Prion Biology in the Context of Bacteria

by

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ABSTRACT

Prions are infectious amyloid aggregates first described in the context of mammalian neurodegenerative diseases collectively known as the transmissible spongiform encephalopathies. Prions have also been uncovered in yeast, where they function as protein-based units of heredity that confer unique phenotypic traits on those cells that harbor them. To date, the discovery of prions and prion-like phenomena has been confined to the Eukarya. The work presented in this thesis seeks to explore the possibility that prion-like mechanisms are operative in the bacterial domain of life.

In what follows, we demonstrate that *Escherichia coli* cells can propagate a model yeast prion in a chaperone-dependent manner, establishing that the bacterial cytoplasmic milieu in general and a molecular machine in particular are poised to support protein conformation-based heredity in bacteria. Moreover, we provide evidence that a bacterial transcription termination factor exhibits prion-like behavior in *E. coli* and yeast. Our work thus suggests that bacterial prions exist and may function as previously unrecognized reservoirs of phenotypic diversity among the most abundant organisms on earth.

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TABLE OF CONTENTS

Abstract 3
Acknowledgements 5
Table of Contents 7

Chapter One: An introduction to prions 9

Chapter Two: Prion propagation can occur in a prokaryote and requires the ClpB chaperone 27
  Abstract 28
  Introduction 29
  Results 33
  Discussion 43
  Materials and Methods 47
  References 51
  Figures 57

Appendix A: A transcription-based reporter for prion propagation in E. coli 82
  Results and Discussion 83
  References 87
  Figures 88

Appendix B: ClpB is the only non-essential chaperone or protease required for prion propagation in E. coli 91
  Results and Discussion 92
  References 94
  Figures 95

Chapter Three: Evidence of a bacterial prion 98
  Abstract 99
  Introduction 100
  Results 102
  Discussion 109
  Materials and Methods 112
  References 116
  Figures 121

Appendix C: A cell-based screen for amyloidogenic proteins of bacterial origin 131
  Results and Discussion 132
  References 136
  Figures 138

Curriculum Vitae 140
CHAPTER ONE

An introduction to prions
History of the transmissible spongiform encephalopathies

The history of prion research represents the quintessential Kuhnian epistemological progression. While we now readily conceive of prions as infectious protein aggregates and protein-based units of heredity, this was – and perhaps is still – not always the case. Accordingly, analysis of the prion paradigm warrants a review of those normal scientific observations, anomalies, retrospections, controversies, and resolutions that led to its establishment (Kuhn, 1962).

Building upon theoretical work presented by Griffith in 1967 (Griffith, 1967), Prusiner coined the term prion in 1982 to describe the nuclease and UV-resistant infectious agent responsible for scrapie, a member of a class of inevitably fatal neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs) (Prusiner, 1982). Scrapie was first characterized as an affliction of sheep, with the earliest documentation of scrapie-like cognitive and motor dysfunctions common to all TSEs dating to the 18th century (Aguzzi and Polymenidou, 2004).

It would not be until the 20th century that evidence of human TSEs would emerge. The first case study of the human prion disease known as Creutzfeldt-Jakob disease (CJD) was reported in the early 1920's (Creutzfeldt, 1920; Jakob, 1921). A second human TSE known as Kuru ("to shiver" in the Fore linguistic family) was described in 1957 as an endemic disease afflicting aboriginal societies of Papua New Guinea (Gajdusek and Zigas, 1957).

Prion disease in humans and animals received global attention in the 1990's when a correlation between the consumption of meat from "mad cows" afflicted
with bovine spongiform encephalopathy (BSE) and the occurrence of a variant form of CJD (vCJD) in humans was noted (Aguzzi, 1996; Bruce et al., 1997; Hill et al., 1997). While BSE was first documented in 1987 (Wells et al., 1987), its relationship to vCJD brought the question of transmissibility – and subsequently, the specificity of transmission between species – to the forefront of prion research and public intrigue.

Prusiner's self-proclaimed "heretical" prion hypothesis was met with criticism and challenged by alternative models of scrapie etiology. Among these models, the most notable was the "virino hypothesis", according to which the infectious scrapie agent represents a protein-associated or -encapsulated nucleic acid (Kimberlin, 1982). The difficulties inherent to experimentally distinguishing between such a virus-like particle and a prion would render the prion hypothesis controversial for several decades after its conception.

**Amyloid and the PrP protein**

Since Prusiner formalized the prion hypothesis, the collective work of many research groups has established that the protein culprit underlying the TSEs of animals and man is the PrP protein, the product of the *PRNP* gene (Prusiner et al., 1984; Aguzzi et al., 2008). PrP is a GPI-anchored extracellular membrane protein found at the surface of neurons and glial cells. Native PrP (PrP<sub>c</sub>) undergoes a dramatic change in conformation upon conversion to the prion state (PrP<sup>Sc</sup>), forming protein aggregates known as amyloids (Diaz-Espinoza and Soto, 2012).
Amyloids are highly-ordered fibrillar assemblies characterized by a cross-β spine. This quaternary structure is typified by X-ray diffraction intensity maxima at 4.7 Å and 10 Å, the distance between adjacent beta strands and opposing beta sheets, respectively, of an amyloid fibril. PrPSc amyloid aggregates are capable of templating the conversion of PrPc and thereby mediate propagation of PrPSc in afflicted individuals as well as transmission between individuals (Legname et al., 2004; Castilla et al., 2005; Wang et al., 2010). Critically, self-propagation is a distinguishing feature of PrPSc amyloids that sets them apart from non-infectious amyloid aggregates underlying neuropathologies like Alzheimer’s, Parkinson’s, and Huntington’s diseases (Chiti and Dobson, 2006). While prion disease can be initiated in animal models via intravenous, intraperitoneal, and intraocular inoculation of infectious material, the mechanistic details of PrPSc transmission, propagation, and pathogenesis – along with the native function of the PrPc protein – are poorly understood and remain the subject of ongoing PrP research (Aguzzi and Calella, 2009).

**Fungal prions**

The discovery of prions in yeast vividly illustrates how scientific progress is more often than not non-linear and often necessitates the reassessment of anomalous observations that pass unnoticed under the guise of normal science (Kuhn, 1962). Specifically, in what would lay the conceptual framework for subsequent fungal prion research, Wickner cleverly invoked prions in 1994 to
account for two non-chromosomal genetic elements that had been described in yeast decades earlier (Wickner, 1994).

The first of these elements (known as [PSI]) was discovered and characterized by Cox in 1965 as a heritable factor increasing the efficiency of stop codon readthrough (Cox, 1965). The second element (known as [URE3]) was uncovered by Aigle and Lacroute in 1972 as a non-chromosomal genetic element that confers upon ura2 mutant cells the ability to utilize ureidosuccinate (Aigle and Lacroute, 1972). The cumulative work of several research groups has now elucidated the molecular biology underlying [PSI] and [URE3]. Specifically, [PSI] (more accurately designated as [PSI\(^+\)]) represents the prion form of the Sup35 protein, an essential translation release factor that – in conjunction with the Sup45 protein – mediates stop codon recognition and translation termination (Stansfield et al., 1995; Paushkin et al., 1997a). [URE3] represents the prion form of the Ure2 protein, a negative regulator of the Gln3 transcription factor, which activates transcription of genes encoding proteins involved in the uptake and catabolism of poor nitrogen sources (Masison and Wickner, 1995; Magasanik and Kaiser, 2002).

Subsequent studies provided critical experimental support for Wickner’s yeast prion hypothesis. Specifically, genetic and cell biological analyses of the [PSI\(^+\)] prion formally established a link between protein aggregation and the inheritance of a phenotypic trait (Patino et al., 1996; Paushkin et al., 1996). Furthermore, biochemical work illuminated the molecular basis of Sup35 aggregation and thereby accounted for the apparent self-perpetuation of a prion conformation (Glover et al., 1997; Paushkin et al., 1997b; Tessier and Lindquist, 2009).
Wickner's 1994 study represented a *bona fide* Kuhnian paradigm shift; that is, it accounted for anomalies of the past and upended our conception of heritability all the while inaugurating a new framework for genetics that was as rigid as it was illuminating. Specifically, Wickner transformed lessons learned from \([URE3]\) and \([PSI]\) into three genetic criteria for prion phenomena: (i) prions exhibit reversible curability, (ii) prion appearance can be induced by overproduction of the prion protein, and (iii) prion-dependent phenotypes resemble those associated with prion protein loss-of-function (Wickner, 1994). These genetic criteria lie at the heart of a paradigm that continues to inform an exciting period of Kuhnian "normal science" in the field of yeast prion biology (Kuhn, 1962). As is the case for any normal science, research at the peripheries of the paradigm – best represented by recent discoveries of prion-like phenomena that satisfy only a subset of Wickner's criteria (Si et al., 2010; Hou et al., 2011; Cai et al., 2014) – continues to threaten the coherence and thereby ensures the progression of prion research.

Like PrP\textsuperscript{Sc}, \([PSI^+]\), \([URE3]\), and the vast majority of yeast prion proteins form amyloid in their prion states (Alberti et al., 2009) As amyloids are recalcitrant to crystallization and insoluble, atomic resolution structures of prion proteins in their amyloid state have remained, for the most part, elusive. However, solid-state NMR techniques have demonstrated success in and continue to hold great promise for defining the structure of prions (Tycko, 2011). In this regard, a prion uncovered in the filamentous fungus *Podospora anserina* known as \([Het-s]\) is particularly notable. \([Het-s]\) corresponds to the prion form of the HET-s protein, which – in conjunction with the HET-S protein – regulates heterokaryon incompatibility (Saupe, 2011).
date, the solid-state NMR structure of recombinant HET-s amyloid, which can be
described as a β-solenoid, represents the only high-resolution view of a prion
domain in its amyloid conformation (Ritter et al., 2005; Wasmer et al., 2008).

One the most significant contributions of yeast prion research to our
understanding of prion biology lie in unequivocal demonstrations of so-called
"protein-only" transmission, a defining feature of the prion hypothesis that
remained experimentally elusive in mammalian models of PrPSc infectivity for
decades. Specifically, the development of protein transformation techniques
permitted the delivery of recombinant Sup35 and HET-s amyloids (generated in
vitro in the absence of cofactors) into Saccharomyces cerevisiae and P. anserina cells,
respectively, and resulted in the emergence of "infected" [PSI+] and [Het-s] cells
(Maddelein et al., 2002; Tanaka et al., 2004; King and Diaz-Avalos, 2004).

Other landmark studies of fungal prions have established that ecologically
diverse wild strains of yeast contain prions, many of which confer upon cells new
phenotypic traits that can – under particular growth conditions – increase cellular
fitness (True and Lindquist, 2000; True et al., 2004; Halfmann et al., 2012; Jarosz et
al., 2014a; Jarosz et al., 2014b). These findings provide experimental support for the
proposal that prion-dependent mechanisms represent bet-hedging strategies that
enable genetically identical cells to adapt to environmental stress (True and
Lindquist, 2000; True et al., 2004; Halfmann and Lindquist, 2010; Halfmann et al.,
2010; Suzuki et al., 2012; Holmes et al., 2013; Newby and Lindquist, 2013). Future
studies of yeast prions and their contributions to environmental adaptation thus
hold great promise for reinforcing the emerging view that prion-like processes can be evolutionarily beneficial.

**Chaperones**

The stable propagation of fungal prions – and thus, the heritability of their associated phenotypes – depends on the activity of chaperone proteins (Chernoff et al., 1995). The critical role that chaperones play in prion biology has been made most apparent in studies of *S. cerevisiae*, where the Hsp100/Clp-family AAA+ disaggregase Hsp104 is strictly required for the propagation of virtually all yeast prions (Liebman and Chernoff, 2012). Hsp104 typically collaborates with Hsp70- and Hsp40-family chaperones to orchestrate the solubilization and refolding of protein aggregates (Parsell et al., 1994; Glover and Lindquist, 1998). However, in the context of prion biology, multiple lines of evidence indicate that Hsp104 functions as a regulator of amyloid fiber appositional growth by fragmenting large prion aggregates into smaller seed particles known as propagons, which can be efficiently partitioned to daughter cells during cell division (Paushkin et al., 1996; Ness et al., 2002; Cox et al., 2003; Satpute-Krishnan et al., 2007).

While Hsp104-, Hsp70-, and Hsp40-mediated amyloid fragmentation provides a general framework for understanding prion heritability, chaperone-mediated prion propagation in yeast is remarkably complex. *S. cerevisiae* contains six Hsp70 paralogs and over a dozen Hsp40-like proteins, a subset of which have been shown to exhibit specific effects on the propagation of distinct prions (Liebman and Chernoff, 2012). Moreover, an atypical yeast prion known as [GAR*]
which confers upon cells the ability to bypass glucose repression-associated metabolic processes, has been shown to depend on Hsp70 chaperones – but not Hsp104 – for its stable propagation (Ball et al., 1976; Brown and Lindquist, 2009).

As the biological complexity underlying chaperone-prion interactions has become increasingly apparent, the study of protein folding and chaperone function remains at the forefront of yeast prion research.

**Bacterial amyloid**

While prion-like epigenetic phenomena may superficially resemble bistable and phase variable biological networks extensively characterized in bacteria (Veening et al., 2008), to date, *bona fide* prion-dependent phenomena have been discovered exclusively in the Eukarya. Furthermore, while amyloids are ubiquitous in living systems and play critical roles in extracellular physiological processes of bacteria (Schwartz and Boles, 2012), it is unclear whether or not bacteria appropriate the amyloid conformation to store intracellular protein-based heritable information.

The founding member of the so-called "functional amyloid" family of bacterial extracellular proteins is the curli fiber produced by *E. coli* and other Enterobacteriaceae (Chapman et al., 2002). Composed of the secreted CsgA and CsgB proteins, curli fibers mediate surface adhesion and have been implicated in biofilm formation as well as immune evasion (Barnhart and Chapman, 2006). Distinct but analogous extracellular functions – particularly those concerning biofilm formation – have been assigned to the TasA and TapA proteins of *Bacillus*
subtilis (Romero et al., 2011) and the FapC protein of *Pseudomonas fluorescens* (Dueholm et al., 2010).

Bacteria also deploy extracellular amyloids in the form of toxins. Microcin E492, for example, is a bactericidal peptide secreted by *Klebsiella pneumoniae* capable of forming inert amyloid fibrils that, in response to changes in pH and osmolarity, dissociate into cytotoxic, pore-forming oligomers that kill neighboring bacteria (Shahnawaz and Soto, 2012). Toxic amyloid oligomers can also play a role in host-pathogen interactions, as evidenced by the plant pathogen *Xanthomonas axonopodis*, which secretes (via a type-III secretion system) a class of amyloidogenic proteins known as harpins into plant cells, eliciting the hypersensitive response (Oh et al., 2007).

As the ability to produce extracellular amyloid is a common feature shared among many bacteria, including those belonging to human microbiomes, bacteria-generated amyloid burdens and their impact on human physiology have begun to receive attention (Hill & Lukiw, 2015). In fact, the recent discovery that the central nervous system is connected to the lymphatic system (Louveau et al., 2015) raises the intriguing – albeit currently unexplored – possibility that amyloid burdens of bacterial origin may influence the progression of amyloid-based human neurodegenerative diseases.

**Do bacterial prions exist?**

It is not obvious how to go about discovering prions – should they exist – in bacteria, where the conspicuous genetic signature of non-Mendelian inheritance is
unobservable. Indeed, the absence of extensive cytoplasmic mixing during bacterial genetic transactions likely prevents – or perhaps necessarily precludes – the observation of prion-like phenomena in bacteria by classic genetic approaches that were and remain essential for the discovery and characterization of fungal prions. Furthermore, experimental tools analogous to cytoduction (Conde and Fink, 1976) and protein transformation (Maddelein et al., 2002; Tanaka et al., 2004) have yet to be established in bacterial systems. These challenges necessitate alternative approaches to conceptualizing and studying prion biology in the context of bacteria, the subject of this dissertation.
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CHAPTER TWO

Prion propagation can occur in a prokaryote and requires the ClpB chaperone

A modified version of this chapter was published previously:


Specific author contributions:

I devised and performed all experiments presented in this chapter. Sean Garrity (Harvard Medical School) made preliminary findings and Entela Nako (Harvard Medical School) provided reagents.
ABSTRACT

Prions are self-propagating protein aggregates that are characteristically transmissible. In mammals, the PrP protein can form a prion that causes the inevitably fatal transmissible spongiform encephalopathies. Prions have also been uncovered in fungi, where they act as heritable, protein-based genetic elements. We previously showed that the yeast prion protein Sup35 can access the prion conformation in Escherichia coli. Here we demonstrate that E. coli cells can propagate the Sup35 prion under conditions that do not permit its de novo formation. Furthermore, we show that propagation requires the ClpB chaperone. Prion propagation in yeast requires Hsp104 (a ClpB ortholog), and several studies have come to conflicting conclusions about the ability of ClpB to participate in this process. Our demonstration of ClpB-dependent prion propagation in E. coli suggests that the cytoplasmic milieu in general and a molecular machine in particular are poised to support protein-based heredity in the bacterial domain of life.
INTRODUCTION

Prions are infectious, self-propagating protein aggregates first described in the context of scrapie (Prusiner, 1982), an example of a class of devastating neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs). Specifically, the prion form of a protein known as PrP is the causative agent of the TSEs, which afflict humans and other mammals. Native PrP (PrPc) undergoes a dramatic change in conformation upon conversion to its prion form (PrPsc), forming distinctive cross-β aggregates termed amyloids (Diaz-Espinoza and Soto, 2012). Highly resistant to denaturation and proteolysis, PrPsc is infectious and templates the conformational conversion of PrPc molecules (Caughey et al., 2009).

Prion-like phenomena have also been described in budding yeast and other fungi. Since Wickner first invoked prions to account for two examples of non-Mendelian genetic elements in *Saccharomyces cerevisiae* (Wickner, 1994; Cox, 1965; Aigle and Lacroute, 1975), the study of fungal prion proteins has resulted in profound advances in our understanding of prion biology, including the first demonstration that purified prion protein aggregates are infectious (Maddelein et al., 2002; King and Diaz-Avalos, 2004; Tanaka et al., 2004). In general, such prion proteins exist in either a native, soluble form or a self-perpetuating, amyloid form, with spontaneous conversion between forms representing a rare event (Allen et al., 2007; Lancaster et al., 2010). However, unlike PrPsc, yeast prions do not normally cause cell death. Instead, they can act as protein-based genetic elements that confer new phenotypes on those cells that harbor them (True and Lindquist, 2000; Tuite
and Serio, 2010; Newby and Lindquist, 2013). Fungal prion proteins have been found to participate in diverse cellular processes (Coustou et al., 1997; True et al., 2004; Suzuki et al., 2012; Holmes et al., 2013). The conversion of these proteins to their prion forms typically results in a dominant loss-of-function phenotype (Cox, 1965; Aigle and Lacroute, 1975). A particularly well-characterized example involves the essential translation release factor Sup35, which confers on cells a heritable nonsense suppression phenotype upon conversion to the prion form (True et al., 2004).

Like other yeast prion proteins, Sup35 has a modular structure, with a distinct prion domain (PrD) that mediates conversion to the prion form, [PSI⁺]. In the case of Sup35, the essential prion determinants, which include a glutamine- and asparagine-rich segment and five complete copies of an imperfect oligopeptide repeat sequence, lie in the N-terminal domain (N), whereas translation release activity resides in the C-terminal domain (C) (Ter-Avanesyan et al., 1993). A highly charged middle region (M) increases the solubility of native Sup35 and enhances the mitotic stability of [PSI⁺] (Liu et al., 2002). Together, Sup35 N and M function as a transferable prion-forming module (NM) that maintains its prionogenic potential when fused to heterologous proteins (Li and Lindquist, 2000). A distinctive property of the Sup35 conversion process in yeast is its dependence on the presence of a pre-existing prion, referred to as a [PSI⁺] inducibility (PIN) factor (Derkatch et al., 1997). Thus, yeast strains containing Sup35 in the non-prion form, [psi⁻], support the spontaneous conversion to [PSI⁺] only if they contain a PIN factor, typically the prion form of the Rnq1 protein (Derkatch et al., 2000). However,
several other yeast prion proteins, including the New1 protein, have the capacity to function as PIN factors in their prion forms (Derkatch et al., 2001; Osherovich and Weissman, 2001).

Importantly, the stable propagation of yeast prions – and thus, the heritability of their associated phenotypes – depends on the function of chaperone proteins (Chernoff et al., 1995). Specifically, the AAA+ disaggregase Hsp104 is strictly required for the propagation of virtually all yeast prions characterized thus far, and several other chaperone proteins have been implicated in this process as well (Liebman and Chernoff, 2012; Winkler et al., 2012). Various lines of evidence support the view that the essential role of Hsp104 with respect to prion propagation stems from its ability to fragment prion aggregates and thereby to generate smaller seed particles known as propagons that can be efficiently partitioned to daughter cells during cell division (Paushkin et al., 1996; Ness et al., 2002; Cox et al., 2003; Kryndushkin et al., 2003; Satpute-Krishnan et al., 2007; Higurashi et al., 2008). Accordingly, depletion or inhibition of Hsp104 in a prion-containing cell leads to prion loss in progeny cells.

The molecular processes underlying prion biology constitute at least two distinct phases, namely, (i) the de novo conversion of a protein from its native to prion form, and (ii) the subsequent propagation of the self-perpetuating prion form over multiple generations. While studies have demonstrated that the bacterial cytoplasm can support the de novo formation of prion-like aggregates (Sabaté et al., 2009; Garrity et al., 2010; Fernández-Tresguerres et al., 2010; Espargaró et al., 2012; Gasset-Rosa et al., 2014), evidence for prion propagation – and thus, protein
conformation-dependent heredity – in bacteria has remained elusive. We previously demonstrated that conversion of Sup35 NM to its prion form in *E. coli* depends on the presence of a transplanted PIN factor derived from the New1 protein, thereby recapitulating aspects of *S. cerevisiae* PIN-dependent prion biology (Garrity et al., 2010). This PIN dependence provides an experimental framework for distinguishing between the initial conversion and subsequent propagation phases of the prion cycle.

Here we show that bacteria can propagate the Sup35 NM prion in an infectious conformation over at least ~100 generations under conditions that do not permit *de novo* prion formation. More specifically, we demonstrate maintenance of the Sup35 NM prion over multiple rounds of restreaking in *E. coli* cells no longer capable of synthesizing New1 protein. Furthermore, we establish that propagation of the Sup35 NM prion in *E. coli* requires ClpB, the bacterial ortholog of Hsp104. The striking parallel between the requirements for both prion formation and prion propagation in yeast and bacteria, which are thought to have diverged more than 2.2 billion years ago, suggests that the paradigm of protein-based heredity may be more ancient than previously inferred (DeSantis et al., 2012).
RESULTS

**E. coli cells can propagate SDS-stable NM aggregates**

Having previously shown that the prionogenic module of Sup35 (hereafter referred to as NM) can adopt an infectious amyloid conformation in the *E. coli* cytoplasm (Garrity et al., 2010), we wished to determine whether or not *E. coli* cells could stably propagate NM in its prion form. To address this question, we took advantage of the fact that conversion of NM to its prion conformation in *E. coli* depends on the presence of a transplanted PIN factor, mirroring the PIN dependence of Sup35 prion formation in *S. cerevisiae* (Figure 1A). More specifically, we envisioned inducing the formation of infectious NM aggregates in the presence of the New1 PrD (hereafter referred to as New1), curing cells of New1-encoding DNA, and monitoring the fate of NM over multiple generations.

To facilitate these experiments, we fused NM and New1 to two monomeric fluorescent proteins (mCherry bearing a C-terminal hexahistidine tag and mGFP, respectively). The two fusion proteins were produced from compatible plasmids under the control of IPTG-inducible promoters. The plasmid encoding New1-mGFP (pSC101\textsuperscript{TS}-\textit{NEW1}) bore a temperature-sensitive origin of replication, enabling us to cure cells of New1-encoding DNA and thereby deplete cells of the New1 fusion protein. As an initial test of our experimental system, we introduced the plasmid encoding NM-mCherry-His\textsubscript{6x} (pBR322-NM) together with either pSC101\textsuperscript{TS}-\textit{NEW1} or an empty vector control (pSC101\textsuperscript{TS}) into *E. coli* cells and induced the synthesis of the fusion proteins at the permissive temperature. After overnight growth, we detected SDS-stable NM aggregates only in cells producing the New1 fusion protein as
assessed by filter retention analysis (Figure 1B, see Materials and Methods). As New1 can independently adopt an amyloid conformation in *E. coli* (Garrity et al., 2010), we also detected SDS-stable New1 aggregates in cells containing both fusion proteins (Figure 1B). Western blot analysis revealed that the intracellular levels of the NM fusion protein were comparable in the presence and absence of the New1 fusion protein (Figure 1C, Supplemental Figure 1A). We also examined the cells by fluorescence microscopy. In cells containing both fusion proteins, NM formed twisted ring structures (Garrity et al., 2010) or large polar foci in a substantial fraction of cells, whereas New1 formed punctate foci in a majority of cells (Figure 1D). In contrast, NM exhibited diffuse fluorescence in cells lacking New1 (Figure 1D).

We then sought to determine whether or not *E. coli* cells could propagate SDS-stable NM aggregates over multiple generations under conditions that did not permit the *de novo* formation of aggregates (that is, in the absence of New1). Our experimental protocol is illustrated in Figure 2 (see also Figure 3A). We first induced fusion protein synthesis in cells transformed with pBR322-NM and either pSC101<sup>TS</sup>-NEW1 (experimental sample) or pSC101<sup>TS</sup> (control sample). These "starter cultures" were grown overnight to allow for the formation of SDS-stable NM aggregates in the experimental sample. The cells were then plated and grown at the non-permissive temperature to cure the cells of pSC101<sup>TS</sup>-NEW1 or pSC101<sup>TS</sup>, thereby generating a set of Round 1 (R1) colonies. Twenty R1 experimental colonies and twenty R1 control colonies were subsequently examined; each was (a) patched onto selective medium to test for loss of pSC101<sup>TS</sup>-NEW1 or pSC101<sup>TS</sup>, (b)
restreaked to generate Round 2 (R2) colonies, and (c) inoculated into liquid medium for overnight growth to test for the presence of SDS-stable NM aggregates. Four separate experimental lineages (L1E-L4E) originating from ancestral R1 experimental colonies containing detectable NM aggregates along with four separate control lineages (L1C-L4C) originating from ancestral R1 control colonies were then followed through Round 3 (R3) and Round 4 (R4). For R2 and each subsequent round, ten experimental colonies and ten control colonies were analyzed.

All R1 experimental and control colonies (20 of each) had lost pSC101<sub>TS-NEW1</sub> or pSC101<sub>TS</sub>, respectively, as assessed by patching on selective medium (data not shown). Moreover, the absence of NEW1 DNA was confirmed by PCR (Figure 3C), and the absence of New1 protein was confirmed by Western blot analysis (Figure 3B). We detected SDS-stable NM aggregates in 8 of 20 experimental samples (Figure 3B) and none of the control samples (Figure 3D). We selected 4 of the 8 aggregate-positive clones (Figure 3B, asterisks) to establish the four experimental lineages and arbitrarily selected four aggregate-negative control clones (Figure 3D, asterisks) to establish the four control lineages. 2 of the 4 experimental lineages (L1E and L3E) retained SDS-stable NM aggregates throughout the course of the experiment (Figure 4A, Supplemental Figure 2B). Of these two lineages, one maintained aggregates in 9 of 10 R4 clones (Figure 4A), and the other maintained aggregates in 7 of 10 R4 clones (Supplemental Figure 2B). We conclude that SDS-stable NM aggregates can be propagated in *E. coli* for at least ~100 generations in the absence of New1 (Figure 5A).
The remaining two experimental lineages (L2\textsubscript{E} and L4\textsubscript{E}) lost detectable SDS-stable NM aggregates at R3 and R4, respectively (Supplemental Figure 2A, Supplemental Figure 2C, Figure 5A). Moreover, the loss of aggregates manifested itself in all ten of the selected colonies at either R3 or R4 (Supplemental Figure 2A, Supplemental Figure 2C, Figure 5A). Curiously, the loss of SDS-stable NM aggregates from a particular lineage coincided with a loss of detectable fusion protein, as assessed by Western blot analysis (Supplemental Figure 2A, Supplemental Figure 2C), suggesting that the loss of aggregates represented an indirect consequence of a radical drop in protein levels (see Discussion). We note that the observed drop in NM-mCherry-His\textsubscript{6} fusion protein levels is not irreversible, as exemplified by L2\textsubscript{E}, which exhibited a loss of detectable NM aggregates coincident with an R3 drop in fusion protein levels and an apparent restoration of fusion protein levels by R4 in all 10 samples (Supplemental Figure 2A). Despite the presence of normal levels of fusion protein, SDS-stable NM aggregates were not recovered in R4 of L2\textsubscript{E}, consistent with the expectation that their propagation requires that protein synthesis be maintained above some threshold level (Supplemental Figure 2A, Figure 5A). Critically, none of the samples (120 in total) from any of the four control lineages contained detectable SDS-stable NM aggregates (Figure 4B, Supplemental Figure 3).

Fluorescence microscopy revealed that cells containing propagated NM aggregates exhibited small foci emanating from large aggregates typically localized at cell poles, a phenotype distinguished from experimental starter culture cells by the lack of twisted ring structures (Figure 5C). However, we observed one instance
of aggregate-positive R1 cells exhibiting twisted ring structures (Supplemental Figure 6C). Whereas we cannot definitively assign the SDS-stable NM aggregates detected by filter retention to those structures detected by fluorescence microscopy, we note that similar structural diversity has been observed for several yeast prions (Derkatch et al., 2001; Zhou et al., 2001). Furthermore, cells from aggregate-negative samples invariably exhibited diffuse fluorescence (Figure 5D).

Propagated SDS-stable NM aggregates are infectious when introduced into [psi–] yeast cells

We next sought to determine whether or not cells that maintained SDS-stable NM aggregates in the absence of New1 contained infectious material capable of converting [psi–] yeast cells to [PSI+]. We prepared bacterial cell extracts from both experimental and control starter cultures, as well as aggregate-positive samples from each of the four experimental lineages. For each experimental lineage, we examined an arbitrarily chosen sample obtained from the last aggregate-positive round. In addition, for L2E and L4E, we examined the R2 and R3 samples (indicated by asterisks in Figure 5E) that gave rise to aggregate-negative clones in the subsequent round. We used these bacterial extracts to transform S. cerevisiae spheroplasts prepared from a [pin–][psi–] strain. The use of a [pin–] recipient strain was critical as transient overproduction of Sup35 (or NM) in [PIN+][psi–] strains significantly stimulates the conversion from [psi–] to [PSI+] (Derkatch et al., 1997), whereas conversion in a [pin–][psi–] background requires the introduction of infectious seed material (Tanaka and Weissman, 2006).
The experimental starter culture yielded \([PSI^+]\) yeast transformants at a frequency of \(\sim 1\%\) (Figure 5E), consistent with our previous findings (Garrity et al., 2010). Similarly, each of the aggregate-positive samples from the four experimental lineages yielded \([PSI^+]\) yeast transformants at a frequency of \(\sim 1\%\); in contrast, the aggregate-negative samples yielded no \([PSI^+]\) yeast transformants (Figure 5E). Among the \([PSI^+]\) transformants we obtained with the experimental samples, we observed both “strong” and “weak” strains (Tanaka et al., 2004; Frederick et al., 2014) (Supplemental Figure 4). We note that \(\sim 1\%\) corresponds only to the frequency of strong \([PSI^+]\) transformants and therefore represents a conservative estimate of \(E.\ coli\) cell extract infectivity; we did not attempt to quantify weak \([PSI^+]\) transformants because they are difficult to distinguish from \([psi^-]\) transformants on the medium utilized to isolate transformants. We conclude that \(E.\ coli\) cells can propagate NM in an infectious prion conformation over at least \(\sim 100\) generations under conditions that do not permit de novo prion formation.

**Propagation of infectious NM aggregates depends on ClpB**

The propagation of \([PSI^+]\) and other prions in yeast requires Hsp104, an Hsp100-family ATP-dependent disaggregase that functions as a ring-shaped hexamer. Specifically, Hsp104 is thought to facilitate prion propagation by fragmenting large aggregates into smaller propagons that are subsequently disseminated during cell division (Paushkin et al., 1996; Ness et al., 2002; Cox et al., 2003; Kryndushkin et al., 2003; Satpute-Krishnan et al., 2007; Higurashi et al., 2008). We therefore investigated whether or not the propagation of NM aggregates in \(E.\ coli\) requires ClpB, the bacterial ortholog of Hsp104. To address this question,
we sought to deplete cells of ClpB specifically during the propagation phase of our experiments. To accomplish this, we modified pSC101\textsuperscript{TS}-\textit{NEW1} such that it also directed the expression of \textit{clpB} under the control of its native promoter. When transformed into \textit{AclpB} cells, pSC101\textsuperscript{TS}-\textit{NEW1-clpB} enabled us to grow starter cultures containing ClpB and subsequently to deplete both ClpB and New1 in cells plated at the non-permissive temperature (Figure 6A).

As expected, we detected SDS-stable NM aggregates in \textit{AclpB} starter culture cells transformed with pBR322-NM and pSC101\textsuperscript{TS}-\textit{new1-clpB} (Supplemental Figure 5A). Furthermore, fluorescence microscopy revealed that these cells contained visible aggregates that were nearly indistinguishable from those in wild-type cells containing pBR322-NM and pSC101\textsuperscript{TS}-\textit{NEW1} (Supplemental Figure 5B). After plating \textit{AclpB} starter culture cells containing pBR322-NM and pSC101\textsuperscript{TS}-\textit{NEW1-clpB} at the non-permissive temperature to cure cells of ClpB- and New1-encoding DNA, we examined 60 R1 colonies for the presence of SDS-stable NM aggregates. In parallel, we examined 60 R1 colonies derived from wild-type starter culture cells containing pBR322-NM and pSC101\textsuperscript{TS}-\textit{NEW1}. As before, every selected colony was patched onto selective medium to test for loss of pSC101\textsuperscript{TS}-\textit{NEW1-clpB} or pSC101\textsuperscript{TS}-\textit{NEW1} and inoculated into liquid medium for overnight growth to test for the presence of SDS-stable NM aggregates. All selected colonies had lost the appropriate temperature-sensitive vector as assessed by patching on selective medium (data not shown), and the absence of New1 and/or ClpB was confirmed by Western blot analysis (Figure 6B, Figure 6C). Whereas 17 of 60 (28\%) wild-type R1 samples tested aggregate-positive, all \textit{AclpB} R1 samples tested aggregate-negative.
Western blot analysis revealed that the wild-type and ΔclpB R1 cells contained comparable amounts of NM fusion protein (Figure 6B, Figure 6C). Furthermore, yeast transformation assays confirmed the presence of infectious material capable of converting $[\psi^-]$ yeast cells to $[\psi^+]$ in ΔclpB starter culture cells transformed with pBR322-NM and pSC101$^{TS}$-NEW1-clpB as well as in an aggregate-positive R1 clone derived from wild-type starter culture cells (Figure 6D). In contrast, a ΔclpB R1 clone derived from ΔclpB starter culture cells containing pBR322-NM and pSC101$^{TS}$-NEW1-clpB as well as an aggregate-negative R1 clone derived from wild-type starter culture cells containing pBR322-NM and pSC101$^{TS}$ lacked detectable infectivity (Figure 6D). We conclude that cells lacking ClpB cannot propagate NM in its infectious prion conformation.

**ClpB disaggregate activity is required for propagation of SDS-stable Sup35 NM aggregates**

To investigate the mechanistic basis for the ClpB dependence of Sup35 NM prion propagation in E. coli, we devised a strategy that enabled us to test the abilities of specific ClpB mutants to support the propagation of SDS-stable Sup35 NM aggregates after their formation in the presence of wild-type ClpB. We tested previously characterized ClpB mutants specifically defective for (i) ATP hydrolysis (ClpB E279A/E678A) (Weibezahn et al., 2003) (ii) substrate threading through the ClpB pore (Y653A) (Weibezahn et al., 2004), and (iii) collaboration with DnaK (E432A) (Oguchi et al., 2012; Seffer et al., 2012; Carroni et al., 2014). Our strategy required us to construct strains in which we could induce the production of a ClpB
mutant specifically during the propagation phase of the experiment while providing wild-type ClpB during the formation phase of the experiment only. To accomplish this, we placed each of the mutant clpB alleles (or the wild-type allele) under the control of the anhydrotetracycline (aTc)-inducible promoter P_{tetO-1} (Lutz and Bujard, 1997), integrated these constructs onto the chromosome of our ΔclpB strain, and transformed the resulting strains with pBR322-NM and pSC101^{TS}-NEW1-clpB.

As expected, we detected SDS-stable Sup35 NM aggregates in starter culture cells of all strains producing plasmid-encoded Sup35 NM-mCherry-His_{6X}, New1-mGFP, and wild-type ClpB (Figure 7A). To determine whether or not each of the mutants could support the propagation of these aggregates following the depletion of New1 and wild-type ClpB, we plated the starter culture cells at the nonpermissive temperature on solid medium lacking or containing increasing concentrations of aTc, generating sets of R1 colonies. We prepared cell extracts from scraped R1 colonies (see Materials and Methods) and examined these extracts for the presence or absence of SDS-stable Sup35 NM aggregates. Whereas SDS-stable Sup35 NM aggregates were detected as a function of increasing aTc concentration in cells carrying the wild-type clpB allele, no aggregates were detected in cells harboring the clpB E279A/E678A, clpB Y653A, or clpB E432A allele at any concentration of aTc (Figure 7A). Western blot analysis revealed that levels of chromosomally-encoded wild-type ClpB and each of the three disaggregase mutants were comparable in cell extracts prepared from colonies scraped off of plates containing 50 ng/ml aTc (Figure 7B). Furthermore, replica plating confirmed that all colonies of R1 cells grown on medium supplemented with 50 ng/ml aTc had been cured of pSC101^{TS-}.
NEW1-clpB (Supplemental Figure 8). We conclude that ATP hydrolysis coupled to substrate translocation through the ClpB central pore and collaboration with DnaK are required for propagation of SDS-stable Sup35 NM aggregates in the absence of New1.
DISCUSSION

Our findings establish that bacteria can propagate a prion. This is, to our knowledge, the first formal demonstration of prion propagation in a non-eukaryote. More specifically, the PIN-dependent de novo conversion of Sup35 NM to its prion form in our E. coli system enabled us to distinguish experimentally between the initial formation phase and subsequent propagation phase of the prion cycle. Under our experimental conditions, two of four cell lineages (L1E and L3E) maintained the prion for the duration of the experiment (i.e., ~100 generations), one lineage (L4E) maintained the prion for ~80 generations, and one lineage (L2E) maintained the prion for ~60 generations. Furthermore, our work demonstrates that prion propagation in E. coli requires ClpB, the bacterial ortholog of Hsp104. We conclude that bacteria can support a chaperone-dependent, protein-based mode of heredity and speculate that the emergence of prion-like phenomena may have predated the evolutionary split between eukaryotes and bacteria.

Stability of prion propagation in E. coli.

As only two of four lineages retained the prion for the full duration of our experiments, propagation of the Sup35 NM prion may be less stable in E. coli than in S. cerevisiae (Cox et al., 1980; DiSalvo et al., 2011). However, as noted above, loss of the prion in two lineages was coincident with a dramatic drop in NM fusion protein levels. Furthermore, this drop was evidently reversible as NM fusion protein levels were restored in R4 of L2E without reappearance of the prion. We do not understand the mechanism underlying this reversible change in protein levels; however, we suggest that stochastic fluctuations in plasmid copy number may set
the stage for such an event. We note that our experiments were performed in recA- cells, which should prevent plasmid rearrangements that might lead to a permanent loss of fusion protein coding capacity.

**Bacterial machinery capable of remodeling NM aggregates.**

The question of whether or not ClpB can substitute for Hsp104 in promoting Sup35 prion propagation has been addressed in a number of studies yielding conflicting indications. On one hand, several *in vitro* studies have provided evidence that Hsp104 (Shorter and Lindquist, 2004; Shorter and Lindquist, 2006; Shorter and Lindquist, 2008, DeSantis et al., 2012), but not ClpB (DeSantis et al., 2012), can fragment amyloid aggregates in the absence of auxiliary factors. Furthermore, whereas the presence of various combinations of S. cerevisiae Hsp70- and Hsp40-family proteins was found to modulate Hsp104 activity on amyloid substrates (Shorter & Lindquist, 2008; DeSantis et al., 2012), ClpB appeared to remain inert even in the presence of bacterial Hsp70 (DnaK), Hsp40 (DnaJ), and nucleotide exchange factor GrpE despite exhibiting robust activity on various disordered protein aggregates *in vitro* (DeSantis et al., 2012).

On the other hand, the results of several *in vivo* studies suggest that ClpB, in the presence of appropriate co-chaperones, is competent to support Sup35 prion propagation in yeast (Tipton et al., 2008; Reidy et al., 2012). Based on an analysis of the *in vivo* activities of Hsp104/ClpB chimeras, Tipton et al. argue that prion replication in yeast requires that Hsp104 collaborate with its cognate Hsp70 chaperone system. A logical inference from their work is that the inability of ClpB to substitute for Hsp104 in supporting Sup35 prion propagation in S. cerevisiae is an
indirect consequence of the inability of ClpB to cooperate with fungal co-chaperones. More recently, Reidy et al. provided direct support for this inference. In particular, Reidy et al. found that ClpB supported prion propagation in yeast provided that DnaK and GrpE were present. Interestingly, the activity of the bacterial disaggregase machinery in yeast was dependent on the fungal Hsp40-family Sis1 protein, consistent with prior work implicating Sis1 as a necessary component of the chaperone network required for prion propagation in yeast (Higurashi et al., 2008; Tipton et al., 2008). Based on these findings, Reidy et al. argue that the amyloid remodeling activity of Hsp104 is an evolutionarily conserved feature of the Hsp100-family chaperones, an inference that is strongly supported by our finding that propagation of the Sup35 NM prion in E. coli requires ClpB.

Despite the apparent prevalence of prions in the fungal kingdom, to date, no bacterial prion has been identified. Notably, the absence of cytoplasmic mixing during conjugation would preclude the discovery of prion-like phenomena by classic genetic approaches, which facilitated the discovery of prions in yeast based on the non-Mendelian inheritance of their associated phenotypes (Wickner, 1994; Cox, 1965; Aigle and Lacroute, 1975). Nevertheless, recent bioinformatics analyses of prokaryotic proteomes have revealed that bacterial and archaeal genomes encode many proteins containing glutamine- and asparagine-rich prion-like domains resembling those found in most confirmed and putative S. cerevisiae prions (Alberti et al., 2009; Espinosa Angarica et al., 2013; A. Yuan, S. Lindquist, and A. Hochschild, unpublished data). Moreover, it is becoming increasingly clear that Q/N-richness at the level of primary amino acid sequence is neither a prerequisite for prion
conversion (Taneja et al., 2007) nor protein amyloidogenesis (Goldschmidt et al., 2010). In fact, several bacteria utilize non-Q/N-rich amyloid-forming proteins to assemble extracellular appendages mediating surface attachment and biofilm formation (Chapman et al., 2002; Romero et al., 2010). These considerations—in conjunction with the work presented here—suggest that prions or prion-like proteins may exist as epigenetic reservoirs of phenotypic diversity in the bacterial domain of life.
MATERIALS AND METHODS

Strains, plasmids, and cell growth

Bacteria experiments were performed with *E. coli* strain DH5αZ1 (Lutz and Bujard, 1997) grown in LB (Miller) medium. To construct DH5αZ1 ΔclpB, a temperature-sensitive plasmid encoding the RecA protein (pSC101<sup>TS</sup>-<i>recA</i>) was constructed and transformed into DH5αZ1 cells. A ΔclpB::Kan<sup>R</sup> allele from strain JW2573 (Keio collection) was transferred to DH5αZ1 cells containing pSC101<sup>TS</sup>-<i>recA</i> via P1 transduction. Cells were subsequently cured of pSC101<sup>TS</sup>-<i>recA</i> by overnight growth and plating in the absence of antibiotic selection at the non-permissive temperature (37°C).

To construct strains harboring chromosomal P<sub>LtetO-1</sub>-<i>clpB</i> alleles, plasmids pAY152, pAY154, pAY155, pAY156, and pAY157 were cloned in strain AY290 and integrated onto the chromosome of strain AY295 at <i>attB(</i>HKO22<i>)</i>. Single-copy integrants were selected on LB agar supplemented with kanamycin (10 μg/ml) and verified by PCR as described (Haldimann and Wanner, 2001).

Yeast experiments were performed with *S. cerevisiae* [pin<sup>−</sup>][psi<sup>−</sup>] strain YJW18 grown in yeast extract peptone dextrose (YPD) medium. For yeast infectivity assays, cell extracts were co-transformed with pRS316 into YJW187 spheroplasts; [PSI<sup>+</sup>] Ura<sup>+</sup> transformants were identified by plating the yeast cells in top agar containing synthetic defined medium lacking uracil and adenine (SD-Ura-Ade) and supplemented with 10 mg/ml adenine hemisulfate (Sunrise Science).

Further details concerning strains and plasmids are provided in Supplemental Table 1.
Propagation experiments

Cells were transformed with pBR322-NM and pSC101{T5}, pSC101{T5}-NEW1, or pSC101{T5}-NEW1-clpB and grown at 30°C on LB agar supplemented with carbenicillin (Carb, 100 µg/ml) and chloramphenicol (Cam, 12.5 µg/ml). Starter cultures were generated by growing transformants at 30°C in 6 ml of LB broth supplemented with Carb (100 µg/ml), Cam (12.5 µg/ml), and 10 µM IPTG to an OD600 of 2.0-2.5. To cure cells of pSC101{T5}-derivatives and generate Round 1 (R1) colonies, starter cultures were diluted (10^{-5}) in pre-warmed (37°C) LB broth supplemented with Carb (100 µg/ml) and 10 µM IPTG. Diluted cells were grown at 37°C on pre-warmed (37°C) LB agar supplemented with Carb (100 µg/ml) and 10 µM IPTG. R1-R4 colonies were, (a) patched on LB agar supplemented with Cam (12.5 µg/ml), (b) restreaked and grown at 30°C on pre-warmed (30°C) LB agar supplemented with Carb (100 µg/ml) and 10 µM IPTG, and (c) inoculated and grown at 30°C in 6 ml LB broth supplemented with Carb (100 µg/ml) and 10 µM IPTG. For analysis of ClpB disaggregate mutants, ~1000 R1 colonies were gently scraped off LB agar plates containing Carb (100 µg/ml), 10 µM IPTG, and a range of aTc concentrations (0-500 ng/ml) in 3 ml LB broth supplemented with Carb (100 µg/ml) and 10 µM IPTG.

Cell cultures and scraped cell suspensions were normalized to 8 ml of an OD600 of 1.0 and pelleted by centrifugation. Cell pellets were resuspended in 166 µl STC Buffer (1 M sorbitol, 10 mM Tris-HCl [pH 7.5], 10 mM CaCl2) supplemented with 10 U of rLysozme (Novagen) and 0.1 U of OmniCleave endonuclease (Epicentre), incubated at room-temperature for 30 min, and incubated on ice for an
additional 30 min. Omnicleave endonuclease was omitted from samples destined for PCR analysis and yeast infectivity assays. Finally, samples were flash frozen and thawed on ice to yield unclarified lysates (used in filter retention assays). To generate partially clarified lysates (used in Western blot analysis and yeast transformation assays), unclarified lysates were subjected to two rounds of low-speed centrifugation, each at 500 RCF for 15 min at 4°C.

**Filter retention assays**

25 µl of unclarified lysates were added to 375 µl of BugBuster protein extraction reagent (Novagen) supplemented with 5 U of rLysozyme and 0.1 U of Omnicleave endonuclease and gently rocked at room-temperature for 30 min. Samples were challenged with 100 µl of 10% (w/v) SDS (2% SDS final concentration) and gently rocked at room-temperature for an additional 30 min. For each sample, 100 µl of undiluted lysate and three 2-fold serial dilutions made in PBS containing 2% SDS were filtered through a 0.2 µm cellulose acetate membrane (Advantec) in a dot-blotting vacuum manifold. Samples on membranes were washed twice with 100 µl of PBS containing 2% SDS and twice with 100 µl of PBS.

**Immunoblotting**

Cellulose acetate membranes (used in filter retention assays) and Hybond-C Extra nitrocellulose membranes (used in Western blot analysis) were blocked for 30 min in PBS containing 3% (w/v) milk. Membranes were probed with one of the following primary antibodies: anti-Sup35 (yS-20, Santa Cruz Biotechnology, 1:5,000), anti-His6x (His-2, Roche, 1:10,000), anti-GFP (Roche, 1:10,000), anti-RpoA
or anti-ClpB (gift from S. Wickner, 1:10,000). Membranes were washed and probed with one of the following HRP-conjugated secondary antibodies: anti-goat IgG (Santa Cruz Biotechnology, 1:10,000), anti-mouse IgG (Cell Signaling, 1:10,000), or anti-rabbit IgG (Cell Signaling, 1:10,000). Proteins were detected with ECL Plus Western blot detection reagents (GE Healthcare) and a ChemiDock XRS+ imaging system (Bio-Rad).

**Yeast infectivity assays**

Protein concentrations of partially clarified *E. coli* cell extracts were determined by the bicinchoninic acid (BCA) assay (ThermoFisher) and normalized to ~1 mg/ml. Protein transformations were performed as previously described (Tanaka and Weissman, 2006; Garrity et al., 2010). Each and every putative [*PSI*⁺] transformant was (a) restreaked on 1/4 YPD agar to assess the [*PSI*⁺] phenotype, (b) restreaked on YPD containing 3 mM GuHCl to cure cells of [*PSI*⁺], and (c) restreaked on 1/4 YPD to assess the [*psi⁻*] phenotype. Only those transformants exhibiting curability were scored as [*PSI*⁺].

**Fluorescence Microscopy**

Cells were spotted onto 1% (w/v) agarose pads consisting of Seakem LE Agarose (Lonza) in PBS and visualized with an UplanFL N 100x/1.30 phase contrast objective mounted on an Olympus BX61 microscope. Images were captured with a CoolSnapHQ camera (Photometrics) and the Metamorph software package (Universal Imaging). All fluorescence images were obtained from 10 ms exposures.
REFERENCES


Figure 1. Conversion of Sup35 NM to its prion form in E. coli requires New1.

A) Cartoon representation of how conversion of soluble Sup35 NM (Sup35_{soluble}) to its amyloid conformation (Sup35_{amyloid}) depends on the presence of New1 in its amyloid conformation (New1_{amyloid}). Sup35 NM and New1 (black) are depicted as fusions to mCherry (red) and mGFP (green), respectively. (B) SDS-stable Sup35 NM aggregates are detected only in cells producing SDS-stable New1 aggregates as assessed by filter retention analysis. For each sample, undiluted lysate and three two-fold dilutions are shown (see Materials and Methods). Sup35 NM and New1 aggregates are no longer detected once boiled. The α-Sup35 antibody recognizes the Sup35 NM-mCherry-His$_{6X}$ fusion protein, and the α-GFP antibody detects the New1-mGFP fusion protein. (C) Intracellular full-length (FL) Sup35 NM fusion protein levels are comparable in the presence and absence of New1 as assessed by Western blot analysis. The α-RpoA antibody recognizes the α subunit of E. coli RNA polymerase. (D) Fluorescence images of representative cells containing Sup35 NM and New1 or Sup35 NM alone. For cells containing both fusion proteins, the mCherry channel, GFP channel, and merged images are shown.
Supplemental Figure 1. α-Sup35 and α-His6x antibodies are interchangeable for detecting the Sup35 NM-mCherry-His6x fusion protein.

(A) Probing nitrocellulose membranes with either α-Sup35 antibody (Figure 1C) or α-His6x antibody results in the detection of similar intracellular Sup35 NM fusion protein products in the presence or absence of New1 as assessed by Western blot analysis. Full-length (FL) Sup35 NM fusion proteins are indicated by arrows. (B) Probing cellulose acetate membranes with either α-His6x antibody (Figure 3B) or α-Sup35 antibody results in the detection of similar aggregate-positive and aggregate-negative samples in starter cultures (ST) and Round 1 (R1) experimental clones as assessed by filter retention analysis. For each sample, undiluted lysate and three two-fold dilutions are shown. Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and negative (N) controls, respectively. (C) Probing cellulose acetate membranes with either α-His6x antibody (Figure 3D) or α-Sup35 antibody results in the detection of similar aggregate-positive and aggregate-negative samples in starter cultures (ST) and R1 control clones as assessed by filter retention analysis.
Starter culture (ST)
Cells contain Sup35 \+ NewI
Sup35 aggregates are formed at 30°C

Cure cells of NewI-encoding plasmid by plating at 37°C

For each of 20 R1 colonies:
(i) inoculate liquid medium for filter retention assay and
(ii) restreak on solid medium

Filter retention assay to detect SDS-stable Sup35 aggregates

Analyze the progeny of 4 retrospectively identified aggregate-positive clones (L1-L4)

x 10 for each lineage (L1-L4)

For each lineage, analyze the progeny of one retrospectively identified aggregate-positive R2 clone

x 10 for each lineage

For each lineage, analyze the progeny of one retrospectively identified aggregate-positive R3 clone

x 10 for each lineage

x 10 for each lineage
Figure 2. Experimental protocol for assessing the ability of *E. coli* cells to propagate SDS-stable Sup35 NM aggregates.

Experiments are initiated with either a starter culture (ST) of cells containing Sup35 NM and New1 (shown) or a starter culture of cells containing Sup35 NM alone (not shown). For each of the 4 lineages (L1-L4), the total number of generations over which Sup35 NM prion propagation is monitored corresponds to the number of cell divisions that occur in the absence of New1 during 4 rounds (R1-R4) of growth on solid medium and an additional round of growth in liquid medium. Growth in the absence of New1 begins at the time the starter culture cells are plated at 37°C (R1). Single R1 colonies were found to contain ~950,000 colony forming units (CFUs), and the liquid cultures contain ~10^8 CFUs per µl. Thus, prion propagation is monitored over 98.7 or ~100 generations.
Figure 3. Converted Sup35 NM can remain in its prion conformation in *E. coli* cells lacking New1.

(A) Cartoon representation of how Sup35 NM can convert to its prion form in the presence of New1 and remain in the prion conformation after cells have been cured of New1-encoding DNA. Sup35 NM and New1 (black) are depicted as fusions to mCherry (red) and mGFP (green), respectively. (B) SDS-stable Sup35 NM aggregates are detected in 8 of 20 Round 1 (R1) experimental clones derived from a starter culture (ST) of cells containing Sup35 NM and New1 as assessed by filter retention analysis. Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and negative (N) controls, respectively. The four aggregate-positive clones selected to establish the four experimental lineages are indicated by asterisks. In all 20 R1 experimental samples, intracellular Sup35 NM fusion protein levels are comparable, and New1 fusion protein is not detectable as assessed by Western blot analysis. The α-His<sub>6</sub> and α-
Sup35 antibodies recognize the Sup35 NM-mCherry-His6x fusion protein (see Supplemental Figure 1B, Supplemental Figure 1C), the α-GFP antibody detects the New1-mGFP fusion protein, and the α-RpoA antibody recognizes the α subunit of *E. coli* RNA polymerase. (C) In all 20 R1 experimental samples, DNA encoding Sup35 NM is detectable whereas DNA encoding the prionogenic module of New1 is not detectable by PCR. (D) SDS-stable Sup35 NM aggregates are not detected in any of the 20 R1 control clones derived from a starter culture of cells containing Sup35 NM alone as assessed by filter retention analysis. The four aggregate-negative clones selected to establish the four control lineages are indicated by asterisks. Intracellular Sup35 NM fusion protein levels are comparable in all 20 R1 control samples.
Figure 4. E. coli cells can propagate the Sup35 NM prion over ~100 generations.

(A) Experimental Lineage 1 (L1E). An aggregate-positive Round 1 (R1) experimental clone derived from a starter culture (ST) of cells containing Sup35 NM and New1 is identified and restreaked to yield progeny Round 2 (R2) clones (gray). All ten R2 clones analyzed contain detectable SDS-stable Sup35 NM aggregates. An aggregate-positive R2 clone is identified and restreaked to yield progeny Round 3 (R3) clones (blue). Again, all ten R3 clones analyzed contain SDS-stable Sup35 NM aggregates. An aggregate-positive R3 clone is identified and restreaked to yield progeny Round 4 (R4) clones (green). 9 of 10 R4 clones analyzed contain SDS-stable Sup35 NM aggregates. The filter retention assay is used to detect SDS-stable Sup35 NM aggregates. Intracellular Sup35 NM fusion protein levels are comparable in all 40 samples as assessed by Western blot analysis. Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and
negative (N) controls, respectively. The α-His₆ₓ and α-Sup35 antibodies recognize the Sup35 NM-mCherry-His₆ₓ fusion protein, and the α-RpoA antibody recognizes the α subunit of *E. coli* RNA polymerase. (B) Control Lineage 1 (L1c). An aggregate-negative R1 control clone derived from a starter culture of cells containing Sup35 NM alone is identified and restreaked to yield progeny R2 clones (gray), an aggregate-negative R2 clone is identified and restreaked to yield progeny R3 clones (blue), and an aggregate-negative R3 clone is identified and restreaked to yield progeny R4 clones (green). No SDS-stable Sup35 NM aggregates are detectable in any sample. Intracellular Sup35 NM fusion protein levels are comparable in all 40 samples as assessed by Western blot analysis.
Supplemental Figure 2. The fate of Sup35 NM in experimental Lineages 2-4.

(A) Experimental Lineage 2 (L2E). An aggregate-positive Round 1 (R1) clone derived from a starter culture (ST) of cells containing Sup35 NM and New1 is identified and restreaked to yield progeny Round 2 (R2) clones (gray). 8 of 10 R2 clones analyzed contain detectable SDS-stable Sup35 NM aggregates as assessed by filter retention analysis. An aggregate-positive R2 clone is restreaked to yield progeny Round 3 (R3) clones (blue). 0 of 10 R3 clones analyzed contain detectable SDS-stable Sup35 NM aggregates. The apparent loss of aggregates coincides with the loss of detectable Sup35 NM fusion protein as assessed by Western blot analysis. Assaying the 10 Round 4 (R4) progeny clones derived from an aggregate-negative R3 clone (green) reveals that fusion protein levels can be restored without the recovery of SDS-stable Sup35 NM aggregates. Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and negative (N) controls, respectively. The α-His$_{6\times}$ and α-Sup35 antibodies recognize the Sup35 NM-mCherry-His$_{6\times}$ fusion protein, and the α-RpoA antibody recognizes the α subunit of *E. coli* RNA polymerase. (B) Experimental Lineage 3 (L3E). 9 of 10 R2 clones analyzed contain detectable SDS-stable Sup35 NM aggregates, which are retained in 8 of 10 R3 progeny clones and 7 of 10 R4 progeny clones. (C) Experimental Lineage 4 (L4E). 8 of 10 R2 clones analyzed contain detectable SDS-stable Sup35 NM aggregates, which are retained in 9 of 10 R3 progeny clones. 0 of 10 R4 clones analyzed contain detectable SDS-stable Sup35 NM aggregates. As in R3 of L2E (A), the apparent loss of aggregates coincides with a dramatic drop in Sup35 NM fusion protein levels.
Supplemental Figure 3. The fate of Sup35 NM in control Lineages 2-4.

(A) Control Lineage 2 (L2c). An aggregate-negative Round 1 (R1) clone derived from a starter culture (ST) of cells containing Sup35 NM alone is identified and restreaked to yield progeny Round 2 (R2) clones (gray). 0 of 10 R2 clones analyzed contain detectable SDS-stable Sup35 NM aggregates as assessed by filter retention analysis. An aggregate-negative R2 clone is restreaked to yield progeny R3 clones (blue). 0 of 10 R3 clones analyzed contain detectable SDS-stable Sup35 NM aggregates. An aggregate-negative R3 clone is restreaked to yield progeny R4 clones (green). 0 of 10 R4 clones analyzed contain detectable SDS-stable Sup35 NM aggregates. Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and negative (N) controls, respectively. The α-His6x and α-Sup35 antibodies recognize the Sup35 NM-mCherry-His6x fusion protein, and the α-RpoA antibody recognizes the α subunit of E. coli RNA polymerase. (B) Control Lineage 3 (L3c). As in L2c (A), no SDS-stable Sup35 NM aggregates are detectable in progeny R2, R3, or R4 clones. (C) Control Lineage 4 (L4c). As in L2c (A) and L3c (B), no SDS-stable Sup35 NM aggregates are detectable in progeny R2, R3, or R4 clones.
Figure 5. Genealogy of *E. coli* cell lineages propagating Sup35 NM in an infectious prion conformation.

(A) The fate of Sup35 NM in four experimental lineages (L1E-L4E) established from a starter culture of cells containing Sup35 NM and New1 is shown. Clones that maintain or lose the Sup35 NM prion are indicated by black or orange lines,
respectively. Rounds 1-4 (R1-R4) are depicted as gray arcs, with R1 situated at the center of the tree. Clones are designated as aggregate-positive if they contain SDS-stable Sup35 NM aggregates that are detectable in the undiluted sample and at least 1 of the 3 two-fold serial dilutions, as analyzed by filter retention. L1E and L3E retain SDS-stable Sup35 NM aggregates for the duration of the experiment (Figure 4A, Supplemental Figure 2B). L2E and L4E lose detectable SDS-stable Sup35 NM aggregates at R3 and R4, respectively. In both cases, the loss of SDS-stable aggregates coincides with a dramatic yet apparently reversible drop in fusion protein levels (Supplemental Figure 2A, Supplemental Figure 2C; see Discussion). Cells from four aggregate-positive L1E-R4 clones visualized by fluorescence microscopy are indicated by asterisks. (B) The fate of Sup35 NM in four control lineages (L1C-L4C) established from a starter culture of cells containing Sup35 NM alone is shown. None of the 120 clones analyzed contain SDS-stable Sup35 NM aggregates (Supplemental Figure 3). Cells from four aggregate-negative L1C-R4 clones visualized by fluorescence microscopy are indicated by asterisks. (C) Fluorescence images of representative cells corresponding to the four aggregate-positive R4 clones indicated by asterisks in (A). (D) Fluorescence images of representative cells corresponding to the four aggregate-negative R4 clones indicated by asterisks in (B). (E) E. coli cell extracts containing propagated, SDS-stable Sup35 NM aggregates are infectious when transformed into S. cerevisiae [psi-] cells. A starter culture (ST) of cells containing Sup35 NM and New1 contain infectious SDS-stable Sup35 NM aggregates capable of converting [psi-] yeast cells to [PSI*]. In contrast, a starter culture of cells containing Sup35 NM alone lacks detectable infectivity. Progeny cell extracts transformed into yeast are identified as RX-Y, where X corresponds to a round number and Y corresponds to a clone number assigned sequentially and clockwise according to (A) and (B). Clones that gave rise to aggregate-negative progeny in the subsequent round are indicated by asterisks. Analysis of these data by Fisher’s exact test indicates that the differences in the frequency of [PSI*] transformants observed with samples containing SDS-stable Sup35 NM aggregates compared with the sample containing soluble Sup35 NM are statistically significant (p < 0.0001). The percentages given refer to strong [PSI*] transformants; samples containing SDS-stable Sup35 NM aggregates (but not samples containing soluble Sup35 NM) also gave rise to weak [PSI*] transformants (Supplemental Figure 4), but these were not quantified (see Results).
Supplemental Figure 4. Bacterial cell extracts containing propagated, infectious Sup35 NM aggregates yield both strong and weak {PSI}+ yeast transformants.

The phenotypes of five representative strong {PSI}+ (A) and weak {PSI}+ (B) strains obtained by transforming S. cerevisiae [psi−] cells with E. coli cell extracts containing propagated, SDS-stable Sup35 NM-aggregates on 1/4 YPD agar before (left) and after (right) passage on YPD agar supplemented with 3 mM GuHCl. For the purposes of comparison, untransformed [psi−], weak [PSI+], and strong [PSI+] yeast strains are shown.
Figure 6. Sup35 NM prion propagation in E. coli requires ClpB.

(A) Cartoon representation of how Sup35 NM can convert to its prion form in the presence of New1 and ClpB but cannot propagate in the prion conformation after cells have been cured of New1- and ClpB-encoding DNA. Sup35 NM and New1 (black) are depicted as fusions to mCherry (red) and mGFP (green), respectively. ClpB is depicted as a purple hexamer. (B) SDS-stable Sup35 NM aggregates are detected in 5 of 20 Round 1 (R1) wild-type (WT) clones derived from a starter culture (ST) of wild-type cells containing Sup35 NM and New1 as assessed by filter retention analysis. In total, 17 of 60 R1 wild-type clones are aggregate-positive.
Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and negative (N) controls, respectively. In all 20 R1 wild-type clones shown, full-length (FL) ClpB is detectable, Sup35 NM fusion protein levels are comparable, and New1 fusion protein is not detectable as assessed by Western blot analysis. The α-His6x and α-Sup35 antibodies recognize the Sup35 NM-mCherry-His6x fusion protein, the α-GFP antibody recognizes the New1-mGFP fusion protein, the α-ClpB antibody recognizes the E. coli ClpB chaperone, and the α-RpoA antibody recognizes the α subunit of E. coli RNA polymerase. Cells from four aggregate-positive R1 wild-type clones visualized by fluorescence microscopy (Supplemental Figure 6C) are indicated by asterisks. (C) SDS-stable Sup35 NM aggregates are not detectable in R1 ΔclpB clones derived from a starter culture of ΔclpB cells containing Sup35 NM, New1, and ectopically produced ClpB as assessed by filter retention analysis. In total, 0 of 60 R1 ΔclpB clones are aggregate-positive (Supplemental Figure 6B). In all 20 R1 ΔclpB clones shown, Sup35 NM fusion protein levels are comparable, and neither ClpB nor New1 fusion protein is detectable as assessed by Western blot analysis. Cells from four aggregate-negative R1 wild-type clones visualized by fluorescence microscopy (Supplemental Figure 6D) are indicated by asterisks. (D) Extract prepared from cells lacking ClpB is not infectious when transformed into S. cerevisiae [psi−] cells. Starter cultures of wild-type cells transformed with pBR322-NM and pSC101TS-NEW1 as well as ΔclpB cells transformed with pBR322-NM and pSC101TS-NEW1-clpB contain infectious SDS-stable Sup35 NM aggregates capable of converting [psi−] yeast cells to [PSI+]. Wild-type starter culture cells containing Sup35 NM alone lack detectable infectivity. An aggregate-positive R1 wild-type clone retains infectious Sup35 NM aggregates. In contrast, an aggregate-negative R1 ΔclpB clone lacks detectable infectivity. Analysis of these data by Fisher’s exact test indicates that the differences in the frequency of [PSI+] transformants observed with samples containing SDS-stable Sup35 NM aggregates compared with the samples containing soluble Sup35 NM are statistically significant (p < 0.0001).
Supplemental Figure 5. ΔclpB cells containing New1 and ectopically produced ClpB can convert Sup35 NM to its prion form.

(A) SDS-stable Sup35 NM aggregates are detected in wild-type (WT) cells producing SDS-stable New1 aggregates as assessed by filter retention analysis. SDS-stable Sup35 NM aggregates are also detected in ΔclpB cells containing SDS-stable New1 aggregates and ectopically produced ClpB. The α-His₆ antibody detects the Sup35 NM-mCherry-His₆ fusion protein, and the α-GFP antibody detects the New1-mGFP fusion protein. A lane cropped from the same immunoblot is indicated by a hash mark. Intracellular levels of full-length (FL) ClpB, Sup35 NM fusion protein, and New1 fusion protein are comparable in the presence and absence of New1 and ectopically produced ClpB as assessed by Western blot analysis. The α-ClpB antibody recognizes the E. coli ClpB chaperone, the α-Sup35 antibody recognizes the Sup35 NM fusion protein, and the α-RpoA antibody recognizes the α subunit of E. coli RNA polymerase. (B) Fluorescence images of representative wild-type cells containing Sup35 NM and New1 and ΔclpB cells containing Sup35 NM, New1, and ectopically produced ClpB. The mCherry channel, GFP channel, and merged images are shown.
Supplemental Figure 6. Propagation of the Sup35 NM prion in E. coli requires ClpB.

(A) SDS-stable Sup35 NM aggregates are detected in 12 of 40 Round 1 (R1) wild-type (WT) clones derived from a starter culture (ST) of wild-type cells containing Sup35 NM and New1 as assessed by filter retention analysis. In total, 17 of 60 R1 wild-type clones are aggregate-positive (Figure 6B). Starter cultures of cells containing Sup35 NM and New1 and cells containing Sup35 NM alone serve as positive (P) and negative (N) controls, respectively. The α-His$_{6X}$ antibody detects the Sup35 NM-mCherry-His$_{6X}$ fusion protein. (B) In contrast, 0 of 40 R1 ΔclpB clones derived from a starter culture of ΔclpB cells transformed with pBR322-NM and pSC101$^{TS}$-NEW1-clpB contain detectable SDS-stable Sup35 NM aggregates. In total, 0 of 60 R1 ΔclpB clones are aggregate-positive (Figure 6C). Cells from four aggregate-negative R1 ΔclpB clones visualized by fluorescence microscopy are indicated by asterisks. The observed difference in the number of aggregate-positive clones of wild-type versus ΔclpB cells is statistically significant (p < 0.0001 as determined by Fisher’s Exact Test). (C) Fluorescence images of representative cells corresponding to the four aggregate-positive R1 wild-type clones indicated by asterisks in (Figure 6B). Notably, wild-type clone R1-14 exhibits twisted ring structures. (D) Fluorescence images of representative cells corresponding to the four aggregate-negative R1 ΔclpB clones indicated by asterisks in (Figure 6C).
Figure 7. Propagation of SDS-stable Sup35 NM aggregates in E. coli requires ClpB disaggregase activity.

(A) SDS-stable Sup35 NM aggregates are detected in starter cultures (ST) of ΔclpB cells containing pBR322-NM, pSC101TS-NEW1-clpB, and one of four aTc-inducible chromosomal clpB alleles. Wild-type (WT) ClpB is depicted as a purple hexamer. ClpB E279A/E678A is unable to hydrolyze ATP, ClpB Y653A is pore-deficient, and ClpB E432A is unable to collaborate with DnaK. Propagated Sup35 NM aggregates are detected in scraped cell suspensions as a function of increasing aTc concentration only for Round 1 (R1) clones producing wild-type ClpB. At no aTc concentration are Sup35 NM aggregates detected in scraped cell suspensions of R1 clones producing ClpB disaggregase mutants or in R1 clones lacking ClpB. Lanes cropped from the same immunoblot are indicated by hash marks. The α-His6X antibody recognize the Sup35 NM-mCherry-His6X fusion protein. (B) Wild-type and mutant ClpB levels along with Sup35 NM fusion protein levels are comparable in R1 clones grown on solid medium supplemented with 50 ng/ml aTc as assessed by
Western blot analysis. The α-Sup35 antibody recognizes the Sup35 NM-mCherry-His6X fusion protein, the α-GFP antibody recognizes the New1-mGFP fusion protein, the α-ClpB antibody recognizes the *E. coli* ClpB chaperone, and the α-RpoA antibody recognizes the α subunit of *E. coli* RNA polymerase.
Supplemental Figure 8. All Round 1 clones producing wild-type ClpB are cured of pSC101<sup>TS</sup>-NEW1-clpB.

All Round 1 (R1) clones derived from a starter culture of ΔclpB cells containing pBR322-NM, pSC101<sup>TS</sup>-NEW1-clpB, and chromosomal aTc-inducible wild-type clpB lose pSC101<sup>TS</sup>-NEW1-clpB as assessed by replica plating from solid medium supplemented with carbenicillin (Carb), chloramphenicol (Cam), IPTG, and 50 ng/ml aTc to solid medium containing either Carb or Cam. pSC101<sup>TS</sup>-NEW1-clpB confers Cam resistance.
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Supplemental Table 1. Strains and plasmids used in this study.
APPENDIX A

A transcription-based reporter for prion propagation in *E. coli*

Specific author contributions:

I devised and performed all experiments presented in this appendix.
RESULTS AND DISCUSSION

The data presented in Chapter Two suggest that ClpB levels are elevated specifically in *E. coli* clones containing SDS-stable Sup35 NM prion aggregates (see Figure 6B of Chapter Two). Taking advantage of this observation, we engineered a $P_{clpB}$-lacZ reporter (present on an F' episome) to determine whether or not increased transcription from the *clpB* promoter can serve as a reliable indicator of the presence or absence of a prion in *E. coli* cells.

Starter cultures of cells containing F' $P_{clpB}$-lacZ, pBR322-NM (encoding the IPTG-inducible synthesis of NM-mYFP-His$_6$ fusion protein), and either pSC101$^{TS}$-NEW1 (encoding the IPTG-inducible synthesis of New1-mCFP-HA$_3$ fusion protein; experimental sample) or pSC101$^{TS}$ (empty vector; control sample) were grown overnight to allow for the formation of the NM prion in the experimental sample (see Figure 2 of Chapter Two for further details). The cells were plated on X-gal-containing indicator plates and grown at the non-permissive temperature to cure the cells of pSC101$^{TS}$-NEW1 or pSC101$^{TS}$. Dark blue Round 1 (R1) colonies were observed at a frequency of up to ~10% for the experimental sample cells. In contrast, all R1 colonies derived from control sample cells appeared pale blue (Figure 1A). Critically, all dark blue colonies consisted of prion-containing cells whereas all pale blue colonies consisted of prion-free cells as assessed by filter retention analysis (Figure 1B).

Upon restreaking at the permissive temperature, dark blue R1 clones yielded a mixture of dark blue and white (apparently *lacZ*-) Round 2 (R2) progeny clones (data not shown). An additional round of restreaking revealed that dark blue R2
clones yielded exclusively dark blue Round 3 (R3) clones whereas white R2 clones yielded exclusively white R3 clones (Figure 1C, sectors 1 and 2). Although we do not yet understand the appearance of white clones derived from experimental sample cells specifically in Round 2 (selection for the F' episome was maintained throughout experiments), we speculate that the presence of the NM prion may promote episomal rearrangements at the non-permissive temperature. Support for this hypothesis comes from the observation that all pale blue R1 clones derived from control sample cells yielded exclusively pale blue R2 and R3 progeny clones (Figure 1C, sectors 3, 4 and 5). Dark blue and white R3 clones that were derived from dark blue R1 colonies contained the NM prion, whereas pale blue R3 clones that were derived from pale blue R1 colonies lacked the NM prion as assessed by fluorescence microscopy (Figure 1D, R3-1, R3-2, and R3-4).

As established in Chapter Two, Sup35 NM prion propagation in E. coli requires the disaggregase activity of the ClpB chaperone. We sought to recapitulate this ClpB-dependence in the context of our transcription-based reporter. To do so, we engineered ΔclpB cells harboring an anhydrotetracycline (aTc)-inducible copy of clpB on the chromosome. Experimental and control starter cultures of these ClpB-depletable cells were grown overnight in the presence of aTc and plated at the non-permissive temperature on X-gal- and aTc-containing indicator plates. Dark blue R1 colonies were observed only among colonies derived from the experimental sample. As before, these dark blue R1 clones yielded a mixture of dark blue and white R2 progeny clones when restreaked on aTc-containing medium (data not shown). However, dark blue R2 clones restreaked on medium lacking aTc yielded pale blue
R3 progeny clones (Figure 1C, sectors 6, 7, and 8) that had been cured of the NM prion as assessed by fluorescence microscopy (Figure 1D, R3-7).

Transcription in bacteria is performed by a multisubunit RNA polymerase (RNAP). To initiate promoter-specific transcription, RNAP must associate with one of several sigma factors. In particular, *E. coli* contains a primary sigma factor known as $\sigma^{70}$ and six alternative sigma factors, including the heat shock sigma factor $\sigma^{32}$. $\textit{clpB}$ transcription is controlled by a $\sigma^{32}$-dependent promoter and is thus upregulated during heat shock (Nonaka et al., 2006). As the activity and stability of $\sigma^{32}$ are negatively regulated by DnaK (Nagai et al., 1994; Rodriguez et al., 2008), which we previously implicated in Sup35 NM prion propagation (see Figure 7A of Chapter Two), we hypothesize that the presence of prion aggregates results in the titration of DnaK away from $\sigma^{32}$, leading to upregulation of $\sigma^{32}$-dependent genes including $\textit{clpB}$ (Figure 1E).

While it is unlikely that the P$_{\text{clpB-lacZ}}$ reporter discriminates between amyloid and non-amyloid protein aggregates, it nevertheless holds great potential in facilitating the discovery of prion-like phenomena. Transient production of a prion protein at high levels can facilitate the establishment of its corresponding prion form, which self-propagates even when the prion protein is subsequently produced at lower levels (Wickner, 1994). Accordingly, we are now situated to screen IPTG-inducible plasmid libraries encoding bacterial proteins for prion-like entities by (i) transiently inducing protein overproduction in library-containing *E. coli* cells at high [IPTG], (ii) plating cells at low [IPTG] on appropriate X-gal and aTc-containing indicator plates, and (iii) identifying clones manifesting signs of a persistent fever.
(i.e., dark blue colonies exhibiting a heat shock response under low [IPTG] conditions) that can be cured by ClpB-depletion as assessed by (iv) restreaking on medium lacking aTc. More generally, our ability to now reliably distinguish between prion-containing and prion-lacking *E. coli* clones will facilitate experimental strategies aimed at identifying environmental conditions and bacterially-encoded factors that influence the stability of prion propagation.
REFERENCES


**Figure 1.** Transcription from the *clpB* promoter reports on Sup35 NM prion propagation in *E. coli.*

(A) Dark blue clones containing an F' P_{clpB-lacZ} reporter are detected among Round 1 (R1) descendants of experimental (E) starter cultures of cells containing NM and New1 whereas all R1 descendants of control (C) starter cultures of cells containing NM alone are pale blue (i.e., 0 out of ~1,000 R1c colonies are dark blue). Cells were plated and grown at the non-permissive temperature (37°C) on medium containing carbenicillin (100 μg/ml), X-gal (40 μg/ml), 500 μM TPEG, and 10 μM IPTG. (B) All 8 dark blue R_{1E} clones tested contain SDS-stable NM aggregates whereas all 50 pale blue R_{1E} clones tested lack NM aggregates as assessed by filter retention analysis. All clones were patched onto medium containing chloramphenicol (25 μg/ml) to confirm loss of the New1-encoding plasmid. Starter cultures (ST) of cells containing NM and New1 and cells containing NM alone serve as positive (P) and negative (N) controls, respectively. For each sample, undiluted lysate and three two-fold dilutions are shown. The α-His6x antibody recognizes the Sup35 NM-mYFP-His6x fusion protein. (C) Dark blue R_{1E} clones yield a mixture of dark blue and white Round 2 (R2) clones, which yield homogenous dark blue (sector 1) and white (sector 2) Round 3 (R3) progeny clones. Pale blue R_{1C} clones yield pale blue R2 and R3 progeny clones (sectors 3, 4, and 5). Dark blue R_{1E} clones that depend on aTc for ClpB production yield a mixture of dark blue and white R2 clones in the presence of aTc (100 ng/ml). Dark blue R2 clones yield pale blue R3 clones (sectors 6, 7, and 8) grown on medium lacking aTc. (D) Fluorescence images of representative cells corresponding to R3 clones identified by sector number in (C). The cells from sectors 1 and 2 contain fluorescent foci characteristic of prion-containing cells, whereas the cells from sectors 4 and 7 exhibit diffuse fluorescence characteristic of cells containing soluble NM fusion protein. (E) A working model of the transcription-based reporter for prion propagation in *E. coli.* In cells containing the Sup35 NM prion (red), ClpB (purple) and DnaKJE (gray) are titrated away from σ^{32}. As σ^{32} is negatively regulated by DnaK, the presence of the prion indirectly activates transcription from the *clpB* promoter.
APPENDIX B

ClpB is the only non-essential chaperone or protease required for prion propagation in *E. coli*

Specific author contributions:

I devised and performed all experiments presented in this appendix.
RESULTS AND DISCUSSION

As discussed in Chapter One, multiple chaperone proteins play critical and complex roles in promoting prion heritability in *S. cerevisiae* (Liebman and Chernoff, 2012). However, the specific effects of yeast chaperones and - based on data presented in Chapter Two – bacterial chaperones on prion propagation remain controversial.

On one hand, *in vitro* experiments performed with purified components suggested that ClpB is unable to fragment prion amyloid aggregates even in the presence of bacterial Hsp70 (DnaK), Hsp40 (DnaJ), and the nucleotide exchange factor GrpE (DeSantis et al., 2012). On the other hand, *in vivo* studies indicated that ClpB could support [PSI+] propagation in yeast provided that DnaK and GrpE were co-produced (Tipton et al., 2008; Reidy et al., 2012). Intriguingly, the ability of ClpB to propagate [PSI+] was strictly dependent on the presence of the yeast Hsp40-family protein Sis1 (Reidy et al., 2012). *E. coli* contains three Hsp40 paralogs (DnaJ, DjlA, and CbpA), and sequence alignment suggests that CbpA is unique in sharing homology with Sis1 over its entire length, raising the possibility that CbpA rather than DnaJ is required for ClpB-dependent Sup35 NM prion propagation in bacteria.

Taken together, these preliminary considerations led us to determine whether or not protein remodeling factors other than the ClpB disaggregase are required for the propagation of a prion in *E. coli* cells. As studies performed in yeast suggest links between ubiquitin-mediated proteolysis and prion behavior (Allen et al., 2007; Kabani et al., 2014; Yang et al., 2014), we included protein degradation factors in our analysis as well. To perform this analysis, we systematically deleted
most non-essential chaperones and proteases in *E. coli* (we note that while *dnaJ* is non-essential, deletion of *dnaJ* in DH5αZ1 – the strain we use to monitor prion propagation in our *E. coli* system [Lutz and Bujard, 1997; Yuan et al., 2014] – renders cells too sick for analysis). Deletion strains were transformed with pBR322-NM (encoding the IPTG-inducible synthesis of NM-mCherry-His6x fusion protein) and pSC101<sup>TS</sup>-NEW1 (encoding the IPTG-inducible New1-mGFP fusion protein). Transformants were grown overnight to allow for the formation of the Sup35 NM prion and subsequently plated at the non-permissive temperature to cure the cells of pSC101<sup>TS</sup>-NEW1. We prepared cell extracts from scraped Round 1 (R1) colonies (see Figure 7A and Materials and Methods of Chapter Two) and tested samples for the presence or absence of SDS-stable Sup35 NM aggregates (Figure 1). Our results establish that ClpB is likely the only non-essential chaperone or protease required for prion propagation in *E. coli* cells.

Although our data confirm that ClpB is the principal mediator of Sup35 NM prion propagation in *E. coli*, we remain intrigued by the possibility that protein remodeling or degradation factors may alter the frequency and stability of aggregate-positive R1 clones and their progeny. With the development of the transcription-based reporter described in Appendix A, we are now situated to quantitatively assess prion propagation in our deletion strain collection.
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Figure 1. ClpB is the only non-essential chaperone or protease required for Sup35 NM prion propagation in *E. coli*.

Genes encoding 15 non-essential protein remodeling factors (purple) and 11 non-essential protein degradation factors (red) were systematically deleted in strain DH5αZ1 by P1 transduction (see Materials and Methods of Chapter Two). Each deletion strain was transformed with plasmids encoding NM and New1. Transformants were grown overnight to allow for the formation of the NM prion and plated at the non-permissive temperature (37°C) on medium containing carbenicillin (100 μg/ml) and 10 μM IPTG. For each strain, 10 Round 1 (R1) clones were patched onto medium containing chloramphenicol (25 μg/ml) to confirm loss of the New1-encoding plasmid before all remaining R1 clones (~1000 colonies) were gently scraped off of plates. Filter retention analysis of cell extracts prepared from scraped cell samples indicates that ClpB is likely the only protein remodeling/degradation factor required for Sup35 NM prion propagation in *E. coli*. Wild-type R1 clones derived from starter cultures of cells containing NM and New1
and cells containing NM alone serve as positive (P) and negative (N) controls, respectively. For each sample, undiluted lysate and three two-fold dilutions are shown. The α-His6 antibody recognizes the Sup35 NM-mCherry-His6 fusion protein, and the α-GFP antibody recognizes the New1-mGFP fusion protein.
CHAPTER THREE

Evidence of a bacterial prion

Specific author contributions:

I devised and performed all experiments and computational analyses presented in this chapter. Alex Lancaster (Whitehead Institute for Biomedical Research) generously shared code for the HMM-based algorithm used to mine all bacterial genomes for candidate prion proteins.
ABSTRACT

Prions are self-propagating protein aggregates. First described in mammals as the causative agent of the fatal transmissible spongiform encephalopathies, prions have also been uncovered in yeast and other fungi, where they function as non-Mendelian genetic elements. We previously demonstrated that the bacterium Escherichia coli can support both the formation and chaperone-dependent propagation of a fungal prion, but whether or not bacterial prion proteins exist in nature remained an open question. Here we provide preliminary evidence that transcription termination factor Rho of Clostridium botulinum may be a bacterial prion-like protein. Our findings suggest that prions exist in the bacterial domain of life and raise the possibility that the phenomenon of protein-based heredity emerged early in evolutionary history.
INTRODUCTION

Prions are infectious protein aggregates that are characteristically transmissible (Prusiner, 1982). A prion protein typically exists in one of two states, either a native soluble form or an aggregated amyloid form. Amyloids are highly-ordered fibrillar structures characterized by a cross-β spine and are associated with many non-transmissible neuropathologies like Alzheimer's, Parkinson's, and Huntington's diseases (Diaz-Espinoza and Soto, 2012; Chiti and Dobson, 2006). Despite intensive investigation, the sequence determinants of amyloidogenesis remain poorly understood, and those features that distinguish non-infectious amyloids from self-propagating prions remain mysterious.

First described in the context of scrapie, the term prion was coined by Prusiner in reference to the nuclease- and UV-resistant particle – subsequently identified as the prion form of the PrP protein – responsible for scrapie, a disease of sheep and goats (Prusiner, 1982; Prusiner et al., 1984). Scrapie belongs to a class of inevitably fatal neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs), which afflict animals and humans (Aguzzi, 2008). Prions have also been discovered in fungi, where they act as protein-based units of heredity that regulate a spectrum of biological processes including translation termination, nitrogen catabolism, heterokaryon incompatibility, sterol biosynthesis, and multicellular growth (Cox, 1965; True et al., 2004; Aigle and Lacroute, 1975; Wickner, 1994; Coustou et al., 1997; Suzuki et al., 2012; Holmes et al., 2013).

To date, prion proteins and prion-like phenomena have been uncovered exclusively in eukaryotes (Aguzzi et al, 2008; Liebman and Chernoff, 2012; Si et al.,
Here we provide evidence that transcription termination factor Rho of *Clostridium botulinum E3 str. Alaska E43* exhibits characteristics of *bona fide* prion proteins. Our findings suggest that prion-like mechanisms may operate in the bacterial domain of life and that protein-based heredity represents a ubiquitous, ancient mode of epigenetic regulation.
RESULTS

A bioinformatics screen identifies bacterial prion candidates

We conducted a large-scale implementation of a hidden Markov model (HMM)-based algorithm developed in the Lindquist lab to mine all sequenced bacterial genomes for proteins containing candidate prion domains (cPrDs) that resemble a training set of 28 glutamine/asparagine-rich yeast prion proteins (Alberti et al., 2009; Lancaster et al., 2014). Our query returned ~ 2,000 high-scoring proteins containing a candidate bacterial prion domain of at least 60 amino acids (the cPrD "core" polypeptide) in length. The majority of computationally predicted bacterial prion proteins were annotated as membrane-associated and/or extracellular proteins, with the greatest number of proteins belonging to the Firmicutes.

To generate a manageable list of putative cPrDs for experimental validation, we focused our attention on cytoplasmic proteins that were represented in more than one bacterial phylum and have an unambiguous ortholog present in E. coli. Taking into consideration the transferable modularity of yeast PrDs (Masison et al., 1997; Li and Lindquist, 2000), we chose to pursue three proteins of known function that exhibit well-defined domain organization: translation initiation factor IF-2 (three orthologs representing two phyla), transcription termination factor Rho (five orthologs representing four phyla), and single-stranded DNA binding protein SSB (eight orthologs representing four phyla) (Figure 1, Supplemental Figure 1).
A subset of cPrDs form extracellular amyloid aggregates at the *E. coli* cell surface

For each candidate bacterial prion protein, we synthesized *E. coli* codon-optimized DNA encoding a previously defined structural domain encompassing the predicted cPrD. As a first test for prion-like properties, we asked whether or not the cPrD of each of the 16 candidate bacterial prion proteins is capable of forming extracellular amyloid at the surface of *E. coli* cells. To do so, we took advantage of a previously described assay (Sivanathan and Hochschild, 2012) that exploits the natural ability of *E. coli* cells to elaborate amyloid structures known as curli fibers at the cell surface (Chapman et al., 2002).

Curli fibers are composed of the CsgA and CsgB proteins, which are first secreted across the *E. coli* inner membrane via the SecYEG translocon and subsequently directed out of the cell via the outer membrane CsgG pore protein, where they assemble as surface-attached amyloid fibrils (Chapman et al., 2002; Hammer et al., 2007; Blanco et al., 2011). CsgA and CsgB share a bipartite N-terminal signal sequence dictating secretion specificity that can be transferred to heterologous proteins (Robinson et al., 2006; Sivanathan and Hochschild, 2012). Furthermore, secretion via the curli export pathway results in the production of extracellular amyloid fibrils specifically for heterologous proteins (such as yeast prion proteins) that have an inherent amyloid-forming propensity (Sivanathan and Hochschild, 2012). Appropriation of the curli secretion system thus serves as the foundation for a cell-based assay of protein amyloidogenicity known as curli-dependent amyloid generator (C-DAG) (Sivanathan and Hochschild, 2013).
The use of C-DAG as a test of protein amyloidogenicity requires that cells be plated on solid medium, presumably to ensure a sufficiently high local concentration of the secreted protein to permit fibril assembly. Two convenient methods can be employed to detect extracellular amyloid material associated with individual colonies. The first of these methods depends on the use of the amyloid-binding dye Congo red (CR). Like natural curli-producing *E. coli* cells, cells engineered to secrete an amyloidogenic polypeptide via the curli secretion pathway typically form red colonies when grown on solid medium containing CR (Sivanathan and Hochschild, 2012). Although the red colony phenotype is not in and of itself sufficiently selective (*i.e.*, false positives are relatively frequent), CR-bound amyloid aggregates characteristically exhibit so-called "apple-green" birefringence when observed by bright-field microscopy between crossed polarizers (Howie et al., 2008; Teng and Eisenberg, 2009). The second method depends on another characteristic of amyloid aggregates, namely, their resistance to denaturation in the presence of SDS. Thus, scraped cell suspensions can be examined for the presence of SDS-stable aggregates by means of the filter retention assay described in Chapter Two.

Of the 16 cPrDs (and 7 of the corresponding 60 amino acid core sequences; see legend to Figure 2A) tested by C-DAG, 12 yielded SDS-stable aggregates as assessed by filter retention analysis (Figure 2A, see Materials and Methods). Among these 12 aggregate-positive samples, 8 exhibited "apple-green" birefringence (Figure 2B, Figure 2C).
**C. botulinum Rho forms SDS-stable aggregates in the *E. coli* cytoplasm**

To monitor the intracellular behavior of cPrDs, we fused each cPrD that tested aggregate-positive in the C-DAG assay (as well as the previously defined structural domain encompassing the corresponding cPrD) to a monomeric fluorescent protein (mYFP bearing a C-terminal hexahistidine epitope tag) and overproduced the resulting chimeras from arabinose-inducible plasmids in the *E. coli* cytoplasm. Among all of the YFP fusion proteins tested, we detected SDS-stable aggregates only for the 68 residue cPrD and the 183 residue N-terminal domain (NTD) of *Clostridium botulinum E3 str. Alaska E43* transcription termination factor Rho (*Cb Rho*) (Figure 3A, Figure 3B). SDS-stability was assessed by two complementary methods: filter retention analysis and semidenaturing detergent-agarose gel electrophoresis (SDD-AGE), which permits the visualization of SDS-stable amyloid polymers as high molecular weight smears (Halfmann and Lindquist, 2008). Furthermore, fluorescence microscopy revealed that the *Cb Rho NTD* fusion protein forms non-diffuse intracellular structures similar to those formed by yeast PrDs produced in *E. coli* cells (Figure 3C) (Garrity et al., 2010; Yuan et al., 2014).

Next, we synthesized *E. coli* codon-optimized DNA encoding full-length *Cb Rho* bearing a C-terminal hexahistidine epitope tag and produced the protein in the *E. coli* cytoplasm. SDD-AGE analysis revealed that full-length *Cb Rho* can form high molecular weight SDS-stable aggregates (Figure 4A). As discussed in Chapter Two, the ability of Sup35 to convert to its prion form in yeast depends on the presence of another prion known as a [*PSI*⁺] inducibility (PIN) factor (Derkatch et al., 1997). Remarkably, like that of Sup35, the ability of full-length *Cb Rho* to form SDS-stable...
aggregates in \textit{E. coli} cells was greatly facilitated by the co-production of the PrD of the yeast PIN factor New1 (Figure 4A, Figure 4B) (Derkatch et al., 2001; Osherovich and Weissman, 2001; Garrity et al., 2010).

\textbf{The \textit{C. botulinum} Rho cPrD behaves as an Hsp104-dependent prion in yeast}

We next asked whether or not the \textit{Cb} Rho cPrD and NTD exhibit prion-like behavior in a yeast Sup35-based assay (Alberti et al., 2010). The well-characterized prion protein Sup35 is an essential translation termination factor, the non-prion and prion forms of which are known as \([\psi^-]\) and \([\Psi^+]\), respectively (Stansfield et al., 1995; Paushkin et al., 1997; Cox, 1965; Wickner, 1994). In \([\psi^-]\) cells, Sup35 is soluble and mediates translation termination at stop codons, whereas in \([\Psi^+]\) cells, Sup35 is aggregated, resulting in stop codon readthrough (True et al., 2004; Baudin-Baillieu et al., 2014). Sup35 is composed of an N-terminal PrD (N), a highly-charged middle region (M), and a C-terminal domain specifying translation release activity (C) (Ter-Avanesyan et al., 1993). Together, Sup35 NM constitutes a prionogenic moiety that is separable from Sup35 C (Li and Lindquist, 2000). The modular organization of Sup35 and other yeast prion proteins permits the characterization of Sup35 C fusion proteins, which can support \([\Psi^+]\)-like states (Sondheimer and Lindquist, 2000; Osherovich and Weissman, 2001). \([\Psi^+]\) and \([\Psi^+]\)-like phenotypes can manifest themselves in an \textit{ade1-14} genetic background. As the \textit{ade1-14} allele encodes a premature stop codon in the adenine biosynthesis gene \textit{ADE1}, \([\psi^-]\) cells are defective for adenine synthesis, grow poorly on adenine-deficient medium, and form red colonies due to the accumulation of phosphoribosylaminoimidazole (Chernoff et al., 2002; Wickner, 2012). In contrast,
[PSI'] and [PSI']-like cells contain a complete adenine biosynthesis pathway, grow well on adenine-deficient medium, and form white colonies.

Taking advantage of these features of [PSI'], we constructed ade1-14 sup35::KanMX4 yeast strains constitutively producing native Sup35 or one of the two Cb Rho domains fused to Sup35C as the sole source of cellular translation release activity (see Materials and Methods). As transient overproduction of a prion protein can increase the frequency of switching to the prion state (Wickner, 1994), we transformed these strains with galactose-inducible plasmids encoding Sup35 NM, the Cb Rho PrD, or the Cb Rho NTD fused to EGFP. Transformants were grown in the presence or absence of galactose, visualized by fluorescence microscopy, and plated on medium lacking adenine. Cells grown in the presence of galactose exhibited non-diffuse intracellular structures similar to those reported previously in studies of yeast prions (Figure 5A) (Alberti et al., 2009; Arslan et al., 2015). Furthermore, transient overproduction of Sup35 NM and the Cb Rho PrD, but not the Cb Rho NTD, increased the frequency of switching to [PSI'] and [PSI']-like prion states as inferred from the number and color of colonies grown on adenine-deficient medium (Figure 5B). As has been observed for other yeast prion protein PrD-Sup35 C chimeras, the Cb Rho NTD-Sup35 C fusion protein is sufficiently aggregation-prone to confer a [PSI']-like state without the need for transient Cb Rho NTD-EGFP fusion protein overproduction (Alberti et al., 2009).

As bona fide prion states are self-perpetuating (Wickner, 2012), we asked whether or not the Cb Rho cPrD-Sup35 C [PSI']-like state established upon galactose-induced overproduction of Cb Rho cPrD-EGFP fusion protein can be stably
propagated under non-inducing conditions. To do so, we tested the ability of Ade+ cells to remain in the [PSI+]\textsuperscript{-}like state over multiple generations. Both [PSI+] and [PSI+]\textsuperscript{-}like white colony phenotypes of cells producing native Sup35 or \textit{Cb} Rho cPrD-Sup35 C, respectively, were maintained over several rounds of restreaking (Figure 5C).

The vast majority of yeast prions, including [PSI+], require Hsp104 for their stable propagation (Liebman and Chernoff, 2012). Yeast cells can therefore be cured of prions upon transient inhibition of Hsp104 activity (Ferreira et al., 2001; Park et al., 2012). To determine whether or not the heritable [PSI+]\textsuperscript{-}like state conferred by \textit{Cb} Rho cPrD-Sup35 C is Hsp104-dependent, we passaged white, Ade+ cells on medium containing the Hsp104-inhibitor guanidine hydrochloride (Kummer et al., 2013). An additional round of restreaking yielded exclusively red colonies, indicating that, as is true for [PSI+], the [PSI+]\textsuperscript{-}like state specified by \textit{Cb} Rho cPrD-Sup35 C can be cured (Figure 5C). These results suggest that the \textit{Cb} Rho cPrD-Sup35 C chimera behaves as an Hsp104-dependent prion in yeast.
DISCUSSION

Prion proteins and prion-like phenomena have been exclusively documented in eukaryotes. Whether or not prions exist in the other two domains of life has remained an open question. We previously demonstrated that E. coli cells can support the formation (Garrity et al., 2010) and chaperone-dependent propagation (Yuan et al., 2014) of a model yeast prion. Here we provide, to our knowledge, the first evidence suggesting that a bacterial protein – the transcription termination factor Rho of Clostridium botulinum E3 str. Alaska E43 – possesses the ability to convert to a prion-like state. Our findings suggest that protein-based heredity is not a unique feature of eukaryotic cells and raise the possibility that prion-like mechanisms underlie epigenetic modes of heredity that are as ancient as they are universal.

Transcription termination in bacteria occurs at intrinsic or Rho-dependent terminators (Boudvillain et al., 2010). Transcription termination factor Rho functions as a hexameric (trimer of dimers) helicase that couples ATP binding and hydrolysis to translocation along nascent RNA associated with the RNA polymerase (RNAP) elongation complex (Skordalakes and Berger, 2003; Gocheva et al., 2015). Rho association with the RNAP elongation complex results in transcription termination via disruption of the RNA-DNA hybrid residing in the RNAP active site (Kim and Patel, 2001; Epshtein et al., 2010).

The Rho NTD consists of an N-terminal basic helix bundle (NHB) and a primary RNA binding domain (PBD) (Bogden et al., 1999). The Cb Rho PrD is located within a so-called N-terminal insertion domain (NID), which lies between
the NHB and PBD (D'Heygere et al., 2013). The length and sequence composition of the NID are highly variable among bacteria and are particularly heterogeneous in the Firmicutes, Bacteroidetes, and Actinobacteria (D'Heygere et al., 2013). Helicase activity resides in the highly-conserved Rho CTD, which contains Walker A and B ATP hydrolysis motifs, a secondary RNA binding site (SBS), and oligomerization determinants (Skordalakes and Berger, 2003).

*E. coli* Rho is a well-characterized, essential gene product (notably, *rho* is not essential in many bacteria and is in fact absent in some species [D'Heygere et al., 2013]). The essentiality of Rho in *E. coli* can be attributed to its ability to prevent the lethal accumulation of R-loops (triple-stranded nucleic acid structures composed of nascent RNA and template DNA), which compromise genome integrity (Washburn and Gottesman, 2011; Leela et al., 2013). Furthermore, it has been suggested that Rho activity is required in *E. coli* to prevent the expression of cryptic prophage elements, many of which encode bacteriocidal toxins (Cardinale et al., 2008); nonetheless, *rho* remains essential even in a strain lacking all cryptic prophage elements (Cardinale et al., 2008; Leela et al., 2013).

Although we have yet to rigorously document prion-like behavior of *Cb* Rho in *E. coli*, we are now well situated to do so. Specifically, our fortuitous finding that the aggregation propensity of full-length *Cb* Rho is dramatically augmented in the presence of New1 provides us with an excellent strategy to distinguish between the *de novo* formation of *Cb* Rho aggregates in the presence of New1 and their subsequent propagation as prion-like particles in the absence of New1. This experimental strategy is analogous to the one successfully implemented in Chapter
Two (see Figure 2 of Chapter Two) and will be greatly facilitated by the transcription-based reporter described in Appendix A.

Although we have thus far relied on *E. coli* as a model system for studying *Cb* Rho, we have initiated experiments in a genetically tractable, non-select agent relative of *C. botulinum, Clostridium difficile*. In collaboration with the Shen lab (University of Vermont), we will attempt to recapitulate *Cb* Rho aggregation behavior in Δrho *C. difficile* cells in order to perform transcription profiling analyses and thereby potentially uncover evidence of transcription read-through, which may have phenotypic consequences in *C. difficile* that can be extrapolated to *C. botulinum* physiology.
MATERIALS AND METHODS

Computational identification of candidate bacterial prion protein

All sequenced bacterial, archaeal, and phage genomes available as of January, 2013 were downloaded and analyzed by custom Java and R scripts (Alberti et al., 2009; Lancaster et al., 2014). Data visualization was performed with the PLAAC application available at http://plaac.wi.mit.edu (Lancaster et al., 2014).

Strains, plasmids, and cell growth

Bacteria experiments to assess intracellular behaviors of cPrDs were performed with *E. coli* strains BW27785 (Khlebnikov et al., 2001) and SG811 (Garrity et al., 2010) grown in LB (Miller) medium. SG811 is a derivative of BW27785 harboring an IPTG-inducible vector encoding the New1-mCFP-HA$_{3X}$ fusion protein integrated at *attB*(λ). Plasmids directing the arabinose-inducible synthesis of cPrD-mYFP-His$_{6X}$ fusion proteins or *Cb* Rho FL were constructed using *E. coli* codon-optimized DNA synthesized as gBlocks® Gene Fragments (IDT). Plasmids were transformed into appropriate cells, which were grown overnight in LB broth containing carbenicillin (Carb, 100 μg/ml) and glucose (0.2%). Overnight cultures were diluted 1:100 in LB broth containing Carb (100 μg/ml) and grown for 1 h prior to induction with arabinose (0.2% final concentration) and, in the case of SG811, 1 mM IPTG. Induced cultures were grown at 37°C for 6 h, normalized to 8 ml of an OD600 of 1.0, and pelleted by centrifugation. Cell pellets were processed and lysed as previously described (see Material and Methods of Chapter Two).
Yeast experiments were performed with *S. cerevisiae* [PIN⁺][psi⁻] strain YRS100 grown in yeast extract peptone dextrose (YPD) medium or synthetic defined (SD) medium. Sup35-based assays for prion behavior were performed as previously described (Alberti et al., 2009). Plasmids pAY354 (encoding native Sup35), pAY389 (encoding *Cb* Rho PrD-Sup35 C), pAY387 (encoding *Cb* Rho NTD-Sup35 C) were transformed into cells and established as the sole source of cellular translation release activity by 5-FOA-mediated plasmid shuffling. Prion induction by transient protein overproduction was achieved by transforming galactose-inducible plasmids pAY353 (encoding Sup35 NM-EGFP), pAY388 (encoding *Cb* Rho PrD-EGFP), and pAY383 (encoding *Cb* Rho NTD-EGFP) into cells of corresponding "shuffled" strains. Transformants were grown for 30 hours in SD-Trp medium containing either 1% galactose (Gal) + 1% raffinose (Raf) (inducing condition) or 2% Raf (non-inducing condition) and spotted on solid medium lacking adenine and containing 2% glucose. Stable propagation and guanidine hydrochloride-mediated curing of resultant [PSI⁺] and [PSI⁻]-like states were monitored as previously described (Yuan et al., 2014).

**C-DAG assay**

The C-DAG assay was performed as previously described (Sivanathan and Hochschild, 2013). In brief, ΔcsgBAC MC4100 cells constitutively overproducing CsgG were transformed with arabinose-inducible export vectors encoding cPrDs and core sequences bearing an N-terminal CsgA signal sequence and a C-terminal hexahistidine epitope tag. Transformants were grown in LB broth containing
carbenicillin (Carb, 100 µg/ml) and chloramphenicol (Cam, 25 µg/ml) and spotted onto LB agar containing arabinose (0.2%), Congo red (CR, 5 µg/ml), Carb (100 µg/ml), and Cam (25 µg/ml). Cells were grown at room temperature for 4-7 days and either scraped off of plates for filter retention analysis or arrayed on glass slides for detection of birefringence.

**Filter retention assays**

Filter retention assays were performed as previously described (Yuan et al., 2014).

**Congo red birefringence**

Cells grown on CR-containing agar were transferred to glass slides with pipette tips. Samples were viewed by bright-field microscopy between crossed polarizers on a Nikon 80i microscope equipped with a Plan Apo 100x 1.4 NA objective. Images were taken through the microscope eyepiece with an iPhone camera.

**Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)**

SDD-AGE was performed as previously described (Halfmann and Lindquist, 2008; Alberti et al., 2010).

**Immunoblotting**

Cellulose acetate membranes (used in filter retention assays) and Hybond-C Extra nitrocellulose membranes (used in SDD-AGE assays and Western blot analysis) were blocked for 30 min in PBS containing 3% (w/v) milk. Membranes
were probed with anti-His6x (His-2, Roche, 1:10,000), anti-GFP (Roche, 1:10,000), or anti-RpoA (NeoClone, 1:10,000) primary antibody. Membranes were washed and probed with anti-mouse IgG (Cell Signaling, 1:10,000) secondary antibody. Proteins were detected with ECL Plus Western blot detection reagents (GE Healthcare) and a ChemiDock XRS+ imaging system (Bio-Rad).

**Fluorescence microscopy**

Fluorescence microscopy was performed as previously described (Yuan et al., 2014). All fluorescence images of bacteria were obtained from 5 ms exposures whereas those of yeast were obtained from 500 ms exposures.
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Figure 1. Computational prediction of a bacterial prion candidate.

Graphical representation of data obtained from a hidden Markov model (HMM)-based prion prediction algorithm for transcription termination factor Rho of *Clostridium botulinum E3 str. Alaska E43* (Cb Rho). The amino acid sequence of Cb Rho is shown in the bottom panel, with residues constituting the cPrD predicted by the prion-like amino acid composition (PLAAC) algorithm (Alberti et al., 2009; Lancaster et al., 2014) or the prion aggregation prediction algorithm (PAPA) (Toombs et al., 2012) highlighted in red or underlined in green, respectively. The top panel shows the probability of each residue belonging to the HMM state "cPrD" (red) or "background" (black). The tracks "MAP" and "Vit" illustrate the Maximum a Posteriori and Viterbi parses of the protein into these two states. Amino acid composition is represented in color, with Q and N residues shown in blue. The middle panel shows averages over a 60 amino acid sliding window of predicted intrinsic protein disorder (FoldIndex, gray) (Prilusky et al., 2005) and cPrD amino acid propensities as assessed by PLAAC (red) or PAPA (green) along with the PAPA cutoff score for predicted prion propensity (dotted green line) (Toombs et al., 2010).
IF-2 (Enterococcus faecalis V583)

IF-2 (Lactobacillus plantarum WCFS1)
Rho (Zanthomonas oryzae pv. oryzae PX099A)

Rho (Fluvicola taffensis DSM 16823)
**Supplemental Figure 1.** Computational prediction of bacterial prion candidates.

Graphical representations of data obtained from a hidden Markov model-based prion prediction algorithm for a subset of candidate bacterial prion proteins characterized in this study (see legend to Figure 1 for further details).
Figure 2. A subset of cPrDs form extracellular amyloid aggregates when exported to the E. coli cell surface via C-DAG.

(A) Filter retention analysis of scraped suspensions of cells engineered to export the indicated cPrD or core polypeptide to the E. coli cell surface via the curli secretion pathway. Sup35 NM serves as a positive control. For each sample, undiluted lysate and three two-fold dilutions are shown. The α-His6 antibody recognizes exported proteins, which contain a C-terminal hexahistidine epitope tag. Ef: Enterococcus faecalis V583, Pc: Pediococcus claussenii ATCC BAA-344, Lp: Lactobacillus plantarum WCFS1, Ft: Fluvicola taffensis DSM 16823, Sg: Sphaerochaeta globus str. Buddy, Xa: Xanthomonas axonopodis pv. citrulino F1, Xo: Xanthomonas oryzae pv. oryzae PX099A, Cb E3: Clostridium botulinum E3 str. Alaska E43, Ps: Pseudoalteromonas sp. SM9913, Sp: Streptococcus pneumoniae ST556, Cc: Campylobacter curvus 525.92, Cg: Corynebacterium glutamicum R, Lm: Listeria monocytogenes HCC23, Pp: Propionibacterium propionicum F0230a. (B) Cells transformed with one of 24 C-DAG export vectors were spotted on medium containing arabinose (0.2%) and Congo red (5 μg/ml) and grown at room temperature for 72 h. The order of spots follow the order of proteins shown in (A), with the Ef IF-2 core corresponding to quadrant A1 and the Pp SSB cPrD corresponding to quadrant D5. Cells producing highly or moderately birefringent material are highlighted in solid or dashed green boxes, respectively. (C) Bright-field image of Cb E3 Rho PrD-secreting cells viewed between crossed polarizers.
Figure 3. The *Cb* Rho cPrD and NTD form SDS-stable aggregates in the *E. coli* cytoplasm.

(A) SDD-AGE analysis of cell extracts containing the indicated protein domain fused to mYFP-His$_{6X}$. Sup35 NM (produced in the presence of New1) and the *Lp* IF-2 cPrD serve as positive and negative controls, respectively. (B) Filter retention analysis of cell extracts containing the indicated *Cb* Rho domain fused to mYFP-His$_{6X}$. For each sample, undiluted lysate and three two-fold dilutions are shown. *Cb* Rho cPrD and NTD aggregates are no longer detected once boiled. The α-His$_{6X}$ antibody recognizes the NM/cPrD/NTD-mYFP-His$_{6X}$ fusion proteins. (C) Fluorescence images of representative cells producing the *Cb* Rho NTD or Sup35 NM fused to mYFP-His$_{6X}$ (top row) and cells co-producing Sup35 NM-mYFP-His$_{6X}$ and New1-mCFP-HA$_{3X}$ fusion proteins (bottom row). For cells containing Sup35 NM and New1, the YFP channel, CFP channel, and merged images are shown.
Figure 4. Full-length Cb Rho forms SDS-stable aggregates in the E. coli cytoplasm.

(A) SDD-AGE analysis of cell extracts containing full-length (FL) Cb Rho bearing a C-terminal hexahistidine epitope tag in the absence or presence of New1. The α-His_{6x} antibody recognizes full-length Cb Rho-His_{6x} protein, and the α-GFP antibody recognizes New1-mCFP-HA_{3x} fusion protein. (B) Filter retention analysis of the same cell extracts analyzed in (A). For each sample, undiluted lysate and three two-fold dilutions are shown. Neither full-length Cb Rho nor New1 aggregates are detected once boiled. (C) Intracellular full-length Cb Rho-His_{6x} protein levels are comparable in the presence and absence of New1 as assessed by Western blot analyses of the same cell extracts used in (A) and (B). The α-RpoA antibody recognizes the α subunit of E. coli RNA polymerase.
Figure 5. The Cb Rho cPrD behaves as an Hsp104-dependent prion in yeast.

(A) Fluorescence images of YRS100-derived yeast cells containing native Sup35 or the indicated Cb Rho domain fused to Sup35 C as the sole source of cellular translation release activity and overproducing Sup35 NM, the Cb Rho cPrD, or the Cb Rho NTD fused to EGFP from galactose-inducible plasmids. For each of the three EGFP fusion proteins, two representative fluorescence images are show. (B) The phenotypes of cells grown for 30 h in liquid medium containing either 1% galactose (Gal) + 1% raffinose (Raf) (top, inducing condition) or 2% Raf (bottom, non-inducing condition) and spotted on solid medium lacking adenine and containing 2% glucose. Transient overproduction of Sup35 NM and the Cb Rho PrD increases the frequency of switching to the corresponding prion state. In the case of the Cb Rho NTD, transient protein overproduction is not required for manifestation of the prion state. (C) The phenotypes of [psi-] cells containing native Sup35 (sector 1), [psi-] like cells containing Cb Rho cPrD-Sup35 C (sector 2), [PSI+] cells isolated following overproduction of Sup35 NM-EGFP (sectors 3 and 4), and [PSI+] like cells isolated following overproducing of Cb Rho cPrD-EGFP (sectors 5 and 6) on 1/4 YPD medium (left). The same cells were passaged once on YPD supplemented with 3
mM GuHCl to inhibit Hsp104 activity and restreaked on 1/4 YPD medium to reveal $[\psi^-]$ and $[\psi^+]$-like progeny (right). We do not yet fully understand why the pre-GuHCl $[\psi^-]$ and $[\psi^+]$-like colonies shown in sectors 1 and 2 appear less red than their corresponding post-GuHCl progeny; however, we note that pre-GuHCl cells are $[PIN^+]$ whereas post-GuHCl cells are $[pin^-]$ and suggest that this distinction may, in part, account for the apparent difference in colony color.
APPENDIX C

A cell-based screen for amyloidogenic proteins of bacterial origin

Specific author contributions:

I devised and performed all experiments presented in this appendix. Sitara Chapman (MIT/Wellesley College) and Shelby Yuan (Harvard College) provided assistance in performing pilot screens for amyloidogenic proteins.
RESULTS AND DISCUSSION

As discussed in Chapter One and Chapter Two, *E. coli* cells are naturally capable of elaborating amyloid fibrillar structures known as curli fibers at the cell surface (Chapman et al., 2002). Curli fibers are composed of the CsgA and CsgB proteins, which are secreted via the inner membrane SecYEG translocon and the outer membrane CsgG pore (Chapman et al., 2002; Hammer et al., 2007; Blanco et al., 2011). The secretion specificity determinants of CsgA and CsgB lie within an N-terminal bipartite signal sequence, which can be transferred to heterologous proteins (Robinson et al., 2006; Sivanathan and Hochschild, 2012). A previously described *E. coli* cell-based curli-dependent amyloid generator (C-DAG) assay appropriates the curli secretion pathway and enables the formation of extracellular amyloid aggregates composed of known amyloidogenic protein domains (Sivanathan and Hochschild, 2012).

We took advantage of the C-DAG assay as a methodology for identifying amyloidogenic polypeptides of bacterial origin. To do so, we first constructed high-complexity libraries encoding in-frame protein fragments derived from gDNA of five model bacteria (*Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae, Bacillus subtilis*, and *Caulobacter crescentus*). The high-resolution crystal structures of the nonameric CsgG transport complex – in conjunction with earlier studies of CsgG-mediated protein secretion – have established approximate size thresholds for substrate export (Van Gerven et al., 2014; Goyal et al., 2014). Accordingly, gDNA fragments were size-selected (100-1,000 bp) to increase the probability of efficient export through the CsgG pore.
Libraries were enriched for in-frame gene fragments by passage through a so-called "ORF-filter" selection system. In brief, the ORF-filtration strategy takes advantage of the cis-splicing activity of the yeast VMA intein and the E. coli twin-arginine transport (TAT) secretion pathway. pInSALecT (gift of S. Lutz), a plasmid encoding the signal sequence of the TorA protein (a TAT substrate) fused to split VMA and β-lactamase (Bla) (Gerth et al., 2004), was modified for the purposes of generating gDNA libraries encoding ssTorA-VMA_{NTD}-X-VMA_{CTD}-Bla chimeras, where X represents a random gDNA fragment. Only if X is in-frame can the complete chimeric protein be produced, resulting in the excision of VMA-X fusion protein and the formation of ssTorA-Bla fusion protein, which confers upon cells resistance to β-lactam antibiotics. Critically, unlike its predecessors, this intein-dependent ORF-filter technology does not select against aggregation-prone polypeptides (Gerth et al., 2004).

ORF-filtered gDNA fragments were recovered and transferred to an arabinose-inducible C-DAG export vector, which encodes the N-terminal signal sequence of CsgA and a C-terminal hexahistidine epitope tag (Sivanathan and Hochschild, 2013). In a pilot screen, we transformed a C-DAG export library containing in-frame E. coli gDNA fragments into ΔcsgBAC E. coli cells harboring a compatible multi-copy plasmid directing the constitutive overproduction of CsgG. In parallel, we transformed pooled, C-DAG-compatible ORF libraries derived from the ASKA E. coli gene collection (Sivanathan and Hochschild, 2012; Kitagawa et al., 2005). Transformants were plated on selective medium containing arabinose and the amyloid-binding dye Congo red (CR).
While amyloid-producing clones can form bright red colonies on CR-containing medium, we have found that CR-binding is an unreliable indicator of extracellular amyloid. In particular, when screening gDNA fragment or ORF libraries for a red colony color phenotype on CR-containing medium, large numbers of false positives are detected. To circumvent this problem, we relied on the fact that CR-bound amyloid aggregates uniquely exhibit "apple-green" birefringence when viewed by bright-field microscopy between crossed polarizers (Howie et al., 2008; Teng and Eisenberg, 2009). Accordingly, following four to seven days of growth at room temperature, bright red colonies were manually picked, arrayed on glass slides, and tested for birefringence. We screened over ~4,000 individual bright red colonies (~2,000 colonies derived from an in-frame *E. coli* gDNA fragment library and ~2,000 colonies derived from an *E. coli* pooled ORF-library) in this manner and identified several birefringent candidates. Subsequent filter retention analysis of scraped cell suspensions revealed that a subset of candidates produced aggregates exhibiting resistance to SDS denaturation, another characteristic of amyloid material (Figure 1A, Figure 1B) (Alberti et al., 2010).

The amyloid conformation represents a low energy state for most polypeptides, and many proteins can form amyloid aggregates under extreme non-physiological conditions *in vitro* (Buell et al., 2014; Teng and Eisenberg, 2009). However, the sequence determinants of amyloidogenesis *in vivo* remain poorly understood. Moreover, those features that distinguish an amyloidogenic protein from a prion protein remain mysterious. Several research groups have developed computational tools to predict amyloidogenic and prionogenic proteins based on
amino acid sequence and composition (Michelitsch and Weissman, 2000; Trovato et al., 2006; Alberti et al., 2009; Maurer-Stroh et al., 2010; Toombs et al., 2012; Espinosa Angarica V et al., 2014).

Among these computational approaches, a structure-guided algorithm developed in the Eisenberg lab is particularly notable and emerged from the observation that hexapeptides of amyloidogenic proteins can crystallize as amyloid-like fibrils in which complementary side-chains of juxtaposed β-sheets form a so-called "steric zipper" (Nelson et al., 2005; Thompson et al., 2006). These short polypeptides exhibit high fibrillation propensities and are thus referred to as "HP segments", which can be found in virtually every protein for which a structure exists (e.g., see Figure 1C). Critically, computational analyses indicate that the position and conformational flexibility of an HP segment in the context of its corresponding native protein dictates whether or not such a protein is prone to form amyloid aggregates (Goldschmidt et al., 2010). Our ongoing identification of bacterial amyloidogenic proteins using the C-DAG assay will facilitate future experimental assessments of HP segment-based computational predictions and their implications for amyloidogenesis and prionogenesis.
REFERENCES


Figure 1. Identification of E. coli-encoded amyloidogenic polypeptides.

(A) Table of candidate amyloidogenic polypeptides identified by C-DAG. 8 of the 9 candidates represent protein fragments that were identified by screening a C-DAG export library enriched for in-frame E. coli gDNA. YmjC was isolated from a C-DAG-compatible ORF library derived from the ASKA E. coli gene collection. Qualitative assessments of Congo red birefringence (BR) as evaluated by bright-field microscopy between crossed polarizers and resistance (R) or sensitivity (S) to 2% SDS as assessed by filter retention analyses are shown. (B) Bright-field image of YmjC-secreting cells viewed between cross polarizers (top). The 60 amino acid YmjC protein (blue) represents the N-terminus of the 212 amino acid NmrA-family protein S1416 (wheat) of Shigella flexneri 2a str. 2457T (Protein Data Bank ID: 3QVO) (bottom). (C) ZipperDB (http://services.mbi.ucla.edu/zipperdb) analysis of YmjC reveals HP-segments exhibiting amyloid-forming potentials beyond a Rosetta threshold of -23 kcal/mol.
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• 2014 Molecular Genetics of Bacteria & Phages Meeting, University of Wisconsin-Madison: "Prion propagation can occur in a prokaryote and requires the ClpB chaperone."

• 2010 Molecular Genetics of Bacteria & Phages Meeting, Cold Spring Harbor Laboratory: "The bacteriophage T4 AsiA protein is the nexus of a protein interaction network at T4 middle promoters."

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