Cold Shock as a Screen for Genes Involved in Cold Acclimatization in Neurospora crassa

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ABSTRACT When subjected to rapid drops of temperature (cold shock), Neurospora responds with a temporary shift in its morphology. This report is the first to examine this response genetically. We report here the results of a screen of selected mutants from the Neurospora knockout library for alterations in their morphological response to cold shock. Three groups of knockouts were selected to be subject to this screen: genes previously suspected to be involved in hyphal development as well as knockouts resulting in morphological changes; transcription factors; and genes homologous to E. coli genes known to alter their expression in response to cold shock. A total of 344 knockout strains were subjected to cold shock. Of those, 118 strains were identified with altered responses. We report here the cold shock morphologies and GO categorizations of strains subjected to this screen. Of strains with knockouts in genes associated with hyphal growth or morphology, 33 of 131 tested (25%) showed an altered response to cold shock. Of strains with knockouts in transcription factor genes, 30 of 145 (20%) showed an altered response to cold shock. Of strains with knockouts in genes homologous to E. coli genes which display altered levels of transcription in response to cold shock, a total of 55 of 68 tested (81%) showed an altered cold shock response. This suggests that the response to cold shock in these two organisms is largely shared in common.

The environmental conditions that life must contend with can vary widely. Organisms have evolved a wide range of mechanisms for contending with these changing conditions. For the filamentous fungus Neurospora, growth continues through nearly the entire range of temperatures (above freezing) that is observed in this environment. Although the rate of tip extension varies linearly with temperature (Watters et al. 2000), the branch density (the statistical distribution of distances between branch sites along a linear growing hypha) remains constant across this range (Watters et al. 2000) allowing the fungus to continue to infiltrate its environment at the same density. Temperatures progressing through this range would be expected to have dramatic impacts on enzyme activity generally (and thus overall metabolism), but also directly on features critical to growth such as membrane fluidity, DNA/RNA stability and the rates of transcription and translation.

In both Neurospora and E. coli, there is a multistage response to cold shock. There is an initial response which is transient in nature, followed by a more long-term response which largely represents a return to normal growth. Neurospora grows via extension at a hyphal tip with periodic branching which is typically lateral (Figure 1A). However, when Neurospora is subjected to cold shock, a multi-phase morphological response is observed (Figure 1B, Watters et al. 2000, Watters 2013). The initial response to cold shock is the growth of a single longer than normal unbranched segment. This was termed the “Lag” phase of the response. This phase is followed by a series of closely spaced apical branch points, termed the “Apical” phase. Apical branch formation has been previously associated with the disruption and attempted reorganization of the normal tip-growth apparatus (Reynaga-Peña et al. 1995, Riquelme & Bartnicki-Garcia 2004), a mechanism distinct from that thought to be involved in lateral branching. Finally, with continued incubation at the lower temperature, the colony returns to lateral branching, termed the “Recovery” phase. Growth in this phase of the response resembles that which would be seen had the colony been grown at 4°C (or any other fixed temperature) continuously (Watters et al. 2000). Thus, the cold shock response appears to be a temporary...
disturbance to a homeostatic system which maintains branch density at a constant, evolutionarily favored, value. The morphological effects of cold shock are the indirect consequence of this stage’s process of adjusting cellular conditions in order to compensate for the new growth temperature.

Homeostasis in the face of temperature changes and more specifically the response to cold shock has been extensively studied in bacterial systems for over 20 years. The effect of cold shock is manifest in multiple cellular systems including: membrane rigidity (Shivaji & Prakash 2010), stability of secondary structures in DNA/RNA (Phadtare 2004), efficiency of protein folding (Phadtare 2004) and ribosome function (Gualerzi et al. 2011). While much remains to be described in these systems, cold shock appears to result in a multi-stage response (Phadtare 2004). First, a lag period in which growth and translation of proteins generally cease. This is followed by an adjustment phase in which specific cold-shock proteins which compensate for the changes brought on by the cold are preferentially translated (Giuliodori et al. 2004). In the final stage, growth continues otherwise normally, but at a reduced rate. DNA microarray transcription profiling of the cold shock response in *E. coli* by Phadtare and Inouye (2004) has shown that several hundred genes respond to cold shock, either being transiently induced/repressed or showing prolonged induction/repression. Analogous responses to cold shock and/or cold acclimation have been observed in diverse organisms including plants (Guy 1999) and animals (Canclini & Esteves 2007). Attempts to uncover cold shock proteins in fungi (Fang & St Leger 2010) have met with mixed success.

It is tempting to draw parallels between what is known about cold shock in bacterial systems and the observed response of *Neurospora* to similar cold shocks. Many of the systems affected during bacterial cold shock would be expected to impact fungal tip growth and branching (e.g., membrane fluidity). In addition, the nature and timing of the two responses are similar. Both can be adjusted by changing the intensity of the cold shock with more mild shocks (lower temperature differences) producing more mild responses and more severe shocks (larger temperature differences) producing more severe responses. Furthermore, the dynamics of the responses parallel each other. In each, there is a multistage response. There is an initial response which is transient in nature, followed by a more long-term response which largely represents a return to normal growth.

The hypothesis of this project was that the observed cold shock response of *Neurospora* is a consequence of a cellular response homologous to that induced by cold shock in bacteria. Under this hypothesis, the observed, transient morphological changes are a consequence of the fungal cell adjusting itself to growth in the cold via a manner which is shared in common with simpler organisms. This hypothesis was tested by screening *Neurospora* knockout strains impacting genes homologous to those identified in *E. coli* which alter their expression patterns in response to cold shock. In addition, a broader collection of selected knockout strains were screened to identify additional genes which play a role in the cold shock response and thus cold acclimatization. Together, the results of this screen provides the first molecular underpinning to the cold shock response in *Neurospora*.

**MATERIALS AND METHODS**

**The Neurospora targeted deletion collection**

As part of the *Neurospora* Genome Project, a collection of strains containing disruptions in presumptive genes was constructed (Colot et al. 2006). Strains representing deletions of most of the genes of the *Neurospora* genome are available from the Fungal Genetics Stock Center (McCluskey 2003) which supplied the knockout strains for this study. The FGSC supplied the knockout strains at a reduced fee in order to support undergraduate research. As each deletion strain has been altered in a single, previously identified, presumptive gene – going from phenotype to sequence is greatly simplified.

The accession numbers listed in Tables 1 and 2 represent the locus number of the gene subject to inactivation in the knockout strain under test. Every annotated gene in *Neurospora crassa* has been assigned a locus number of the form NCU####. The gene identities reported in the tables are those associated with the genes as annotated on the FungDiB database as of July 2017: fungidb.org/fungidb/. The gene identities reported are based solely on the annotations currently associated with those strains and have not been independently confirmed by the authors of this study. Gene Ontologies reported are those determined by pantherdb.org (Mi et al. 2017) as of December 2017.

**Knockout sets selected to be subjected to screen**

A screen of the entire library was determined to be impractical. We instead screened an abbreviated subsection of the library chosen to be more likely to yield positive responses. These fall into three basic sets.

The first set are knockouts of genes homologous to those which show altered transcription in *E. coli* when subjected to cold shock (Phadtare
Table 1 Of 344 knockouts screened 118 were observed to alter the phenotype of the cold shock response. For each knockout strain tested ("ID/NCU####") we report the Cold Shock phenotype, the annotated gene function and gene abbreviation, the set of mutants the knockout came from (E. coli cold shock mutant ortholog, the Morphological or Hyphal growth plates from the FGSC, or the Transcription Factor plates from the FGSC) and the Gene Ontology categorizations for both Molecular Function and Biological Process. FGSC# is the strain number at the Fungal Genetics Stock Center.

| ID     | FGSC# | CS Phenotype | Gene Function | Gene   | Knockout set | GO: Molecular Function | GO: Biological Process |
|--------|-------|--------------|---------------|--------|--------------|------------------------|------------------------|
| NCU03938 | 11228 | burst        | alternative oxidase-5 | aod-5  | Morph/Hyph   |                         | Biological Regulation   |
| NCU03070 | 11109 | burst        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU01782 | 11375 | burst        | Ras guanyl-nucleotide exchange factor RasGEF superoxide dismutase-1 | sod-1  | Morph/Hyph   | antioxidant/bind-    |                         |
| NCU02133 | 11215 | burst        | E. coli CS Orth   |         | Binding       | Catalytic Activity     |                         |
| NCU01213 | 11205 | burst        | superoxide dismutase-2 ubiquitin-conjugating enzyme E | sod-2  | E. coli CS Orth | Binding                |                         |
| NCU03623 | 11226 | burst        | Binding/Catalytic Activity |         | Biological Regulation | E. coli CS Orth |                         |
| NCU04242 | 11230 | burst/dense  | period-6         | prd-6  | Morph/Hyph   |                         |                         |
| NCU07728 | 11268 | burst/thin   | siderophore regulation | sre    | Morph/Hyph   |                         |                         |
| NCU03901 | 11305 | cot-like     | peroxin 14       | pex14  | Morph/Hyph   |                         |                         |
| NCU07617 | 11254 | dense        | aconidi-ate-3    | acon-3 | Morph/Hyph   |                         |                         |
| NCU05410 | 16183 | dense        | arginine-5       | arg-5  | E. coli CS Orth | Binding               |                         |
| NCU02114 | 11571 | dense        | G1/S-specific cyclin Cln1 |        | Morph/Hyph   |                         |                         |
| NCU00144 | 11340 | dense        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU03120 | 11036 | dense        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU03356 | 11128 | dense        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU03417 | 11083 | dense        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU03905 | 11131 | dense        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU03962 | 11112 | dense        | hypothetical protein |        | Transc Factors |                         |                         |
| NCU06990 | 11032 | dense        | hypothetical protein | sub-1  | Transc Factors |                         |                         |
| NCU01154 | 11127 | dense        | protopenthecia-1 |        | Transc Factors |                         |                         |
| NCU04899 | 11742 | dense        | tca-15           |        | E. coli Orth  | Catalytic Activity     | Metabolic Process       |
| NCU03415 | 11821 | fail         | aldehyde dehydrogenase |        | CBS-3       | E. coli Orth  | Catalytic Activity/ transporter Activity |
| NCU11289 | 23565 | fail         | aldo-keto reductase |        | E. coli Orth  |     |                         |
| NCU00097 | 11110 | fail         | BEAK-1           | bek-1  | Transc Factors |                         |                         |
| NCU02017 | 11108 | fail         | CBF/NFY family transcription factor | ada-2  | Transc Factors |                         |                         |
| NCU00056 | 21444 | fail         | condensing enzyme with mitochondrial function | cem-1  | E. coli Orth  |                         |                         |
| NCU00467 | 11284 | fail         | COP9 signalosome-5 | csn-5  | Morph/Hyph   | Binding                | Metabolic Process       |
| NCU06068 | 11063 | fail         | fungal specific transcription factor | col-25 | Transc Factors |                         |                         |
| NCU07788 | 11031 | fail         | fungal specific transcription factor | col-26 | Transc Factors |                         |                         |
| NCU07945 | 11056 | fail         | fungal specific transcription factor | tah-4  | Transc Factors |                         |                         |
| NCU07947 | 13023 | fail         | glycolipid transfer protein HET-C2 |        | Morph/Hyph   |                         | Localization/Metabolic Process |
| NCU05927 | 20010 | fail         | GTP-binding protein GUF1 |        | E. coli Orth  |                         |                         |
| NCU00528 | 12080 | fail         | hyphal anastomosis-4 | ham-4  | E. coli Orth  |                         |                         |
| NCU07561 | 11114 | fail         | hypothetical protein |        | Transc Factors |                         |                         |

(continued)
| ID     | FGSC#  | CS Phenotype | Gene Function | Gene | Knockout set | GO: Molecular Function | GO: Biological Process |
|--------|--------|--------------|---------------|------|--------------|------------------------|------------------------|
| NCU09120 | 11964  | fail         | lysine-specific histone demethylase Aof2 | E. coli CS Orth | Binding | Cellular Process/Metabolic Process |
| NCU09830 | 11263  | fail         | menadione-induced gene-12 | mig-12 | Morph/Hyph | Catalytic Activity | Cellular Process/Metabolic Process |
| NCU09842 | 11321  | fail         | mitogen activated protein kinase-1 | mak-1 | Morph/Hyph | Catalytic Activity/signal transducer activity | Biological Regulation/Cellular Process/Response to Stimulus/Metabolic Process |
| NCU03314 | 11296  | fail         | mob-2-like-a | mob-2a | Morph/Hyph | Binding/Catalytic Activity | Cellular Process |
| NCU09975 | 14572  | fail         | multidrug resistance protein 3 | E. coli CS Orth | | | |
| NCU08294 | 11007  | fail         | nitrogen assimilation transcription factor nit-4 | nit-4 | Transc Factors | | |
| NCU03277 | 11333  | fail         | peroxin 10 | pex10 | Morph/Hyph | | |
| NCU02636 | 11221  | fail         | peroxin 4 | pex4 | Morph/Hyph | | |
| NCU01004 | 22657  | fail         | phosphate-asparagine decarboxylase proenzyme CHOL-15 | E. coli CS Orth | Catalytic Activity | Cellular Process/Metabolic Process |
| NCU07832 | 20832  | fail         | pre-mRNA processing splicing factor 8 | msp-39 | E. coli CS Orth | Catalytic Activity | Cellular Component Organization or Biogenesis/Cellular Process/Localization |
| NCU06028 | 11034  | fail         | quinic acid utilization activator | qa-1F | Transc Factors | | |
| NCU06205 | 11372  | fail         | regulator of conidiation-1 | rco-1 | Morph/Hyph | | |
| NCU06145 | 12557  | fail         | RING-6 | RING-6 | Morph/Hyph | | |
| NCU02214 | 11068  | fail         | TAH-2 | tah-2 | Transc Factors | | |
| NCU10008 | 22177  | fail         | tricarboxylic acid-14 | tca-14 | E. coli CS Orth | | |
| NCU02356 | 11712  | fail         | white collar 1 | wc-1 | Transc Factors | | |
| NCU02173 | 11440  | fail         | zinc finger transcription factor-52 | znf-52 | Transc Factors | | |
| NCU05591 | 11239  | thin         | ABC transporter CDR4 | Morph/Hyph | Catalytic Activity / transporter Activity | Cellular Process/Metabolic Process |
| NCU03013 | 11223  | thin         | anchored cell wall protein-10 | acw-10 | Morph/Hyph | antioxidant/binding/catalytic Activity | Cellular Process/Response to Stimulus |
| NCU02333 | 11217  | thin         | arginase-1 | aga-1 | Morph/Hyph | Binding/Catalytic Activity | Cellular Process/Metabolic Process |
| NCU03184 | 11357  | thin         | C2H2 conidiation transcription factor FlbC | Morph/Hyph | | | |
| NCU07075 | 11249  | thin         | calcium exchanger | cax | Morph/Hyph | transporter activity | Biological Regulation/Cellular Process |
| NCU05770 | 11532  | thin         | catalase-2 | cat-2 | E. coli CS Orth | antioxidant/binding | Cellular Process/Response to Stimulus/Metabolic Process |
| NCU05051 | 11097  | thin         | COL-23 | col-23 | Transc Factors | | |
| NCU08345 | 11286  | thin         | ctr copper transporter | tct-1 | Morph/Hyph | | |
| NCU08216 | 22525  | thin         | cystathionine beta-synthase | MET-11 | E. coli CS Orth | Binding | Cellular Process/Metabolic Process |
| NCU03076 | 11294  | thin         | delta-1-pyrroline-5-carboxylate dehydrogenase | Morph/Hyph | | | |
| NCU08968 | 22160  | thin         | dimethyladenosine transferase | E. coli CS Orth | | | |

(continued)
Table 1, continued

| ID       | FGSC# | Phenotype | Gene Function                          | Gene   | Knockout set | GO: Molecular Function                          | GO: Biological Process                          |
|----------|-------|-----------|----------------------------------------|--------|--------------|------------------------------------------------|------------------------------------------------|
| NCU01772 | 22283 | thin      | DNA-directed RNA polymerase III polypeptide | rpo-10 | E. coli CS Orth | Cellular Process/Metabolic Process               | Biological Regulation/Cellular Process/Metabolic Process |
| NCU02542 | 11220 | thin      | emblen-meyerhof pathway-1              | emp-1  | Morph/Hyph   | Catalytic Activity                               |                                                    |
| NCU01744 | 22231 | thin      | enhancer-2 of am                     | en(am)-2| E. coli CS Orth | Cellular Process/Metabolic Process               |                                                        |
| NCU04264 | 11232 | thin      | extracellular developmental signal biosynthesis protein FluG |        | Morph/Hyph   | Binding                                          |                                                        |
| NCU04140 | 11562 | thin      | FK506 resistant-2                     | fkr-2  | E. coli CS Orth | Binding/Catalytic Activity                       | Cellular Process/Metabolic Process               |
| NCU09930 | 21617 | thin      | folic acid synthesis protein          | fol-9  | E. coli CS Orth | transporter activity                             | Biological Regulation/Metabolic Process          |
| NCU05606 | 13744 | thin      | glucosidase 2 subunit beta            | GHX-4  | E. coli CS Orth | Binding/Catalytic Activity                       |                                                    |
| NCU01528 | 22515 | thin      | glyceraldehyde-3-phosphate dehydrogenase-1 | gpd-1  | E. coli CS Orth | Catalytic Activity                               |                                                    |
| NCU06005 | 13543 | thin      | glycerol kinase                       | GLK-1  | E. coli CS Orth |                                                |                                                    |
| NCU02630 | 11952 | thin      | heat shock protein 78                 | hsp78  | E. coli CS Orth |                                                |                                                    |
| NCU07156 | 20700 | thin      | histidine-6                          | his-6  | E. coli CS Orth |                                                |                                                    |
| NCU02556 | 11840 | thin      | histone acetyl transferase-2          | hat-2  | E. coli CS Orth |                                                |                                                    |
| NCU01629 | 11102 | thin      | hypothetical protein                  |        | Transc Factors |                                                |                                                    |
| NCU04669 | 11307 | thin      | hypothetical protein homologous to Bactericidal permeability-increasing protein |        | Morph/Hyph   |                                                |                                                    |
| NCU04561 | 11136 | thin      | melanization defective-1             | mid-1  | Transc Factors |                                                |                                                    |
| NCU09767 | 18564 | thin      | membrane transporter                  |        | E. coli CS Orth |                                                |                                                    |
| NCU04791 | 18772 | thin      | menadione-induced gene-1             | mig-10 | E. coli CS Orth |                                                |                                                    |
| NCU05151 | 13482 | thin      | phosphoketolase                       | PHK-1  | E. coli CS Orth |                                                |                                                    |
| NCU06342 | 20075 | thin      | phospholipase D                       | PLA-5  | E. coli CS Orth |                                                |                                                    |
| NCU05295 | 11593 | thin      | proteasome catalytic alpha-5          | pca-5  | Morph/Hyph   |                                                |                                                    |
| NCU09366 | 11603 | thin      | proteasome catalytic beta-6           | pcb-6  | Morph/Hyph   | Catalytic Activity                               |                                                    |
| NCU01613 | 11291 | thin      | protoperithecia-2                    | pp-2   | Morph/Hyph   | Catalytic Activity                               |                                                    |
| NCU02260 | 11586 | thin      | regulatory particle, ATPase-like-3    | rpt-3  | Morph/Hyph   |                                                |                                                    |
| NCU02055 | 13283 | thin/fail | uridine nucleosidase Urh1             |        | E. coli CS Orth |                                                    |                                                    |
| NCU07705 | 11029 | thin/fail | C6 finger domain-containing protein   | NUS-1  | Transc Factors |                                                    |                                                    |
| NCU08000 | 11005 | thin/fail | cutinase transcription factor 1 alpha | far1   | Transc Factors |                                                    |                                                    |
| NCU05536 | 11027 | thin/fail | hypothetical protein                  |        | Transc Factors | Binding/Catalytic Activity                       | Cellular Component Organization or Biogenesis/Metabolic Process |
| NCU08651 | 11012 | thin/fail | zinc binuclear cluster-type protein   | col-27 | Transc Factors |                                                    |                                                    |
Table 1, continued

| ID   | FGSC#   | Phenotype | CS | Gene Function | Gene | Knockout set | GO: Molecular Function | GO: Biological Process |
|------|---------|-----------|----|---------------|------|--------------|------------------------|-----------------------|
| NCU07732 | 14014   | thin/fail | arginine-2 | arg-2 | E. coli CS Orth | Binding/Catalytic Activity | Cellular Process/Metabolic Process |
| NCU04117 | 21178   | thin/fail | ATP-dependent permease | MDL2 | E. coli CS Orth |  |  |
| NCU06659 | 12287   | thin/fail | GTP-binding protein | GTP-3 | E. coli CS Orth |  |  |
| NCU08693 | 14197   | thin/fail | heat shock protein 70-5 | hsp70-5 | E. coli CS Orth | Binding |  |
| NCU10760 | 12539   | thin/fail | jumonji domain-containing protein 5 |  | E. coli CS Orth |  |  |
| NCU08858 | 14492   | thin/fail | MFS alpha-glucoside transporter | SUT-1 | E. coli CS Orth | transporter activity |  |
| NCU00793 | 15944   | thin/fail | trehalose phosphate synthase | GT20-2 | E. coli CS Orth | Catalytic Activity |  |
| NCU08336 | 22591   | thin/fail | tricarboxylic acid-12 | tca-12 | E. coli CS Orth | Catalytic Activity |  |
| NCU00771 | 19376   | thin/fail | UBX domain-containing protein 7 |  | E. coli CS Orth |  |  |
| NCU04583 | 12407   | weak | acetyltransferase | ada-1 | E. coli CS Orth | Catalytic Activity |  |
| NCU00499 | 11120   | weak | all development altered-1 | arg-6 | E. coli CS Orth | Binding |  |
| NCU03033 | 16296   | weak | asparagine synthetase 2 | asn-1 | E. coli CS Orth | Binding/Catalytic Activity |  |
| NCU00919 | 16502   | weak | ATP-dependent RNA helicase rok-1 | drh-16 | E. coli CS Orth |  |  |
| NCU08933 | 23868   | weak | cellular nucleic acid-binding protein |  | E. coli CS Orth |  |  |
| NCU01408 | 11275   | weak | COP9 signalosome-3 | csn-3 | Eucalyptus Orth | Morph/Hyph |  |
| NCU01625 | 15732   | weak | DNA repair helicase RAD3 | DNR-10 | E. coli CS Orth | Binding/Catalytic Activity | Cellular Process/Metabolic Process |
| NCU07027 | 20154   | weak | glycosylphosphorylation | GYP-1 | E. coli CS Orth | Binding/Catalytic Activity |  |
| NCU06523 | 23841   | weak | glycosylhydrolysis family 13-4 | gh13-4 | E. coli CS Orth | Catalytic Activity |  |
| NCU01589 | 13671   | weak | heat shock protein 60 | hsp60 | E. coli CS Orth | Binding |  |
| NCU05909 | 11104   | weak | hypothetical protein |  |  |  |  |
| NCU08439 | 15564   | weak | leptomycin B resistance protein pmr1 | ABC-2 | E. coli CS Orth | transporter activity |  |
| NCU00565 | 18702   | weak | lipoic acid synthetase | LIA-1 | E. coli CS Orth | Catalytic Activity |  |
| NCU04339 | 16454   | weak | ribokinase | RIK-8 | E. coli CS Orth | Catalytic Activity |  |
| NCU03894 | 11325   | weak | serine/threonine protein kinase-4 | stk-4 | E. coli CS Orth | Binding/signal transducer activity |  |
| NCU06017 | 13547   | weak | thiosulfate sulfurtransferase | TST-1 | E. coli CS Orth |  |  |
| NCU10053 | 21996   | weak | thymidylate synthase | pyr-8 | E. coli CS Orth |  |  |
| NCU08658 | 11059   | weak | zinc finger transcription factor-50 | znf-50 | E. coli CS Orth | Transcription Factors |  |
| ID      | Gene Function                        | Gene                     | Knockout set | GO: Molecular Function          | GO: Biological Process                  |
|---------|--------------------------------------|--------------------------|--------------|---------------------------------|-----------------------------------------|
| NCU00017| hypothetical protein                 | 11075                    | Transc Factors|                               |                                         |
| NCU00019| Fork head protein homolog 1          | 11437                    | FKH1         | Transc Factors                  |                                         |
| NCU00038| zinc finger transcription factor-32  | 11483                    | znf-32       | Transc Factors                  |                                         |
| NCU00081| DNA topoisomerase 3-beta             | 15983                    | dnt-3        | E. coli CS Orth                 |                                         |
| NCU00090| pH-response transcription factor pacC/RIM101 | 11397                | pacc-1       | Transc Factors                  |                                         |
| NCU00105| ribosome biogenesis-58               | 15796                    | rgb-58       | Morph/Hyph                      | Cellular Component Organization or Biogenesis |
| NCU00135| Phosphatidyl synthase, phosphatidyl synthase, variant 1 | 16021                | gpl-1        | Morph/Hyph                      | Cellular Process/Metabolic Process      |
| NCU00157| COP9 signalosome-1                   | 11282                    | csn-1        | Morph/Hyph                      |                                         |
| NCU00204| hypothetical protein                 | 12199                    | Morph/Hyph   |                                 |                                         |
| NCU00217| hypothetical protein                 | 11020                    | Morph/Hyph   |                                 |                                         |
| NCU00233| glycosyl hydrolase family 16-15     | 11117                    | gh16-15      | Transc Factors                  |                                         |
| NCU00285| hypothetical protein                 | 11118                    | Transc Factors|                               |                                         |
| NCU00289| tall aerial hyphae-1                | 11085                    | tah-1        | Transc Factors                  |                                         |
| NCU00329| vegetative asexual development-1     | 11119                    | vad-1        | Transc Factors                  |                                         |
| NCU00355| catalase-3                           | 1202                    | cat-3        | Morph/Hyph                      | Antioxidant Activity/Binding/Catalytic Activity |
| NCU00396| pre-mRNA-splicing factor re-1       | 11612                    | msp-5        | Morph/Hyph                      | Response to Stimulus/Cellular Process/Metabolic Process |
| NCU00406| velvet                               | 11323                    | vel          | Morph/Hyph                      | Biological Regulation/Developmental Process/Response to Stimulus/Cellular Process |
| NCU00554| Aspartate-semialdehyde dehydrogenase | 16113                    | hom-1        | Morph/Hyph                      |                                         |
| NCU00609| initiation-specific alpha-1,6-    | 16119                    | och-1        | Morph/Hyph                      | Cellular Process/Metabolic Process      |
| NCU00631| chromatin remodelling factor 9-1    | 11738                    | crf9-1       | Transc Factors                  |                                         |
| NCU00634| Ribosomal protein L14               | 16123                    | crp-47       | Morph/Hyph                      | Structural Molecule Activity            |
| NCU00694| hypothetical protein                 | 11103                    | Transc Factors|                               |                                         |
| NCU00749| conidiation at high carbon dioxide-1| 11438                    | chc-1        | Transc Factors                  |                                         |
| NCU00768| mRNA binding post-transcriptional regulator | 15724                | Morph/Hyph   |                                 |                                         |
| NCU00808| zinc finger transcription factor-48  | 11122                    | znf-48       | Transc Factors                  | Cellular Process/Metabolic Process      |
| NCU00810| Beta-galactosidase                   | 11285                    | gh2-3        | Morph/Hyph                      | Biological Regulation/Cel-               |
| NCU00824| histone deacetylase-3               | 11614                    | hda-3        | Morph/Hyph                      | lular Component Organization or Biogenesis/Cellular Process |
| NCU00902| zinc finger white collar protein WC2 | 11124                    | wc-2         | Transc Factors                  |                                         |
| NCU00923| topogenesis of outer membrane beta barrel protein 37 | 11273                | tob37        | Morph/Hyph                      |                                         |
| NCU00945| fungal specific transcription factor | 11064                    | col-20       | Transc Factors                  |                                         |
| NCU00959| succinate dehydrogenase iron-sulfur protein | 16505                | tca-10       | E. coli CS Orth                 | Cellular Process/Metabolic Process      |
| NCU01020| hypothetical protein                 | 13009                    | Morph/Hyph   |                                 | Binding                                 |
| NCU01033| hypothetical protein related to regulatory protein wetA | 11204                | Morph/Hyph   |                                 |                                         |
| NCU01037| hypothetical protein                 | 13038                    | Transc Factors|                               |                                         |
| NCU01097| hypothetical protein                 | 11038                    | Transc Factors|                               |                                         |
| NCU01122| hypothetical protein                 | 11125                    | Transc Factors|                               |                                         |
| ID       | Gene                | Function                                                            | Knockout set | GO: Molecular Function                                      | GO: Biological Process                                      |
|----------|---------------------|----------------------------------------------------------------------|--------------|-------------------------------------------------------------|-------------------------------------------------------------|
| NCU01181 | 11287 acyl-CoA dehydrogenase family member 11             | acd-3                                                   | Morph/Hyph   | Antioxidant Activity/Binding/Catalytic Activity             | Developmental Process                                        |
| NCU01197 | 11288 cell wall biogenesis protein phosphatase Ssd1       | gul-1                                                   | Morph/Hyph   |                                                             |                                                             |
| NCU01213 | 11206 superoxide dismutase-2                            | sod-2                                                   | Morph/Hyph   |                                                             |                                                             |
| NCU01225 | 11207 ubiquitin conjugating enzyme - 13                   | uce-1                                                   | Morph/Hyph   |                                                             |                                                             |
| NCU01368 | 11582 proteasome component 11C                          | pcb-4                                                   | Morph/Hyph   | Catalytic Activity                                          | Cellular Process/Metabolic Process                          |
| NCU01478 | 11002 fungal specific transcription factor domain-containing protein | Transc Factors                                             |              |                                                             |                                                             |
| NCU01642 | 11211 hypothetical protein homologous to Neurofibromin  |                                                        | Morph/Hyph   |                                                             |                                                             |
| NCU01833 | 11213 Two-component histidine kinase CHK-1                | nik-2                                                   | Morph/Hyph   |                                                             |                                                             |
| NCU02057 | 11342 transcription factor-1                             | tcf-1                                                   | Transc Factors|                                                             |                                                             |
| NCU02094 | 11060 vegetative asexual development-2                    | vad-2                                                   | Transc Factors| Binding                                                    | Cellular Process/Metabolic Process                          |
| NCU02111 | 11611 myosin-5                                            | myo-5                                                   | Morph/Hyph   | Binding/Structural Molecule Activity/Catalytic Activity     | Cellular Component Organization or Biogenesis/Localization Process/Cellular Process |
| NCU02142 | 11071 hypothetical protein                               | rac-1                                                   | Transc Factors| Binding/Signal Transducer Activity/Catalytic Activity       | Biological Regulation/Cellular Component Organization or Biogenesis/Developmental Process/Response to Stimulus/Cellular Process/Metabolic Process |
| NCU02160 | 11525 small GTPase RAC                                    |                                                        | Morph/Hyph   |                                                             |                                                             |
| NCU02226 | 16056 methylthioribose-1-phosphate isomerase             | met-23                                                  | Morph/Hyph   | Catalytic Activity                                          | Cellar Process/Metabolic Process                            |
| NCU02250 | 16168 ATP synthase subunit ATP9                          | oli                                                      | Morph/Hyph   | Transporter Activity/Catalytic Activity                     | Cellar Process/Metabolic Process                            |
| NCU02265 | 11554 period clock protein FRQ                           | frq                                                      | Morph/Hyph   |                                                             |                                                             |
| NCU02307 | 11054 hypothetical protein                               |                                                        | Transc Factors|                                                             |                                                             |
| NCU02387 | 11219 nuclear import and export protein MsnS             |                                                        | Morph/Hyph   | Binding/Transporter Activity                                |                                                             |
| NCU02406 | 16076 nuclear protein                                     |                                                        | Morph/Hyph   | Binding                                                     |                                                             |
| NCU02498 | 11289 Culin-3                                            | cul-3                                                   | Morph/Hyph   | Binding                                                     |                                                             |
| NCU02576 | 11072 zinc finger transcription factor-39                 | znf-39                                                  | Transc Factors|                                                             |                                                             |
| NCU02604 | 11659 U3 small nucleolar RNA-associated protein 10        | rbg-7                                                   | Morph/Hyph   | Binding                                                     |                                                             |
| NCU02639 | 16474 Argininosuccinate synthase                          | arg-1                                                   | E. coli CS Orth | Catalytic Activity                                        | Cellar Process/Metabolic Process                            |
| NCU02666 | 11344 zinc finger transcription factor-58                  | znf-58                                                  | Transc Factors|                                                             |                                                             |
| NCU02667 | 11345 cutinase G-box binding protein                      | msn-1                                                   | Transc Factors|                                                             |                                                             |
| NCU02699 | 11347 zinc finger transcription factor-14                  | znf-14                                                  | Transc Factors|                                                             |                                                             |
| NCU02712 | 15714 acetate-10                                          | ace-1                                                   | E. coli CS Orth |                                                             |                                                             |

(continued)
| ID        | Gene Function                  | Gene    | Knockout set | GO: Molecular Function | GO: Biological Process          |
|-----------|--------------------------------|---------|--------------|------------------------|--------------------------------|
| NCU02713  | conidial separation-1          | csp-1   | Transc Factors | Binding                | Cellular Process/Metabolic Process |
| NCU02724  | transcription factor-21        | tcf-21  | Transc Factors |                        |                                |
| NCU02752  | zinc finger transcription factor-47 | znf-47  | Transc Factors |                        |                                |
| NCU02768  | transcription factor-20        | tcf-20  | Transc Factors |                        |                                |
| NCU02794  | Fso1                           | so      | Morph/Hyph   |                        |                                |
| NCU02826  | sodium/calcium exchanger protein | tmr-16  | Morph/Hyph   | Transporter Activity   |                                |
| NCU02896  | all development altered-3      | ada-3   | Transc Factors |                        |                                |
| NCU02934  | hypothetical protein           | ncf-4   | Transc Factors |                        |                                |
| NCU02948  | non-anchored cell wall protein-4 | ncf-4   | E. coli CS Orth | Catalytic Activity     |                                |
| NCU02957  | hypothetical protein           | tcf-26  | Transc Factors |                        |                                |
| NCU02994  | hypothetical protein           | tcf-26  | Transc Factors |                        |                                |
| NCU03033  | transcription factor-26        | tcf-26  | Transc Factors | Binding                | Biological Regulation/Response to Stimulus |
| NCU03043  | C2H2 finger domain-containing protein FlbC | acon-4  | Transc Factors |                        |                                |
| NCU03073  | DNA polymerase epsilon, subunit D | pole-4  | Transc Factors |                        |                                |
| NCU03077  | hypothetical protein           | pole-4  | Transc Factors |                        |                                |
| NCU03096  | bromodomain associated domain-containing protein | pole-4  | Transc Factors |                        |                                |
| NCU03110  | hypothetical protein           | chromatin-like domain-containing protein | Transc Factors |                        |                                |
| NCU03125  | NIMA-interacting protein TinC | paa-5   | Morph/Hyph   | Binding/Catalytic Activity | Metabolic Process |
| NCU03164  | two-component system response regulator | col-21  | Transc Factors |                        |                                |
| NCU03206  | zinc finger transcription factor-22 | znf-22  | Transc Factors |                        |                                |
| NCU03320  | endoribonuclease ysh-1         | paa-5   | Morph/Hyph   | Binding/Catalytic Activity | Metabolic Process |
| NCU03479  | oxidase assembly protein 2     | tah-3   | Transc Factors |                        |                                |
| NCU03489  | transport of copper-2          | tcf-20  | Transc Factors |                        |                                |
| NCU03576  | conidiphore development protein hymA | hym-1   | Morph/Hyph   | Binding                |                                |
| NCU03593  | homeobox domain-containing protein | kal-1   | Transc Factors |                        |                                |
| NCU03643  | fatty acid regulation-2        | far-2   | Transc Factors |                        |                                |
| NCU03669  | AdoMet-dependent RNA methyltransferase sbp1 | rmt-3   | E. coli CS Orth | Catalytic Activity     | Cellular Component Organization or Biogenesis/Cellular Process/Metabolic Process |
| NCU03686  | oxidase assembly protein 2     | tah-3   | Transc Factors |                        |                                |
| NCU03699  | zinc finger transcription factor-13 | znf-13  | Transc Factors |                        |                                |
| NCU03702  | rRNA 2'-O-methyltransferase fibrillarin | rbg-16  | Morph/Hyph   |                        |                                |
| NCU03725  | vegetative incompatibility blocked-1 | vib-1   | Morph/Hyph   | Binding                | Biological Regulation/Cellular Process/Metabolic Process |
| NCU03931  | all development altered-5      | ada-5   | Transc Factors |                        |                                |
| NCU04001  | female fertility-7             | ff-7    | Transc Factors |                        |                                |
| NCU04096  | serine/threonine-protein kinase 3 | prk-9    | Morph/Hyph   | Binding/Signal Transducer Activity/Catalytic Activity | Biological Regulation/Developmental Process/Multicellular Organismal Process/Response to Stimulus/Cellular Process |
| NCU04142  | heat shock protein 80          | hsp80   | E. coli CS Orth |                        |                                |
| NCU04179  | C2H2 transcription factor       | sa-1    | Transc Factors |                        |                                |
| NCU04211  | hypothetical protein           | sa-1    | Transc Factors |                        |                                |
| NCU04302  | ubiquitin-conjugating enzyme E | nup-22  | Morph/Hyph   |                        |                                |
| NCU04359  | hypothetical protein           | nup-22  | Transc Factors |                        |                                |
| ID       | Gene Function                          | Gene    | Knockout set | GO: Molecular Function                | GO: Biological Process            |
|----------|----------------------------------------|---------|--------------|--------------------------------------|-----------------------------------|
| NCU04390 | fungal specific transcription factor    | 11134   | col-22       | Transc Factors                       |                                   |
| NCU04513 | ubiquitin conjugating enzyme Ubc14     | 11234   | uce-14       | Morph/Hyph                           | Catalytic Activity                | Metabolic Process                |
| NCU04533 | DUF1881 domain-containing protein      | 11298   | app          | Morph/Hyph                           |                                   |
| NCU04619 | hypothetical protein                   | 11137   | Transc Factors|                                      |                                   |
| NCU04628 | hypothetical protein                   | 11138   | Transc Factors|                                      |                                   |
| NCU04731 | Sterol regulatory element binding protein sah-2 | 11139 | sah-2        | Transc Factors                       |                                   |
| NCU04733 | UvrD/REP helicase                      | 11737   | mus-50       | E. coli CS Orth                      |                                   |
| NCU04834 | sensor histidine kinase/ response regulator | 11236 | phy-1        | Morph/Hyph                           |                                   |
| NCU04851 | hypothetical protein                   | 11089   | Transc Factors|                                      |                                   |
| NCU04866 | all development altered-6              | 11022   | ada-6        | Transc Factors                       | Transporter Activity/Catalytic Activity | Cellular Process/Metabolic Process |
| NCU05046 | calcium-transporting ATPase 3          | 11237   | ena-1        | Morph/Hyph                           |                                   |
| NCU05210 | postreplication repair E3 ubiquitin-protein ligase rad-18 | 11444 | uvs-2        | Transc Factors                       | Catalytic Activity                | Response to Stimulus/Cellular Process/Metabolic Process |
| NCU05242 | zinc finger transcription factor-25    | 11364   | znf-25       | Transc Factors                       |                                   |
| NCU05250 | nuclear division-76                    | 11492   | div-76       | Transc Factors                       | Binding                           | Biological Regulation/Cel-ular Component Organization or Biogenesis/Localization Process/Response to Stimulus/Cel-ular Process/Metabolic Process |
| NCU05294 | zinc finger transcription factor-40    | 11074   | znf-40       | Transc Factors                       |                                   |
| NCU05383 | fungal specific transcription factor    | 11019   | col-24       | Transc Factors                       |                                   |
| NCU05411 | pathway-specific nitrogen regulator    | 11040   | Transc Factors|                                      |                                   |
| NCU05637 | hypothetical protein                   | 11365   | Transc Factors|                                      |                                   |
| NCU05767 | zinc finger transcription factor-10    | 11051   | znf-10       | Transc Factors                       |                                   |
| NCU05790 | phytochrome-like histidine kinase 2    | 11241   | phy-2        | Morph/Hyph                           |                                   |
| NCU05854 | hypothetical protein                   | 11314   | Morph/Hyph   | Catalytic Activity                   |                                   |
| NCU05858 | fatty acid oxygenase                   | 11242   | fam-2        | Morph/Hyph                           |                                   |
| NCU05891 | arid/bright domain-containing protein  | 11904   | Morph/Hyph   | Binding                              |                                   |
| NCU05956 | Beta-galactosidase                     | 11310   | gh2-2        | Morph/Hyph                           | Catalytic Activity                |                                   |
| NCU05993 | hypothetical protein                   | 11078   | Transc Factors|                                      |                                   |
| NCU05994 | transcription factor-10                | 11025   | tcf-10       | Transc Factors                       | Binding                           |                                   |
| NCU06049 | DNA damage response protein RcaA       | 12674   | nbs1         | Morph/Hyph                           | Binding/Catalytic Activity         |                                   |
| NCU06145 | RING-6                                 | 12558   | Morph/Hyph   |                                   |                                   |
| NCU06173 | hypothetical protein                   | 11366   | Transc Factors|                                      |                                   |

(continued)
| ID      | Gene Function                          | Gene   | Knockout set | GO: Molecular Function | GO: Biological Process |
|---------|---------------------------------------|--------|--------------|------------------------|------------------------|
| NCU06175 11244 | Peroxisomal membrane protein           | pex3   | Morph/Hyph   |                         |                        |
| NCU06186 11369 | hypothetical protein                   |        | Transc Factors |                         |                        |
| NCU06205 11371 | transcriptional repressor rco-1        | rco-1  | Transc Factors |                         |                        |
| NCU06213 11373 | zinc finger transcription factor-9    | znf-9  | Transc Factors |                         |                        |
| NCU06265 11245 | Hyphal anastamosis-13 protein         | ham-13 | Morph/Hyph   |                         |                        |
| NCU06407 11017 | zinc finger transcription factor 1     | vad-3  | Transc Factors |                         |                        |
| NCU06411 11116 | vegetative asexual development-4      | vad-4  | Transc Factors | Binding/Signal Transducer Activity/Catalytic Activity | Metabolic Process |
| NCU06419 11319 | map kinase kinase                     | mek-1  | Transc Factors | Binding/Catalytic Activity | Biological Regulation/Developmental Process/Response to Stimulus/Cellular Process |
| NCU06429 11835 | alpha-actinin                          |        | Morph/Hyph   |                         |                        |
| NCU06440 11595 | proteasome component PRE6             | pca-4  | Morph/Hyph   | Catalytic Activity      | Cellular Process/Metabolic Process |
| NCU06454 15833 | Rho-type GTPase                       | cdc42  | Morph/Hyph   | Binding/Signal Transducer Activity/Catalytic Activity | Biolog
celular Regulation/Cel-lular Component Organization or Biogenesis/Developmental Process/Response to Stimulus/Cytoskeletal Process/Metabolic Process |
| NCU06503 11377 | zinc finger transcription factor-24   | znf-24 | Transc Factors |                         |                        |
| NCU06531 11312 | hypothetical protein                   |        | Transc Factors |                         |                        |
| NCU06605 11184 | DNA damage-binding protein 1           | dim-8  | Morph/Hyph   | Binding                 | Response to Stimulus/Cytoskeletal Process/Metabolic Process |
| NCU06650 11247 | secretory phospholipase A2            | spp-3  | Morph/Hyph   |                         |                        |
| NCU06656 11013 | transcriptional activator protein acu-15 | acu-15 | Transc Factors |                         |                        |
| NCU06695 15946 | cytochrome c oxidase polypeptide VI   | cox-6  | Morph/Hyph   | Transporter Activity/Catalytic Activity | Cellular Process/Metabolic Process |
| NCU06714 12653 | para-aminobenzoic acid synthetase     | pab-1  | Morph/Hyph   |                         |                        |
| NCU06744 11379 | hypothetical protein                   |        | Transc Factors |                         |                        |
| NCU06764 11597 | 20S proteasome subunit Y7             | pca-2  | Morph/Hyph   | Catalytic Activity      | Cellular Process/Metabolic Process |
| NCU06799 11001 | fungal specific transcription factor   | vad-5  | Transc Factors |                         |                        |
| NCU06845 12617 | short chain dehydrogenase/reductase   |        | Morph/Hyph   |                         |                        |
| NCU06910 15950 | Cell wall integrity and stress response component 1 | wsc-1 | Morph/Hyph   |                         |                        |
| NCU06919 11105 | hypothetical protein                   |        | Transc Factors |                         |                        |
| NCU06971 11066 | transcriptional activator xlnR         | xfr-1  | Transc Factors |                         |                        |
| NCU07007 11006 | submerged protoperithecia-2           | sub-2  | Transc Factors |                         |                        |
| NCU07039 11381 | GATA type zinc finger protein Asd4    |        | Transc Factors |                         |                        |
| NCU07139 11055 | BEAK-2                                | bek-2  | Transc Factors |                         |                        |
| NCU07221 11251 | two-component system protein A         | hcp-1  | Morph/Hyph   |                         |                        |
| NCU07237 23704 | hypothetical protein                   |        | E. coli CS Orth |                         |                        |
| NCU07281 14469 | glucose-6-phosphate isomerase         | gpi-1  | E. coli CS Orth | Catalytic Activity      | Metabolic Process |
| NCU07374 11016 | hypothetical protein                   |        | Transc Factors |                         |                        |
| NCU07378 11252 | serine threonine protein kinase        | stk-12 | Morph/Hyph   | Catalytic Activity      | Biological Regulation/Cytoskeletal Component Organization or Biogenesis/Response to Stimulus/Cytoskeletal Process/Metabolic Process |
| NCU07379 11383 | transcription factor-5                 | tcf-5  | Transc Factors |                         |                        |
| NCU07392 11041 | transcriptional regulatory protein pro-1 | adv-1 | Transc Factors |                         |                        |
| NCU07420 11844 | eIF4A                                 | eIF4A  | Morph/Hyph   |                         |                        |

(continued)
| ID      | Gene Function          | Gene     | Knockout set | GO: Molecular Function          | GO: Biological Process                          |
|---------|------------------------|----------|--------------|--------------------------------|------------------------------------------------|
| NCU07535| 11094 SAH-3            | sah-3    | Transcription| Catalytic Activity             | Cellular Process/Metabolic Process              |
| NCU07589| acetyltransferase      |          |              |                                |                                                |
| NCU07591| 12816 Integral membrane protein | Morph/Hyph |              |                                |                                                |
| NCU07605| hypothetical protein   |          |              |                                |                                                |
| NCU07621| zinc-regulated transporter 1 | tzn-1 | Morph/Hyph | Transporter Activity           | Cellular Process                                |
| NCU07900| hypothetical protein   |          | Transcription|                                |                                                |
| NCU07952| zinc finger transcription factor-37 | znf-37 | Transcription|                                |                                                |
| NCU08049| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08050| hypothetical protein   |          | Morph/Hyph |                                |                                                |
| NCU08055| zip-like-1             | zip-1    | Transcription| Binding                        | Response to Stimulus/Cellular Process/Metabolic Process |
| NCU08063| kinetochore protein-18 | kpr-18   | Transcription| Catalytic Activity            | Cellular Process/Metabolic Process              |
| NCU08093| hypothetical protein   |          | Morph/Hyph | Transporter Activity/Catalytic Activity | Celluar Process/Metabolic Process |
| NCU08147| Na or K P-type ATPase  | ph7      | Morph/Hyph | Transporter Activity/Catalytic Activity | Cellular Component Organization or Biogenesis/Cellular Process/Metabolic Process |
| NCU08148| H+/nucleoside cotransporter | E. coli CS Orth | Transporter Activity |                                | Localization Process/Cellular Process |
| NCU08225| high affinity nickel transporter nic1 | trn-34 | Morph/Hyph |                                |                                                |
| NCU08289| DNA methylation modulator-2 | dmm-2 | Transcription| Binding                        | Biological Regulation/Cellular Component Organization or Biogenesis/Response to Stimulus/Cellular Process/Metabolic Process |
| NCU08290| Ku70/Ku80 family protein | mus-51 | E. coli CS Orth | Binding                        |                                                |
| NCU08443| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08516| aldose 1-epimerase     | aep-1    | E. coli CS Orth | Catalytic Activity            | Metabolic Process                                |
| NCU08634| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08652| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08726| fluffy                 | fl       | Transcription|                                |                                                |
| NCU08741| Hyphal anastamosis protein 3 | ham-3 | Morph/Hyph |                                |                                                |
| NCU08744| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08791| catalase-1             | cat-1    | Morph/Hyph | Antioxidant Activity/Binding/Catalytic Activity | Response to Stimulus/Cellular Process/Metabolic Process |
| NCU08848| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08875| Cullin binding protein CanA |         | Morph/Hyph |                                |                                                |
| NCU08881| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08890| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08901| hypothetical protein   |          | Transcription|                                |                                                |
| NCU08927| dihydroceramide delta(4)-desaturase | dcd | Morph/Hyph | Catalytic Activity            | Cellular Process/Metabolic Process              |
| NCU08992| hypothetical protein   |          | Morph/Hyph | Binding                        |                                                |
| NCU09033| zinc finger transcription factor-46 |        | Transcription|                                |                                                |
| NCU09068| nitrogen catabolic enzyme regulatory protein | nit-2 | Transcription|                                |                                                |
The protein sequences of *E. coli* genes identified by were retrieved from the *E. coli* database (ecocyc.org/). These amino acid sequences were then fed into a BLAST search on the NIH NCBI site (blast.ncbi.nlm.nih.gov/Blast.cgi) with the output limited to *Neurospora* sequences in order to identify their nearest *Neurospora* homologs. These homologs were then searched on FungiDB to determine which had homologs were then searched on FungiDB to determine which had

| ID       | Gene Function                  | Gene     | Knockout set | GO: Molecular Function               | GO: Biological Process                           |
|----------|--------------------------------|----------|--------------|-------------------------------------|-------------------------------------------------|
| NCU09071 | AGC/NDR protein kinase         | dbf2     | Morph/Hyph   | Catalytic Activity                  | Biological Regulation/Cellular Component Orga-    |
|          |                                |          |              |                                     | nization or Biogenesis/Response to Stimulus/    |
|          |                                |          |              |                                     | Cellular Process/Metabolic Process               |
| NCU09123 | Ca/CaM-dependent kinase-1      | camk-1   | Morph/Hyph   |                                     |                                                 |
| NCU09201 | hypothetical protein           | vad-6    | Transc Factors|                                    |                                                 |
| NCU09205 | nitrate assimilation regulatory | tcf-27   | Transc Factors|                                    |                                                 |
| NCU09248 | transcription factor-27        |          | Transc Factors|                                    |                                                 |
| NCU09252 | hypothetical protein           |          | Transc Factors|                                    |                                                 |
| NCU09315 | phosphorus acquisition-con-    | nuc-1    | Transc Factors|                                    |                                                 |
|          | trolling protein               |          |              |                                     |                                                 |
| NCU09333 | Zinc finger transcription fact  | ace-1    | Transc Factors|                                    |                                                 |
|          | or-1                          |          |              |                                     |                                                 |
| NCU09364 | Hsp30-like protein             | hsp30    | Morph/Hyph   |                                     | Response to Stimulus/                           |
| NCU09423 | secreted protein related to    |          |              |                                     | Metabolic Process                                |
|          | phopholipase A2                |          |              |                                     |                                                 |
| NCU09450 | 26S proteasome regulatory      | rpn-2    | Morph/Hyph   | Catalytic Activity                  | Cellular Process/Metabolic Process               |
|          | subunit Rpn2                   |          |              |                                     |                                                 |
| NCU09494 | hypothetical protein           |          | Morph/Hyph   |                                     |                                                 |
| NCU09529 | hypothetical protein           |          | Transc Factors| Binding                             | Cellular Process/Metabolic Process               |
| NCU09549 | zinc finger transcription factor-51 | znf-51 | Transc Factors|                                    |                                                 |
| NCU09655 | hypothetical protein           |          | Morph/Hyph   |                                     |                                                 |
| NCU09739 | all development altered-7      | fid      | Transc Factors|                                    |                                                 |
| NCU09804 | zinc finger transcription      | znf-43   | Transc Factors|                                    |                                                 |
|          | factor-43                      |          |              |                                     |                                                 |
| NCU09829 | hypothetical protein           |          | Transc Factors|                                    |                                                 |
| NCU09866 | thyroid hormone receptor       |          | Morph/Hyph   |                                     |                                                 |
|          | interactor 12                  |          |              |                                     |                                                 |
| NCU09882 | metacaspase-1A                 | mcp-1    | Morph/Hyph   |                                     |                                                 |
| NCU10006 | hypothetical protein           |          | Transc Factors|                                    |                                                 |

and Inouye 2004). The protein sequences of *E. coli* genes identified by were retrieved from the *E. coli* database (ecocyc.org/). These amino acid sequences were then fed into a BLAST search on the NIH NCBI site (blast.ncbi.nlm.nih.gov/Blast.cgi) with the output limited to *Neurospora* sequences in order to identify their nearest *Neurospora* homologs. These homologs were then searched on FungiDB to determine which had knockout strains available. From this final list, 68 were selected for screening in this study. This set was selected to determine the degree of relationship between the cold shock response in *E. coli* and *Neurospora*. Second, two previously organized sets of knockouts generally associated with hyphal growth and morphology and available from the FGSC were included in this screen. One set (identified as “plate 29 – morphologicals” by the FGSC) contained strains with knockouts known to cause morphological changes. The second set (identified as “Hyphal Growth Set” by the FGSC) contained strains with knockouts in genes homologous to genes in yeast known to affect polar growth. A total of 131 strains from these two sets were screened.

The last set consists of knockouts of known transcription factors in *Neurospora*. This collection is available as a set from the Fungal Genetics Stock Center (McCluskey 2003). It was selected for this screen to determine which transcription factors play a role in signaling to the cell that cold adaptation genes must be activated. A total of 145 strains from this set were screened.

Media

Media and culturing procedures were those described in Davis & deSerres (1970). Growth described as being on “minimal” was in plates containing Vogel’s minimal medium (Davis & deSerres 1970) with 2% agar.

Screen

The selected knockout strains were subjected to a screen looking for altered responses to cold shock. Wild-type *Neurospora* progresses through a three-stage response following a shift into the cold. To induce the cold shock response, we initially grew strains at 33°C and shifted to 4°C. We selected 33°C as our “normal” temperature as the cold shock response has previously been demonstrated to be dependent on the degree of the temperature shift the hypha are subjected to (Watters et al. 2000). The larger temperature shift used here would be expected to result in tighter branching during the apical phase. We decided this was desirable as it would make any variations from the normal cold shock response more visible and easier to identify in the screen. Strains were inoculated by dropping a suspension of conidia onto Vogel’s Minimal Medium and incubated overnight at 33°C. The next morning plates were moved to 4°C. After an overnight incubation at 4°C, the strain’s response to cold shock was photographed and evaluated. Variations in the cold shock response from that of wild-type *Neurospora* were judged...
Photomicroscopy
Growing cultures were examined and photographed using a Motic 10MP digital camera attached to a Wolfe Beta Elite trinocular microscope. Photographs were taken of well separated, leading hyphae. All photomicrographs were taken using 40x magnification.

Phenotypes scored
The morphology of strains following cold shock was scored visually by comparing collections of photographs of cold shock in a given strain to the response seen with a wild type strain (Neurospora crassa Oak Ridge). Those with altered responses were then further categorized visually into the groups reported in Table 1 “CS phenotype.”

Undergraduate Student Involvement in Research
Valparaiso University is an undergraduate institution. All of the experiments reported here were conducted by undergraduate students under the supervision of the corresponding author. Students came to the lab under a variety of circumstances. Six of the student co-authors engaged the project as students in our Bio 496 (Independent Research) course in which students conduct research in the lab of a faculty member under their supervision. Two were upperclassmen working in the lab as paid assistants while being supported by a grant by the Indiana Space Grant Consortium (INSGC). The INSGC also supported a student from the local community college who contributed to this study. An additional student was supported by a separate grant from the INSGC with the purpose of bringing freshmen into research labs for a true research experience.

This project was chosen specifically to be one which would work well in the undergraduate university environment. The choice of organism as well as the project are suitable to a setting where funds are limited (or at times, unavailable). The study of morphology is one which students can easily grasp, and which they find relatively easy to score. Applying these questions to the knockout library allows us to take advantage of this tool and turn a quick screen into a collection of mostly identified gene functions associated with the trait. The work is technically straightforward, so undergraduate students can involve themselves with the actual conduct of the project after fairly little training in the basics of media preparation, sterile technique, basic microbiological techniques and the use of the microscope and camera.

The corresponding author was responsible for the design of the project. Undergraduates were then organized into teams incorporating both newer and older student researchers so the more experienced students could help guide the newer ones. Within these groups, students were responsible for dividing up aspects of the day-to-day activity of the project into segments and assigning individuals to be responsible for carrying out that day’s activity. This allowed them to dovetail the research activities into their normal class and work schedules. For example in a given week, one student would be responsible for making media, another for inoculating plates, and another for photomicroscopy. Scoring and categorization of the mutant phenotypes was conducted by students by examining photographs and confirmed by the corresponding author.

Data availability
The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS AND DISCUSSION
During the initial study of the cold shock response in Neurospora (Watters et al. 2000), it was observed that two classical morphological mutants (most notably “granular” and “delicate”) produced altered responses to cold shock (not reported), demonstrating that mutants could be obtained which influenced this process. We chose to screen mutants from the Neurospora knockout library for their cold shock response in order to provide a genetic grounding to this process which has, thus far, been lacking. We chose to use the mutants of the knockout library instead of the products of a random mutagenesis as the knockouts allow an immediate identification of gene function in most cases.

Knockout strains displaying an altered morphological response to cold shock were classified according to the specific variation they displayed. Examples are shown in Figure 1. The “burst” phenotype was defined as displaying a large number of growing tips which stop growing, swell and then structurally fail leaving a pool of cytoplasm at the tip. The “fail” phenotype was defined as failing to display the apical branch phase characteristic of cold shock. In the “fail” response, growth proceeds normally with lateral branching following cold shock. The “thin” phenotype was defined by a very rapid decrease in hyphal diameter following cold shock. It was common to observe “thin” in combination with other altered cold shock responses. The “dense” phenotype was defined by displaying apical branching with visibly shorter distances between branch points following cold shock relative to the response in wild-type. The “weak” phenotype was defined as the opposite – an apical branch phase with visibly longer distances between branch points relative to wild-type following cold shock. Finally, the “cot-like” phenotype was characterized by a lack of apical branching, but a shift to tightly spaced lateral branches which morphologically resembled the growth of the traditional cot mutants at the restrictive temperature.

Screen of E. coli cold shock gene homolog knockout set
A total of 68 Neurospora strains with knockouts of genes homologous to E. coli genes which alter transcription in response to cold shock (Phadtare and Inouye 2004) were screened. A total of 55 (81%) showed altered morphology to cold shock (Knockouts presenting alterations to the cold shock response are reported together in Table 1, sorted by phenotype). The knockouts displaying altered response to cold shock represent a variety of cellular functions. Phadtare and Inouye report genes which respond to cold shock by altering their transcription levels. Comparisons (χ² not shown) between these transcription changes in E. coli and the cold shock phenotype displayed by these genes orthologs in Neurospora do not suggest there are any clear associations between transcription changes and cold shock morphology.

The screen of cold shock orthologs provides a test of the hypothesis that E. coli and Neurospora share a great deal of their cold shock response in common. The very high percentage of overlap between genes playing a role in these two widely separated organisms suggests that the two responses are functionally related.

Screen of Morphological/Hyphal plate knockout sets
A total of 131 selected mutant strains from the Neurospora knockout library were previously segregated into two collections. The “Morphological” collection resulted in known morphological variations in the knockout strains. The “Hyphal” collection consisted of knockouts of genes previously suspected to play a role in hyphal growth. These two collections were screened for alterations to their response to cold shock. In total, 33 (25%) strains were identified (Table 2) that displayed variant cold shock responses. The altered responses fell into several phenotypic categories (Table 1).
The morphological/hyphal knockouts were previously screened for temperature-dependent branch density (Watters et al. 2011). Comparing the strains identified above with alterations to their cold shock response to those previously determined to show temperature-dependent branching we find only a modest overlap with the following strains showing altered phenotypes in both: NCU02333, NCU00830, NCU04242, NCU02114, NCU04264, and NCU03076. Examining the overlap statistically via $\chi^2$ (calculations not shown) yields a p value greater than 0.9, strongly suggesting that the overlap is random. This suggests that these two screens (cold shock vs. temperature sensitive branching during steady-state growth) are independent. This leads us to conclude that the cold shock response and temperature-dependent branching are independent aspects of cold adaptation, highlighting the different genes involved in short-term adaptation to the cold as opposed to those required for sustained growth in cold environments. Additional screens of the knockout library for strains displaying growth rate dependent branching, and comparing them to those with an altered cold shock response will allow us to further examine the apparent independence of these two morphological screens.

Screen of transcription factor knockout set
A total of 145 Neurospora strains with knockouts in genes which function as transcription factors were screened for their response to cold shock. In all, 30 (20%) showed altered morphology to cold shock (Table 1).

As with the knockouts of orthologs of E. coli cold shock responding genes, the mutant strains identified in the additional screens show no observed correlations between the phenotypes observed and the annotated functions of the genes with a variety of functions being associated with the observed cold shock variations.

Frequency of knockouts yielding alterations in cold shock was dependent on the category: the knockout
As detailed above, mutants screened represented three different categories of knockouts: E. coli cold-shock responding orthologs, Neurospora morphological/hyphal growth mutants, and Neurospora transcription factors. These three groups displayed altered cold shock responses at different rates with the majority (81%) of the E. coli orthologs showing altered responses and much lower frequencies (23% and 20% respectfully) of the morph/hyphal and transcription factor knockouts showing altered responses (Table 1). Additionally, the phenotypes of the altered cold shock response showed a non-random distribution with regard to the knockout set the mutant was associated with using $\chi^2$. Comparing knockout set vs. cold shock phenotype among those with alterations yields a $\chi^2$ of 32.2 and an associated p value < 1%. Much of the significance is coming from an over-representation of “dense” cold shock responses among otherwise unidentified (i.e., “hypothetical protein”) transcription factors.

Cold shock phenotype was not correlated to GO categorization of the knockouts
The cold shock phenotype of knockouts was compared to their gene ontology categorizations via $\chi^2$ analysis. Comparing cold shock phenotype to either its Molecular Function or Biological Process categorization failed to produce significant differences (p values of ~0.75 and ~0.5 respectfully). Thus, particular GO categorizations are not associated with specific altered phenotypes in the cold shock response.

The data were also examined to determine if there was a non-random association between knockouts which show any alteration to their cold shock response (regardless of the specific phenotype) and those that show the wild type response vs. their GO categorization. For both “Molecular Function” and “Biological Process” GO categories, no significant association was seen (via $\chi^2$, $P = 0.4$ and 0.5 respectfully), similarly failing to support the possibility that knockouts with specific GO categorizations are tied to the cold shock response.

Cold shock phenotype was weakly associated With growth rate at 25° among transcription factor knockouts
Linear growth rates at 25° for the transcription factor knockouts reported by Carrillo et al. (2017) were compared via T-test for knockouts showing altered cold shock responses vs. those showing no alteration to the response. One possible association between growth rate at 25° and altered cold shock phenotype was found for the knockouts displaying a dense phenotype which showed statistically faster growth rates at 25° than those with no alterations to cold shock (T-test, $P = 0.019$). This is consistent with previous observations between growth rate and cold shock (Watters et al. 2000), however the opposite association (slow growth rates at 25° among mutants displaying weak cold shock responses or failure to respond) is not observed, as would be expected if growth rate was a key factor among the knockouts. Taken together, there appears to be, at best, a weak association between growth rate at 25° and alterations to the cold shock phenotype among the transcription factor knockout mutants. This stands in contrast to the observation in wild type Neurospora (Watters et al. 2000) that the morphology of the cold shock response was directly dependent on growth rate changes. This suggests that the altered morphologies observed among the knockout mutants are due to changes in gene activity associated with the knockouts and not simply the consequence of changes in growth rates in these mutants.

In conclusion, the gene functions highlighted by these screens (Table 1) are diverse. It is unclear how the diverse gene network, partially exposed here, coordinates for the function of temperature acclimatization. The results presented here demonstrate a strong relationship between the cold shock responses of E. coli and Neurospora crassa. The phenotype under examination here (morphological response to cold shock) appears to be influenced by a diverse network of genes. Similar diversity of function has been observed in other examinations of morphogenesis in Neurospora (Seiler & Plamann 2003). Further work on cold acclimatization should help clarify these connections.

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