolizing bacteria. Live bacteria constitute both the main food source and the commensal gut flora of nematodes in nature and in the laboratory and can significantly affect the animal's lifespan (Larsen and Clarke, 2002). *B. subtilis*, which is a natural food for *C. elegans*, is significantly better at promoting longevity than is *E. coli* (Garsin et al., 2003), although the mechanism by which it does so remains unknown. Here, we present evidence that directly implicates a bacterial metabolite (NO) in the wellbeing of *C. elegans*. NO synthesized by bacteria diffuses into the worm's tissues and initiates a signaling cascade that results in a specific transcriptional response. This in turn increases thermotolerance and extends the lifespan (Figure 5E). Our results argue for the advantage of a "NO-rich" bacterial diet that renders nematodes more adaptive to daily heat fluctuations in their natural environment (Figure 4).

Comparative gene profiling reveals the principal role of *daf-16* and *hsf-1* in the NO-mediated transcriptional response (Table 2). Whereas DAF-16 has been known to control a broad spectrum of genes that are important for aging (Kenyon, 2010; Murphy et al., 2003), the role of HSF-1 in this respect has been mostly attributed to small HSPs (Walker and Lithgow, 2003), some of which are also under DAF-16 control (Hsu et al., 2003; McColl et al., 2010). Our results place several known longevity targets of DAF-16 (*ctl-2*, *gei-7*, *nlp-29*, and T19B10.2) (Halaschek-Wiener et al., 2005; Murphy et al., 2003; Samuelson et al., 2007) under HSF-1 control (Table 2) and thus expand the role of *hsf-1* in aging. In fact, *hsf-1* is necessary for the full induction of at least 46 genes (out of 65) in response to NO (Table 2). Because we used the partial loss-of-function *hsf-1* mutant for RNA-seq, one might expect that most, if not all, of the NO-responsive genes require fully functional HSF-1. However, because the majority of these genes have not been characterized, their individual contributions to longevity remain to be determined.

Interestingly, NO extends the lifespan regardless of the animal's caloric diet. Glucose stimulates proaging ILS activity and shortens lifespan (Lee et al., 2009; Schulz et al., 2007). However, NO augments longevity irrespective of glucose supplementation (Figure 2A; Table 1), which suggests that the ILS pathway per se is not a direct target for NO signaling.

NO is an unusual signaling molecule because of its ability to diffuse freely across membranes, whereupon it can directly or indirectly (e.g., via S-nitrosylation) alter the activities of numerous cellular and extracellular proteins (Foster et al., 2003; Thomas et al., 2008). These unique qualities of NO could allow the early evolutionary establishment of bacteria-to-nematode signaling (Figure 5). Eukaryotes likely acquired NOS from bacteria (the latter have the most ancient version of NOS) (Gusarov et al., 2008) by horizontal gene transfer, resulting in its autonomous expression in various tissues. It is also possible that *C. elegans* lost their NOS because they had a sufficient supply of NO from bacteria. Considering the high evolutionary conservation of HSF-1 and FOXO from worms to humans, it is likely that NO also functions in the activation of mammalian cytoprotective HSPs and other genes. For example, induction of the metallothionein gene by NO has been demonstrated in glomerular mesangial cells (Datta and Lianos, 2006). Our results may explain why induction of eNOS is required for the antiaging phenotypes of calorie-restricted mice (Nisoli et al., 2005). Moreover, NO derived from human microbiota and probiotics may induce a beneficial response in the gastrointestinal tract similar to that observed in *C. elegans*.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

WT *C. elegans* (N2), PS3551 (hsf-1^{-} (sy441) I), IK597 (gcy-23(nj37) gcy-8(oy44) gcy-18(nj38) IV), OH8 (ttx-3(mg158) X), CF1038 (daf-16(mu86) I), CF1553 (muIs84[pAD76(sod-3::GFP)]), MQ130 (clk-1(qm30)III), CX3553 (lin-15B(n765) kyIs104 X), and TJ375 (hsp-16.2::GFP (gpIs1)) strains were obtained from the Caenorhabditis Genetics Center. The HSP70 reporter strain AM446 rmIs223 (C12C8.1::GFP) was a gift from Richard Morimoto at Northwestern University (Chicago, IL, USA). Nematodes were handled according to standard methods (Brenner, 1974). Strains were grown on NGM or NGMga agar plates. NGMga has 2% glucose and 0.5 mM arginine. To make a 0.5 M stock solution, arginine was dissolved in 1 M K_3PO_4 buffer pH 6.0 and sterilized by filtration. Bacterial cultures were grown in lysogeny broth (LB) overnight, and then 50 μl of each culture was spread on top of agar plates. The plates were incubated for ~20 hr at 25°C and then for at least 2 hr at 20°C before worms were transferred onto them.

Self-Brood Size and Rate of Egg Production

These experiments were performed essentially as described (Wong et al., 1995). Eggs from strain N2 nematodes were isolated, treated with hypochlorite, and incubated for 20 hr at 20°C in S-buffer without cholesterol (Fabian and Johnson, 1994). A synchronized population of L1 arrested worms was then placed on NGMga agar plates seeded with WT or Δnos *B. subtilis*. Five stage L4 animals were picked manually and transferred to a new plate. The worms were then transferred twice a day to prevent overcrowding until egg laying ceased. The progeny were counted 3 days after removal of the parents. The experiment was performed in quadruplicate.

Postembryonic Development and Adult Size

These experiments were performed essentially as described (Wong et al., 1995). Strain N2 worms were grown on NGMga agar plates seeded with WT or Δnos *B. subtilis*. Unstaged eggs were placed at 20°C and allowed to hatch for 4 hr. Larvae that hatched during this period were placed singly on fresh plates and monitored every 5 hr until they reached stage L4.

To measure the worms' size, ~20 randomly picked worms fed on WT or Δnos *B. subtilis* were anesthetized with sodium azide, straightened, and photographed. Their length in pixels was then compared with a 1 mm scale bar.

Lifespan Analysis

Lifespans were monitored at 20°C as described previously (Apfeld and Kenyon, 1999; Dillin et al., 2002). To transfer worms to *B. subtilis* strains, embryos were isolated by treating adult hermaphrodites with alkaline hypochlorite and allowing their embryos to develop for 3 days. Worms were grown at 20°C for at least one generation before use for lifespan analysis. All experiments were repeated at least three times and ~100 worms per bacterial strain were used for each experiment. In all cases, stage L4 worms were used at t = 0 for lifespan analysis. Worms were judged to be dead when they ceased pharyngeal pumping and did not respond to prodding with a platinum wire. Worms with internal hatching were removed from the plates and not included in lifespan calculations. Data were analyzed and survival Boltzmann sigmoid curves were generated using the OriginPro statistical analysis software

Table 2. NO-Mediated Transcriptional Response in *C. elegans*

Gene Name	Fold Change			RNAi Phenotype[a]	Proposed Function/Regulation[a]
	WT	hsf-1	daf-16		
B0564.4, best-2[b]	112.7059	21.69379			bestrophin
F42C5.3[b]	66.5806	4.46194		Cd^{2+} hypersensitive	
H20E11.2[b]	51.00653	5.78593	10.96109		
F14F9.3[c]	40.53932	–	–		
cnc-4[c]	28.59042	–	–		Caenacin/up by fungus infection
F44G3.2[b]	26.15636	11.34394	5.04492		
T26F2.3[b]	24.15517	3.50431	–		
Y47H9C.1[b]	23.61727	7.34845	28.96342		
F22G12.1[b]	22.97288	4.6469	–		
ZK970.7[c]	21.72629	–	–		Hypodermal antigen/up by ionizing radiation and bacterial infection
clec-174[c]	17.7085	–	–		C-type lectin
numr-1[b]	13.00478	3.66535	–		nuclear localized metal responsive/up by heavy metals
asns-2[b]	12.13069	4.05603	–		asparagine synthetase
K11G9.1[c]	11.2531	–	–		
T05H4.4	11.24717	12.44039	13.5106		cytochrome b5 reductase
F44E5.4[c]	10.61704	–	–	Cd^{2+} hypersensitive	HSP-70 family
F20G2.5[b]	10.10418	2.3716	–		
F44E5.5[c,d]	9.72353	–	–	Cd^{2+} hypersensitive	HSP-70 family
F22B8.7	9.26968	4.95121	4.95591		
gst-35	9.15564	7.5618	6.00145		glutathione S-transferase
F21C10.9[b]	8.43864	3.02262	3.92108		
B0507.8[c]	7.99163	–	–		
mtl-1[c,d,e]	7.93483	–	–	shortened lifespan, Cd^{2+} hypersensitive	Metallothionein/up by Cd^{2+} or HS
W09G12.7[c]	7.73859	–	–		
hsp-16.1[c,d,e]	6.54254	–	–	shortened lifespan, Cd^{2+} hypersensitive, pathogen susceptibility	AlphaB-crystallin family of HSP/up by HS
Y37E11B.7[b]	6.52872	2.83957	–		
fip-6[c]	6.52786	–	–		fungus-induced protein
Y58A7A.5	6.39853	3.8848	–		
hsp-16.11[c,d,e]	6.32357	–	–	shortened lifespan, Cd^{2+} hypersensitive, pathogen susceptibility	alphaB-crystallin family of HSP/up by HS and other stresses
F54B8.4	6.10525	4.57506	–		Death-Associated Protein 1 (DAP-1)
C42D4.3[c]	5.91702	–	–		
21ur-14767, B0507.10[c]	5.84932	–	–		
nlp-29[c,d]	5.51832	–	–		antimicrobial neuropeptide-like protein/up by infection
21ur-15446,ugt-24[c]	5.35156	–	–		UDP-glucuronosyl transferase
hmit-1.1[c]	5.32289	–	–	NaCl hypersensitive	proton (H^+)-dependent myoinositol transporters
cyp-14A5[b]	5.23293	2.12681	–		cytochrome P450/up by ionizing radiation and bacterial infection
hsp-16.48[c,d]	4.89714	–	–	shortened lifespan	alphaB-crystallin family of HSP/up by HS and other stresses
hsp-16.49[c,d,e]	4.89714	–	–	shortened lifespan	alphaB-crystallin family of HSP/up by HS and other stresses
tts-1[d]	4.51601	2.35973	–		transcribed telomerase-like sequence

(*Continued on next page*)

Table 2. *Continued*

Gene Name	Fold Change WT	hsf-1	daf-16	RNAi Phenotype[a]	Proposed Function/Regulation[a]
C15B12.1,C15B12.8[c]	4.33139	–	–		
cth-1	4.32086	3.20586	–		cystathionine gamma-lyase
F35B3.4[c]	4.13123	–	–		
gst-4	4.08816	2.70628	–		prostaglandin D synthase/up by paraquat
gpdh-1[c]	4.02085	–	–		glycerol 3-phosphate dehydrogenase/up by hypertonicity
catp-3[c]	3.93884	–	–		cation transporting ATPase
F53E10.1	3.82715	2.68753	–		
tag-297	3.8219	1.83366	–		
col-109	3.79753	2.47966	–		cuticular collagen
col-14	3.76487	1.96309	–		
F53B2.8	3.7495	2.26495	–		
Y58A7A.4[c]	3.56262	–	–		
col-104	3.2877	1.88388	–		collagen
ugt-31	3.24107	2.71891	–		UDP-glucuronosyl transferase
Y47D7A.13	3.09998	–	–		
ctl-2[c,e,f]	3.08308	–	–	shortened lifespan, paraquat hypersensitive	catalase
dpy-5	2.99322	2.15157	–		procollagen
C53A3.2[c]	2.97659	–	–	dauer constitutive	
col-138	2.94086	2.03359	–		collagen
col-97	2.91164	2.10267	–		cuticular collagen
col-73	2.84636	1.95342	–		collagen
T19B10.2[c,g]	2.78333	–	–	shortened lifespan of *daf-2*	
glb-1	2.57088	5.92675	8.16357		globin/up by anoxia, down by daf-2
gei-7[c,e]	2.49843	–	–	shortened lifespan	isocitrate lyase/malate synthase
col-135[c]	0.32228	–	–		collagen
C33G8.3[c]	0.20551	–	–		

List of genes regulated by NO treatment in WT (N2), *hsf-1* (SY441), and *daf-16* worms ($q < 0.05$) (see Tables S4, S5, and S6 for more details). Stage L4 *C. elegans* were treated with MAHMA. Four hours after treatment, RNA was isolated and subjected to RNA-seq according to Illumina guidelines. Differentially expressed genes were determined as described in Experimental Procedures. Genes are sorted by fold change of expression with respect to WT. Footnotes d–g indicate genes previously associated with aging.

[a]Information from http://www.wormbase.org.

[b]Genes whose expression was decreased >2-fold in the *hsf-1* mutant.

[c]Genes that require both DAF-16 and HSF-1 activities.

[d]Halaschek-Wiener et al. (2005).

[e]Murphy et al. (2003).

[f]Dong et al. (2007).

[g]Samuelson et al. (2007).

package. Mean lifespans were compared using Student's t test, assuming one-tailed distribution and two-sample equal variance. All lifespan plots represent the composites of all independent experiments tabulated in Table S2.

Exposure to NO

Because the NO donor MAHMA NONOate (Sigma) has a very short half-life at pH = 6 (~1 min), we developed a procedure to achieve optimal exposure of *C. elegans* to NO. Freshly prepared NGM agar plates were placed open in a tissue culture hood for 30–40 min to evaporate excess liquid and to ensure rapid absorption of added solutions. The plates were then transferred to 20°C and allowed to equilibrate for 2 hr. A freshly prepared solution of 250 mM NO donor in water was applied to NGM agar plates to achieve a final concentration of 1 mM. Immediately afterward, 50 μl of an overnight bacterial culture was spread atop the plate, and then ~40 worms were quickly transferred to the plate. For control experiments, the NO donor was substituted with an equal amount of distilled water. For measurements of the effect of NO on lifespan, worms were exposed to NO three times at stage L4 (day 0), stage A2 (day 2), and stage A4 (day 4).

Detection of Bacterial NO Production in *C. elegans*

WT worms were fed *E. coli* TOP10 strains harboring either empty vector or $pNOS_{Ban}$ plasmid (Gusarov et al., 2008) at 20°C on NGM plates supplemented with 100 μg/ml carbenicillin. On the 9th day of adulthood, they were transferred to plates containing 2% arabinose, 5 mM arginine, and 20 μM CuFl. For a background fluorescence control, worms were transferred to the same plate without CuFl. Fresh CuFl was prepared as described previously (Gusarov

et al., 2008). After 2 hr of incubation, the worms were anesthetized with a drop of 5 mM levamisole. Images of ~20 worms for each variant were captured at a fixed exposure time using a Zeiss Discovery stereomicroscope equipped for fluorescence illumination. The fluorescence intensity of individual worms was quantified using ImageJ.

Detection and Measurement of Bacterial NO Production In Vitro

NO produced by cells is oxidized to nitrite and nitrate in aerated media. Hence, the concentrations of nitrite and nitrate are directly proportional to the level of NO production (Gusarov et al., 2008). To measure nitrite and nitrate, cell culture supernatants were clarified by centrifugation and then filtered through Microcon YM-3 membrane filters (Millipore). Nitrite and nitrate were measured in the flow-through with a fluorometric and colorimetric nitrate/nitrite assay kit (Cayman Chemicals).

HS Reporter Assays

Worms were exposed to control conditions (growth at ambient temperature), treated with NO as described above, or heat shocked (30°C for 30 min). NO-treated and heat-shocked worms were allowed to recover before analysis (recovery times for each experiment are indicated in the figure legends). The worms were anesthetized and processed for image capturing as described above.

Thermotolerance Assays

Thermotolerance assays were performed essentially as described (McColl et al., 2010). Worms were allowed to develop and grow on agar plates at 20°C until they reached stage L4. They were then exposed to the NO donor as described above and allowed to recover for 4 or 15 hr at 20°C. After recovery, they were shifted to 34°C. In a typical experiment, three to four plates of worms, containing ~30–40 worms per plate, were used. Single plates were removed from the high temperature at 1 hr intervals and worms were scored for signs of life as described in "Lifespan Analysis" above. To calculate the median survival time, each data set was fitted to a Boltzmann sigmoid curve; the average ±SE is presented in the figures.

Bacterial Growth

Overnight cultures of WT and *nos* deletion bacteria were inoculated in liquid NGM, NGMga, or LB media. Cell growth was monitored in a Bioscreen C automated growth analysis system at 30°C. All growth curves were determined in triplicate, and the average was reported.

B. subtilis Sporulation Assay

Overnight cultures of *B. subtilis* WT and *nos* deletion strains were plated on NGM and NGMga agar plates. Plates were incubated at 25°C overnight (~20 hr) and then shifted to 20°C. Every 24 hr beginning at the time of plating, bacteria were scraped from the plate, resuspended in saline, and split in half. Serial dilutions were plated from one half to determine the total colony-forming units (cfu). The other half was incubated for 20 min at 80°C before plating. Because only spores survive at 80°C, the percentage of spores was calculated as the ratio of the heat-treated fraction to the total cfu.

RNA Isolation, Next-Generation Sequencing, and Differential Expression Analysis

Approximately 300 stage L4 worms of each strain were treated with NO donor (1 mM MAHMA). Worms were collected 4 hr later and washed in S-buffer, and total RNA was isolated as described in Reinke et al. (2000). A TrueSeq RNA Sample Preparation Kit v2 (Illumina) was used to prepare 1 μg of total RNA for RNA-seq. Two independent biological replicates were done for each experimental condition. The reference genome and annotation data for *C. elegans* (Ensembl assembly based on WS220 build) were downloaded from the Illumina website (ftp://igenome:G3nom3s4u@ussd-ftp.illumina.com/Caenorhabditis_elegans/Ensembl/WS220/Caenorhabditis_elegans_Ensembl_WS220.tar.gz). To estimate the expression level of transcripts and test for differential expression between different experimental conditions, the Tophat/Cufflinks/Cuffdiff pipeline was used (Trapnell et al., 2012). Briefly, the RNA-seq reads were trimmed of adaptor sequences and then mapped to the *C. elegans* transcriptome with Tophat software package (Trapnell et al., 2009) using the bowtie2 aligner and default parameters. The transcripts were assembled and their abundances estimated using the Cufflinks package (Roberts et al., 2011). A statistical test for differential gene expression was performed using the Cuffdiff tool in the Cufflinks package with a q value (p value adjusted for multiple testing (Storey and Tibshirani, 2003; threshold of 0.05). Analysis and visualization of the differential expression data were performed with the R software package (version 2.15.1) using the cummeRbund library (version 2.0).

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GSE43614.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.12.043.

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