Eukaryotic Initiation Factor 2 (eIF2) Signaling Regulates Proinflammatory Cytokine Expression and Bacterial Invasion*

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**Background:** eIF2 is a critical point of stress-induced regulation of translation in eukaryotic cells.

**Results:** eIF2 signaling is activated by bacterial pathogens and regulates two key infection-associated processes.

**Conclusion:** Regulation of translation in eukaryotic cells is involved in innate immune responses.

**Significance:** These findings enlarge the possible targets for therapeutic interventions against bacterial pathogens.

In eukaryotic cells, there are two well characterized pathways that regulate translation initiation in response to stress, and each have been shown to be targeted by various viruses. We recently showed in a yeast-based model that the bacterial virulence factor YopJ disrupts one of these pathways, which is centered on the α-subunit of the translation factor eIF2. Here, we show in mammalian cells that induction of the eIF2 signaling pathway occurs following infection with bacterial pathogens and that, consistent with our yeast-based findings, YopJ reduces eIF2 signaling in response to endoplasmic reticulum stress, heavy metal toxicity, dsRNA, and bacterial infection. We demonstrate that the well documented activities of YopJ, inhibition of NF-κB activation and proinflammatory cytokine expression, are both dependent on an intact eIF2 signaling pathway. Unexpectedly, we found that cells with defective eIF2 signaling were more susceptible to bacterial invasion. This was true for pathogenic *Yersinia*, a facultative intracellular pathogen, as well as for the intracellular pathogens *Listeria monocytogenes* and *Chlamydia trachomatis*. Collectively, our data indicate that the highly conserved eIF2 signaling pathway, which is vitally important for antiviral responses, plays a variety of heretofore unrecognized roles in antibacterial responses.

Various types of cellular stress result in a transient reduction in general protein synthesis. In eukaryotic cells, one component of this translational inhibitory response is centered on the eIF2 initiator factor, which, together with the initiator methionine tRNA and GTP, forms the ternary complex. Phosphorylation of the α-subunit of eIF2 at Ser-51 by any one of the four so-called eIF2α kinases (GCN2, PERK, protein kinase R (PKR),2 and HRI) negatively affects the GDP-GTP exchange activity of the β-subunit of eIF2, thus reducing the cellular levels of active ternary complexes. Upon the stress-induced activation of the eIF2α kinases and the subsequent phosphorylation of eIF2α, specific mRNAs are selectively translated (by virtue of their 5’-UTRs), and their encoded proteins contribute to cellular recovery (1). The best characterized of these mRNAs is that encoding the basic leucine zipper transcription factor ATF4 (Gcn4 in budding yeast), which, upon its synthesis, translocates to the nucleus, where it transcriptionally activates the expression of a number of stress-adaptive genes, including those encoding ATF3 and CHOP.

Viral infection is one such type of cellular stress that activates eIF2-mediated translation control. The eIF2α kinase PKR becomes activated by virus-derived dsRNA, which has the effect of reducing general protein synthesis and consequently reducing viral replication. Indicative of its importance in anti-viral cellular responses, several viruses have independently evolved the means to obstruct the activation of PKR (2). PKR has been shown to be required for LPS-induced activation of STAT1 and the induction of apoptosis in bacterially infected cells (3), suggesting that antiviral and antibacterial response pathways may overlap. There have also been various reports indicating that eIF2 signaling may be active during bacterial infections, the majority of which consist of uncharacterized “hits” from microarray-based screens (4–6). Recently, it has been reported that there is enhanced expression of several endoplasmic reticulum stress proteins (associated with PERK) in lung tissue of mice infected with *Mycobacterium tuberculosis* (7). It has also been shown that LPS- and Toll-like receptor-mediated signaling is functionally intertwined with HRI- and PERK-mediated stress responses, further suggesting that eIF2 signaling may be involved in antibacterial responses (8–10).

By performing a yeast-based mutagenesis screen for eukaryotic factors that are responsive to the bacterial virulence factor YpkA (*Yersinia* protein kinase A) (11), we discovered that eIF2 signaling is specifically activated by the kinase activity of YpkA and that this response reduces the cellular toxicity of YpkA (12). Unexpectedly, we found that the cellular activity of an additional *Yersinia* virulence factor, YopJ, which is encoded on the same transcript as YpkA, is entirely dependent on a properly functioning eIF2 signaling pathway (12). YopJ has no apparent phenotype in unstressed yeast cells. However, YopJ-expressing yeast cells are extremely sensitive to various types of stresses (e.g. osmotic, oxidative, and nutritional). Interestingly, YopJ

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*This work was supported, in whole or in part, by United States Public Health Service Grant AI53459 (to K. S.) from NIADD.† To whom correspondence should be addressed: Dept. of Microbiology and Immunology, University of Miami Miller School of Medicine, 1600 NW 10th Ave., Miami, FL 33173. E-mail: kschesser@med.miami.edu.‡ The abbreviations used are: PKR, protein kinase R; MEF, mouse embryonic fibroblast; qPCR, real-time quantitative PCR; Luc, luciferase; rTNFα, recombinant TNFα; m.o.i., multiplicity of infection.
also altered the eIF2-mediated cellular stress response induced by the kinase activity of YpkA.

Although our yeast-based findings indicate that the interactions between YopL and eIF2 signaling can shape the stress response, it was not immediately obvious how these interactions would affect the infection process. Here, we examined eIF2 responses in mammalian cells following infection with bacterial pathogens and determined whether those responses are sensitive to bacterially encoded virulence factors. We describe two separate findings that were not foreseen from our prior yeast-based studies: that eIF2 signaling is linked to infection-induced expression of cytokines as well as to bacterial invasion.

EXPERIMENTAL PROCEDURES

Infection-based Experiments—Macrophage-like RAW 264.7 cells (or wild-type and eIF2α(S51A) mouse embryonic fibroblasts (MEFs) (13)) were seeded in 24-well plates at 2 × 10^5 cells/well and infected the next day. For the Yersinia infections, the Yersinia pseudotuberculosis wild-type (YP111/pIB102) (14) and ΔyopJ (YP111/pIB223) (15) strains were grown in tissue culture medium, diluted to A_{595} = 0.1, and propagated for 2 h at 26 °C and then for 1 h at 37 °C. The bacterial strains were further diluted to achieve the desired titer and added to cultured cells.

For the translocation assay, a plasmid encoding a hybrid protein consisting of YopE (residues 1–130) and a 40-residue Elk tag (16) was transformed into a multiple copy strain of Y. pseudotuberculosis (17); the resulting strain was then used to infect wild-type and eIF2α(S51A) MEFs. After a 2-h infection period, cells were lysed, and the resulting lysates were analyzed using anti-phospho-Elk (Ser-383) and anti-Elk antibodies (Cell Signaling) to detect phosphorylated and total Elk, respectively.

For gene expression assays, at the indicated times, total RNA was extracted using a ZR RNA MiniPrep kit (Zymo Research), and cDNA was prepared using an Omniscript RT kit (Qiagen). Real-time quantitative PCR (qPCR) was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems) using TaqMan probes (Applied Biosystems). Signals of specific mRNAs were normalized with mouse GAPDH as the housekeeping gene, and the relative -fold changes were determined by calculating 2^{-ΔΔCt}.

For the Yersinia internalization assay, infection was initiated as described in the figure legends, and the fraction of internalized bacteria was determined by calculating the ratio of the number of bacteria recovered from the gentamycin–containing wells at 1 h to the number of bacteria recovered from the untreated wells. Each data point is the average of three independent experiments, and the results are representative of three separate experiments.

Listeria monocytogenes was obtained from American Type Culture Collection (DMX 09-082) and grown to stationary phase in brain-heart infusion broth (BD Biosciences) at 37 °C. Bacteria were diluted in tissue culture medium and added to cells. The infection proceeded as described in the figure legends and was terminated by removing the medium from the wells and lysing the cells with water, and the resulting lysates were plated on standard LB medium to determine their titers. The Chlamydia trachomatis infection assays (18) were performed as described in the figure legends.

Transfection-based Experiments—10^6 HEK 293T cells were seeded in 24-well plates and transfected the next day. Transfection mixtures contained Lipofectamine 2000 (Invitrogen), the pTK-ATF4-Luc reporter (provided by David Ron, University of Cambridge, Cambridge, United Kingdom), pRL-TK (encoding Renilla luciferase, which serves as a transfection control), and YopJ-encoding sequences cloned into Retro-X Tet-Off (Clontech). YopJ expression in transfected cells was verified by qPCR using YopJ-specific primers as described below. No signals were obtained in non-reverse transcriptase controls, indicating that YopJ-specific CDNAS were derived from mRNAs. In some experiments, the transfected cells were treated the next day with either 10 ng/ml thapsigargin (Calbiochem) or arsenic trioxide (Sigma) at the concentrations indicated in the figure legends, and 4 h later, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase reporter system assay (Promega). Firefly luciferase signals were normalized against the Renilla-derived signals. PKR /−/− and PKR /+/− MEFs (gift of J. Durbin) were transfected with the IFN-β-Luc reporter (19), pRL-TK, and the YopJ-encoding expression plasmid. The following day, cells were supertransfected with 6 μg of poly(I:C) (GE Healthcare) in Lipofectamine 2000, and 6 h later, cells were lysed, and luciferase levels were determined as described above. Salubrinal, the NF-κB-Luc reporter plasmid, and recombinant TNFα were purchased from Tocris Bioscience, Stratagene, and R&D Systems, respectively.

RESULTS

eIF2 Signaling in Yersinia-infected Cells—To determine whether eIF2 signaling is activated following exposure to a bacterial pathogen, the levels of phosphorylated eIF2α were evaluated in cultured murine macrophage-like cells infected with the bacterial pathogen Y. pseudotuberculosis. There was a notable increase in eIF2α phosphorylated at Ser-51 in cells following a 60-min infection compared with uninfected cells that was comparable with that observed in cells exposed to arsenite (Fig. 1A). To further evaluate eIF2 signaling in infected cells, we analyzed the transcript levels of endogenous Atf3, which is an immediate downstream target gene of ATF4 and a commonly used readout for eIF2 signaling in mammalian cells (20). In human HEK 293 cells infected with Yersinia, the kinetics of inductive ATF3 expression were comparable with those in cells treated with thapsigargin (see Fig. 2B below) (data not shown). In murine macrophage-like cells, inductive Atf3 expression increased over a 4-h infection period in contrast to TNFα expression, which reached a plateau after 30–60 min of infection (Fig. 1B).

To determine whether infection-induced Atf3 expression is in fact dependent on the phosphorylation of eIF2α, we used MEF cells expressing a non-phosphorylatable mutant, eIF2α(S51A). Like RAW 264.7 cells, wild-type MEFs exhibited both higher levels of eIF2α phosphorylation and Atf3 transcript levels in response to infection with Yersinia; this inductive expression did not occur in eIF2α(S51A) MEFs (Fig. 1C and D). Interestingly, whereas the ATF4 target gene Chop was significantly induced in response to thapsigargin (data not shown), it was not induced in Yersinia-infected cells (Fig. 1D). The rel-
Bacterial Virulence Factor YopJ Impacts eIF2 Signaling—In yeast cells, the activity of the Yersinia-encoded virulence factor YopJ is dependent on an intact eIF2 signaling pathway (12). To determine whether YopJ affects eIF2 signaling in mammalian cells, Atfβ expression was assayed in cells infected with either the yap/J+ or ΔyopJ strain of Yersinia. Atfβ expression was greater in cells infected with the ΔyopJ strain compared with the yap/J+ strain (Fig. 2A), showing that YopJ, when delivered into cells by the bacterial type 3 secretion system, negatively affects eIF2 signaling. This inhibitory activity of YopJ toward eIF2 signaling (Fig. 2A) is reminiscent of its previously reported negative effect on the expression of proinflammatory cytokines such as TNFα (21, 22).

To determine whether YopJ affects other previously characterized inducers of eIF2 signaling, cells were cotransfected with YopJ-encoding expression plasmids and a reporter plasmid that constitutively expresses a transcript consisting of the ATF4 5′-UTR control region upstream of sequences encoding firefly luciferase (23). As mentioned in the Introduction, stress-induced activation of eIF2α signaling results in the preferential translation of the ATF4-Luc transcript. HEK 293T cells expressed transfected wild-type YopJ and an inactive variant, YopJ(C172A), at comparable levels (data not shown). Treatment of cells with either thapsigargin or arsenite induce ATF4 expression by activating the eIF2α kinases PERK and HRI, respectively (Fig. 2, B and C) (24, 25). Wild-type YopJ reduced both thapsigargin- and arsenite-induced ATF4 expression in YopJ-expressing cells, in terms of fold increase, was similar to that observed in cells lacking PKR. Collectively, these data indicate that eIF2α expression occurs in response to bacterial infection.
infection with the \( \text{yop}^+ \) strain of \( \text{Yersinia} \), and this inductive expression was markedly enhanced in wild-type MEFs infected with the \( \Delta \text{yop} \) strain (Fig. 3A). In striking contrast, in eIF2\( \alpha \) (SS1A) MEFs, there was no increase in TNF\( \alpha \) transcript levels following infection (Fig. 3A), indicating that eIF2 signaling regulates the infection-induced expression of this cytokine.

We also tested whether pharmacological disruption of eIF2 signaling affects infection-specific TNF\( \alpha \) expression by treating cells with the small molecule salubrinal, which specifically inhibits the regulatory subunit PPP1r15ab/GADD34 of the cells with the small molecule salubrinal, which specifically inhibits the regulatory subunit PPP1r15ab/GADD34 of the translating ribosome-penetrating eIF2 system (28). Salubrinal treatment of culture macrophages resulted in a several-fold reduction in infection-induced expression of TNF\( \alpha \) (Fig. 3B). There were no detectable differences in cell viability between untreated and salubrinal-treated cells (data not shown). Salubrinal has similarly been shown to abrogate \( \beta \)-amyloid- and free fatty acid-induced eIF2\( \alpha \) signaling (29, 30), although it is not currently known how salubrinal-induced enhancement of phosphorylated eIF2\( \alpha \) levels negatively affects eIF2 signaling. However, considered together with the results derived from the eIF2\( \alpha \) (SS1A)-expressing cells, these data indicate that perturbing eIF2\( \alpha \)-mediated signaling negatively affects infection-induced cytokine expression.

A number of experiments were performed to corroborate the unexpected linkage of infection-induced eIF2 signaling and cytokine expression. Because inductive cytokine expression is regulated by NF-kB, we determined whether NF-kB activation is sensitive to genetic or pharmacological disruption of eIF2 signaling. Wild-type and eIF2\( \alpha \) (SS1A) MEFs were transfected with a NF-kB-Luc reporter plasmid and then treated with 0, 1, and 10 ng/ml rTNF\( \alpha \) for 5 h, at which time, reporter gene expression was determined. B, cells were cotransfected with a NF-kB-Luc reporter plasmid and treated 6 h later with either vehicle or the indicated doses of salubrinal (Sal) for 18 h. Cells were then treated or not with rTNF\( \alpha \). Cells were harvested 3 h later, and reporter gene expression was determined and normalized to the untreated sample. Inset, HEK 293T cells were treated with salubrinal at the indicated concentrations for 18 h and then pulsed with rTNF\( \alpha \) for 10 min. Cell lysates were prepared, and IkB protein levels determined by Western analysis. All results shown are representative of at least three independent experiments. *, \( p < 0.05 \) between the indicated groups; **, \( p > 0.05 \) between the indicated groups.

NF-kB-regulated reporter gene and was not observed in the transfection control gene (Fig. 4B). Salubrinal treatment did not notably affect the basal expression levels of the NF-kB reporter gene. Additionally, there was a reduction in the rTNF\( \alpha \)-induced degradation of endogenous IkB in salubrinal-treated cells (Fig. 4B, inset). These data are consistent with the reports cited above that similarly showed that salubrinal negatively affects \( \beta \)-amyloid- and free fatty acid-induced activation of NF-kB (29, 30). Although the mechanistic basis of how eIF2 signaling is linked to NF-kB is currently not known, the data shown here raise the possibility that the inhibitory effect of Yop on the NF-kB activation and subsequent cytokine expression that occur during a \( \text{Yersinia} \) infection (22) is regulated by eIF2.

eIF2 Signaling Opposes \( \text{Yersinia} \) Invasion—We noted that, upon prolonged infection with \( \text{Yersinia} \) (~4 h), the eIF2\( \alpha \) (SS1A) MEFs rounded up and detached from the substra-
levels of YopE between wild-type and eIF2
MEFs was due to higher levels of intracellular YopE. Using this
C
normalized signals of three separate wells relative to the uninfected controls. *, at least three independent experiments. * and **,
inclusion-forming units (shown in
extracellular bacteria. The number of internalized bacteria was then determined 30 min later and is shown relative to the internalization levels of the wild-type
MEFs. Each data point represents the average of three independent wells and is representative of three separate experiments (p < 0.05 between the two
indicated groups). D, wild-type and eIF2α(S51A) MEFs were seeded on coverslips and infected with
C. trachomatis
L2 at m.o.i. = 1 for either 1.5 h (4 °C) or 1 h (37 °C). Excess bacteria were then removed, and after 24 h of infection at 37 °C, cells were fixed and stained for
C. trachomatis, and the total number of inclusion-forming units (shown in D) per coverslip was determined by direct microscopic counting and is plotted in E. All results shown are representative of at least three independent experiments. * and **, p < 0.05 between co-labeled groups.

**FIGURE 6. eIF2 signaling is activated and opposes invasion by intracellular bacterial pathogens.** A, RAW 264.7 cells were left untreated (Mock), exposed to thapsigargin (Tg) or sodium arsenite (As), or infected with
L. monocytogenes
at m.o.i. = 50. The resulting whole cell lysates were examined by Western analysis using antibodies specific for eIF2α phosphorylated at Ser-51 (P-eIF2α) and actin. B, RAW 264.7 cells were infected with
L. monocytogenes
at m.o.i. = 50, and at the indicated times, cells were collected, and Atf3 and TNFα transcript levels were determined by qPCRs. Shown are the average GAPDH-normalized signals of three separate wells relative to the uninfected controls. *, p < 0.05 between the indicated groups and transcript levels in uninfected cells. C, wild-type and eIF2α(S51A) MEFs were infected with
L. monocytogenes, and after a 30-min attachment period, gentamycin was added to the wells to kill extracellular bacteria. The number of internalized bacteria was then determined 30 min later and is shown relative to the internalization levels of the wild-type
MEFs. Each data point represents the average of three independent wells and is representative of three separate experiments (p < 0.05 between the two
indicated groups). D, wild-type and eIF2α(S51A) MEFs were seeded on coverslips and infected with
C. trachomatis
L2 at m.o.i. = 1 for either 1.5 h (4 °C) or 1 h (37 °C). Excess bacteria were then removed, and after 24 h of infection at 37 °C, cells were fixed and stained for
C. trachomatis, and the total number of inclusion-forming units (shown in D) per coverslip was determined by direct microscopic counting and is plotted in E. All results shown are representative of at least three independent experiments. * and **, p < 0.05 between co-labeled groups.

tum compared with the wild-type MEFs, which appeared relatively much less affected under similar infection conditions. 

*Yersinia*-directed cytotoxicity is primarily a result of the translocation of YopE into the host cell cytoplasm (31). A translocation assay (16) was employed to determine whether the enhanced cytotoxicity observed in the infected eIF2α(S51A) MEFs was due to higher levels of intracellular YopE. Using this assay, there was no detectable differences in the intracellular levels of YopE between wild-type and eIF2α(S51A) MEFs (Fig. 5A). To determine whether the enhanced toxicity of the eIF2α(S51A) MEFs to

*Yersinia* infection could be due to differences in bacterial invasion, the number of internalized bacteria was compared in wild-type and eIF2α(S51A) MEFs after a brief infection period. Using a standard bacterial invasion assay (32), the number of internalized bacteria recovered from eIF2α(S51A) MEFs was much greater (>25-fold) than the number of internalized bacteria recovered from wild-type MEFs (Fig. 5B). Because we could detect no differences in bacterial adhesion between wild-type and eIF2α(S51A) MEFs (data not shown), these data suggest that the internalization of

*Yersinia* is regulated by eIF2 signaling.

**eIF2 Signaling Opposes Invasion by Professional Intracellular Pathogens.—**The bacterial pathogens
L. monocytogenes
and
C. trachomatis
actively invade and proliferate within eukaryotic cells. With similar kinetics as in *Yersinia*-infected cells, infection of murine macrophage-like cells with
L. monocytogenes
induced phosphorylation of eIF2α (Fig. 6A) and expression of Atf3 (Fig. 6B). These findings are consistent with those recently reported by Pillich et al. (33), who showed that
L. monocytogenes
infection is associated with enhanced levels of phosphorylated eIF2α. *L. monocytogenes* also induced phosphorylation of eIF2α in wild-type MEFs with similar kinetics as in macrophages (data not shown). In cell invasion assays, the number of intracellular
L. monocytogenes
cells recovered from eIF2α(S51A) MEFs was severalfold greater than that recovered from wild-type MEFs (Fig. 6C). There was a similar level of enhanced invasion of eIF2α(S51A) MEFs by
C. trachomatis
(Fig. 6, D and E). Interestingly, although the number of
C. trachomatis-containing inclusion-forming units was significantly greater in eIF2α(S51A) MEFs compared with wild-type MEFs (4.9-fold at 4 °C and 7.5-fold at 37 °C), the inclusions themselves were visibly comparable (i.e. contained similar numbers of bacterial cells) between the two cell types (Fig. 6D). Additionally, the progeny recovered for both MEF lines were equally infectious as determined by standard titering on HeLa cells (data not shown), indicating that a functioning eIF2 signaling pathway is not required for the intracellular development of
C. trachomatis. Collectively, these data show that there is a host cell mechanism involving eIF2 signaling that plays a role in opposing invasion by bacterial pathogens.

**DISCUSSION**

Recently, we discovered that, in yeast cells, eIF2 signaling is specifically activated and regulated by the bacterial virulence factors YpkA and YopJ (12), suggesting that this pathway may participate in antibacterial responses. Here, we have shown that eIF2 signaling in mammalian cells is activated following bacterial infection and that this activation is negatively regulated by YopJ (Fig. 7). An unexpected finding supported by five independent experiments (Fig. 3, A and B, and Fig. 4, A, B, and inset) is that eIF2 signaling is required for the infection-specific activation of NF-κB and proinflammatory gene expression. These finding suggest that cytokines should be classified as "stress
eIF2 and Bacterial Pathogens

Invasion (-) eIF2α (+) cytokines
YopJ (+YpkA?)

**FIGURE 7. eIF2α functions during bacterial infection.** Bacterial infection activates eIF2 signaling required for cytokine expression as well as opposing bacterial invasion. In cells infected with *Yersinia*, the virulence factor YopJ is translocated into the cytosol, where it inhibits eIF2-mediated functions. YopJ may collaborate with the coexpressed YpkA virulence factor (12).

recovery" genes, analogous to other genes that are expressed in an eIF2α-dependent manner in response to oxidative, osmotic, thermal, and endoplasmic reticulum stress.

The linking of cytokine expression to eIF2 signaling provides a clear rationale for why this signaling pathway would be targeted by a bacterially encoded virulence factor. It is currently thought that YopJ blocks NF-κB by directly modifying signaling proteins (*e.g.* IκB) that prevent its activation (34, 35). These latter studies were based on transfection expression systems that so far have not been extended to infection models. Because we have shown that YopJ inhibits eIF2 signaling and that infection-induced eIF2 signaling is required for NF-κB activation and proinflammatory gene expression, we propose that YopJ acts through eIF2 to repress proinflammatory signaling pathways.

Activation of eIF2 signaling leads to the expression of stress recovery genes, which, as far as we are aware, have not been linked to morphology-based activities. By comparing cells possessing intact *versus* defective eIF2 signaling, we made the unanticipated discovery that eIF2 signaling is linked to bacterial internalization. This property was observed in what is considered a facultative intracellular pathogen, *Yersinia* (Fig. 5), as well as two pathogens, *Listeria* and *Chlamydia* (Fig. 6), which actively invade cells as part of their infection cycle. To our knowledge, this is the first demonstration of a direct linkage between eIF2 signaling and cellular events at the morphological level. In the case of the Gram-negative *Yersinia*, the YpkA-mediated activation of eIF2 signaling (which is tempered by YopJ) may contribute to the well documented antiphagocytic activity of *Yersinia* (36, 37). With regard to the professional intracellular pathogens *Listeria* (Gram-positive) and *Chlamydia* (phylogenetically distinct, Gram-negative), invasion is clearly to the detriment of the host cell. The fact that there was a consistent pattern between these three very diverse pathogens indicates that eIF2 signaling is part of a general antibacterial defense system.

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