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Folding optimization in vivo uncovers new chaperones

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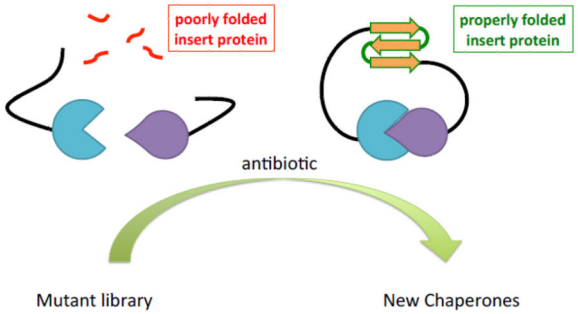
Abstract

By employing a genetic selection that forces the cell to fold an unstable, aggregation prone test protein in order to survive, we have generated bacterial strains with enhanced periplasmic folding capacity. These strains enhance the soluble steady state level of the test protein. Most of the bacterial variants we isolated were found to overexpress one or more periplasmic proteins including OsmY, Ivy, DppA, OppA, and HdeB. Of these proteins, only HdeB has convincingly been previously shown to function as chaperone in vivo. By giving bacteria the stark choice between death and stabilizing a poorly folded protein, we have now generated designer bacteria selected for their ability to stabilize specific proteins.

Graphical abstract



Keywords

chaperone discovery; protein folding; folding sensor; periplasm; proteostasis

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Introduction

Although all the information necessary for the folding of a protein is contained in its amino acid sequence, cells have evolved molecular chaperones to assist in the proper folding of proteins within the crowded cellular environment [1]. Chaperones have traditionally been identified as genes induced upon exposure to some type of stress, such as heat shock [2]. While these types of approaches have been tremendously useful in understanding how cells deal with a particular stress, they do not directly monitor how proteins like chaperones affect in vivo protein stability. In addition, protein folding also requires assistance in the cell in the absence of stress, and consequently a variety of chaperones are not heat inducible [3]. Because of these limitations, our current understanding of factors governing protein stability in vivo is almost certainly incomplete. As a result, attempts to optimize the in vivo folding environment for the expression of unstable heterologous proteins is generally a trial and error process.

Overproduction of previously identified molecular chaperones has in some cases resulted in the stabilization of unstable heterogeneous proteins, but there is no universal solution to the problem of protein instability. According to a recent review although “molecular chaperones and folding catalysts appear to present a panacea for problems of heterologous protein folding …. painstaking investigation of chaperone overproduction has however, met with mixed – and largely unpredictable – results to date” [4]. These observations may be because the substrate specificity of even well studied chaperones and proteases are not sufficiently known to be able to predict how they act on heterologous proteins.

We have recently developed a genetic selection that directly links increased in vivo protein stability to increased antibiotic resistance. This strategy allows one to optimize protein folding and enhance protein stability in vivo, and, most importantly, to generate an alternate unbiased route to chaperone discovery (Figure 1A) [5, 6, 50]. In this selection, a test protein is sandwiched between two domains of a selectable marker, such as β-lactamase, forming a tripartite fusion. Our logic is that if the test protein is stable and properly folded, this should bring the two halves of β-lactamase close together to form a functional enzyme; cells containing this stable folding biosensor will therefore be resistant to high levels of the antibiotic ampicillin. However, if the test protein is unstable, it should be cleaved by the variety of proteases present in the cell, or drive the folding biosensor into aggregates, resulting in less functional β-lactamase and lower levels of resistance to β-lactam antibiotics. Increasing antibiotic concentrations will require higher levels of functional β-lactamase, in effect forcing the cell to either fold the unstable protein or die.

We previously showed that this selection could enable us to improve the cellular folding environment simply through the isolation of chromosomal mutations that improved the antibiotic resistance of strains containing our folding biosensors. In response to a selection in which cells were subject to random chromosomal mutagenesis and then forced to fold an unstable mutant of the well studied protein folding model immunity protein 7 (Im7), we discovered that a novel periplasmic chaperone, Spy, was overproduced in these folding enhanced strains which resulted in up to 700 fold overproduction of normally unstable variants of Im7 [7]. In this current study, we used our selection to find strains that would

stabilize a protein that Spy was inactive against (unpublished data), namely, a highly destabilized mutant of the maltose binding protein (MBP). The strains isolated in this selection overproduced OsmY, HdeB, DppA, OppA, and/or Ivy. We show here that all five of these proteins individually can enhance the activity of our test proteins in vivo and have chaperone activity in vitro. Therefore, through direct selection for maintenance of an unstable test protein, we can evolve bacteria that have an optimized folding environment through the expression of chaperones including a number not previously characterized.

Results and Discussion

Selection for folding optimized bacteria

We sought to enhance the ability of bacteria to fold unstable proteins by using a split β-lactamase protein stability biosensor. In contrast to the highly stable native MBP, $MBP_{G32D/I33P}$ is highly unstable and has a strong tendency to aggregate [8]. As expected, insertion of $MBP_{G32D/I33P}$ into β-lactamase results in *Escherichia coli* that are substantially less ampicillin resistant when compared to the same fusion protein with native MBP inserted [5] (Figures 1A and 1B). As a separate genetic handle, we took advantage of a second property of our test protein MBP in our selection for folding enhanced bacteria. Cells expressing $MBP_{G32D/I33P}$ (both alone and in the fusion context) fail to specify a Mal^+ phenotype on maltose MacConkey agar plates (Figure 1C), which requires maltose transport across the inner-membrane by MBP. The Mal^- phenotype specified by $MBP_{G32D/I33P}$ appears to be a direct reflection of its instability in the periplasm since $MBP_{G32D/I33P}$ purified from inclusion bodies and refolded has native affinity for maltose [8]. We therefore reasoned that reversal of the Mal^- phenotype by stabilization of $MBP_{G32D/I33P}$ should provide a convenient secondary screen for in vivo protein folding. We reasoned that this second genetic handle should also be useful in helpings us screen out host mutants that enhance ampicillin resistance for reasons unrelated to alterations in the protein folding capacity of the strain such as by decreasing permeability to the antibiotic or enhancing its export.

To generate folding enhanced strains, chromosomal DNA of *malE* deleted *E. coli*, (MT419) was randomly mutagenized with ethyl methanesulfonate. Next, two plasmids were simultaneously transformed into ethyl methanesulfonate treated cells, one plasmid (pCWL2) with the tripartite β-lactamase-$MBP_{G32D/I33P}$ fusion under arabinose control and a second plasmid (pCWL4) carrying the unfused $MBP_{G32D/I33P}$ protein under isopropyl β-D-1-thiogalactopyranoside (IPTG) control. This second plasmid was present in order to allow us to rapidly screen out via western analysis strains that stabilized the β-lactamase-$MBP_{G32D/I33P}$ fusion but failed to stabilize the $MBP_{G32D/I33P}$ protein itself. Both the $MBP_{G32D/I33P}$ protein and the β-lactamase-$MBP_{G32D/I33P}$ fusion react to anti-MBP antibody but run with very different motilities on an SDS gel. Presumptive folding enhanced strains were isolated by selecting for increased ampicillin resistance and/or screening for Mal^+ activity as described in 'Materials and methods'. The characterization of two independently isolated strains (MT704 and MT710) that show increased resistance to ampicillin and utilization of maltose are illustrated in Figure 2. Plasmids from our putative folding enhanced strains were then isolated and retransformed into our unmutagenized parental

strain background (MT419) to ascertain that the phenotypes were not plasmid encoded. Finally, we conducted western blots to determine the levels of soluble $MBP_{G32D/I33P}$ protein and β -lactamase-$MBP_{G32D/I33P}$ fusion protein. We found that the expression levels increased substantially in our selected strains far above that present in MT426 (an unmutagenize background strain containing the plasmid expressing the unstable β -lactamase-$MBP_{G32D/I33P}$ fusion) where no MBP fusion is visible, but below the level of the wild type β -lactamase-MBP fusion found in MT425 (Supplementary Figure 1).

Folding optimized strains overexpress the periplasmic proteins OsmY, DppA, OppA, Ivy, and HdeB

Chaperones are typically required in large amounts to be effective both in vitro and in vivo [1]. A very straightforward way to increase the effectiveness of a chaperone is therefore to increase its concentration in the appropriate cellular compartment. Since β-lactamase and MBP are periplasmic proteins, we focused on abundant periplasmic proteins whose expression was markedly increased in our folding enhanced (MT695-MT803) strains as compared to the nonmutagenized parent strain carrying the same plasmids (MT426). We excised these bands from denaturing gels, and identified the proteins by using electrospray ionization mass spectrometry. Using this approach, we identified five proteins that were overexpressed at least two-fold in several independently isolated folding-enhanced Amp^R/Mal^+ strains (Figure 3, Supplementary Figure 2, Supplementary Table 1). The highest degree of overexpression was observed for OsmY and Ivy, which were both ≥ 2-fold overexpressed in 18 out of 26 of the strains examined and upregulated as much as 30–40 fold in some of our folding enhanced strains (Supplementary Table 1). OsmY is an abundant protein of unknown function that is induced by osmotic stress and Ivy is an inhibitor of vertebrate lysoszyme [9, 10]. In addition, we found that the polypeptide binding proteins DppA, and OppA and the acid stress chaperone HdeB, were also ≥ 2-fold overexpressed in about half of our folding-enhanced strains (Supplementary Table 1). We noted that the strains typically overexpressed more than one of these proteins. The simplest possibility is that each of these proteins can independently and directly function as a chaperone and that it's enhanced expression acts to improve the folding environment for our biosensor. A closely related possibility is that these proteins are part of a chaperone machine that requires at least two components to be overexpressed in order to improve the folding environment. Alternatively, these proteins could be acting indirectly to improve the folding environment or may even themselves be inactive in this in vivo assay but are co-regulated with other undetected proteins, which are responsible for the observed phenotype. A mutation for instance might knock out a protease that allows for the simultaneous overproduction of a number of unstable proteins including our test protein. In fact we did notice that OsmY, Ivy and DppA were often simultaneously overexpressed (Supplementary Figure 3), suggesting either that the three proteins are under a similar regulatory mechanism of control or that a fixed ratio of the three proteins best answers the selection conditions.

All five identified proteins individually improve the function of the β-lactamase-$MBP_{G32D/I33P}$ fusion in vivo

To test if the individual overexpression of OsmY, DppA, OppA, Ivy, or HdeB could improve the in vivo function of the β-lactamase-$MBP_{G32D/I33P}$ fusion, we transformed the

respective plasmids or the empty vector controls into MT422, an unmutagenized MT419 parent strain background strain that contains the plasmid encoding the tripartite β-lactamase-$MBP_{G32D/I33P}$ fusion. In parallel, we tested if overexpression of any of these proteins was sufficient to allow strains containing the $MBP_{G32D/I33P}$ protein to ferment maltose. As before, we measured resistance to ampicillin as read-out for the increased stability of our tripartite fusion system, and maltose utilization as a read-out for MBP-activity. When grown on plates containing 100 μg/ml ampicillin, overexpression of OsmY alone allows for up to a 50-fold increase in colony forming units compared to the vector controls (Figure 4). Overexpression of DppA, OppA, Ivy, or HdeB alone also improves ampicillin resistance to varying degrees, increasing the colony forming units by 5–10-fold (Figure 4). Consistent with these results, we found that OsmY, DppA, OppA, and HdeB overexpression alone was sufficient to substantially improve the Mal^+ activity of $MBP_{G32D/I33P}$, with OsmY showing the strongest effect (Supplementary Figure 4). We note that expression of any protein tested alone was not sufficient to give as strong a Amp^R/Mal^+ phenotype as the strongest originally isolated chromosomal mutants, consistent with our observation that the strains answering our selection overexpress several different proteins. We therefore tested whether simultaneous overexpression of OsmY together with DppA, OppA, Ivy, or HdeB would further increase β-lactamase-$MBP_{G32D/I33P}$ activity. Although our folding enhanced strains often overexpress several of the above proteins together, we did not observe additional antibiotic resistance when expressing OsmY plus one additional protein (data not shown). This, however, might be partially due to our screening the incorrect combinations of protein overexpression (an exhaustive screening would involve testing 5! = 120 different protein overexpression combinations), or our inability to adequately mimic the expression level of these proteins that was found in our chromosomal mutant strains, or due to the presence of other mutations, for example mutations in the proteostasis machinery of the cell, that contribute to the phenotype. Additionally, it is possible that mutations in other cellular pathways not directly related to proteostasis may also influence to the observed phenotypes of our folding enhanced strains.

OsmY functions as effective chaperone in vitro

Although one can imagine a host of ways that the overexpression of these five proteins could directly or indirectly increase the soluble levels of our unstable test protein, the most obvious is that these proteins function as molecular chaperones. Indeed, there is good evidence that DppA from *Rhodobacter* has chaperone-like activity [40], and HdeB from *E. coli* has recently been shown to function as a chaperone at the moderately acidic pH of 4 [11]. Moreover, there is preliminary evidence that OppA may have weak chaperone activity in vitro [12]. To investigate whether indeed all of our five identified proteins function as molecular chaperones, we purified the proteins, and determined their individual influence on inhibiting the aggregation of model chaperone substrates α-lactalbumin (α-LA), lactate dehydrogenase (LDH), and luciferase in a purified system. We started with OsmY and Ivy because they showed the highest degree of induction in vivo and because neither of them had previously been reported to have chaperone activity. We therefore measured the influence of OsmY on the thermal aggregation of two model substrates, LDH and luciferase, as well as on the dithiolthreitol induced aggregation of α-LA. We found that purified OsmY effectively inhibits the aggregation of LDH and α-LA, (Figure 5A and Supplemental Figure

5A). OsmY also inhibits the aggregation of luciferase but less effectively (Supplemental Figure 5D). These results demonstrate that our selection approach had succeeded in uncovering at least one new periplasmic chaperone in *E. coli*.

Prior to this study, OsmY did not have any assigned molecular function. It was originally identified as a protein expressed in stationary phase [13] and in response to hyperosmotic stress [10]. Our discovery that OsmY is a chaperone is consistent with the regulation of this protein; OsmY is induced by several stresses known to cause protein misfolding, including heat shock, acidic pH, and bile salts [14–20]. In addition, OsmY is overexpressed ~18-fold and ~4-fold, respectively, in strains evolved for increased tolerance to both ethanol and oxidative stress [21, 22], two well-known folding stresses. Interestingly, OsmY protein levels were found to be 10-fold higher in a *dsbA* null strain relative to the wild-type strain, making OsmY one of the most abundant proteins in the periplasm in a *dsbA* null strain [23]. Importantly, *dsbA*$^{-}$ strains are unable to catalyze disulfide bond formation [24] and suffer from widespread protein misfolding [23]. These results suggest that OsmY expression is induced in response to protein misfolding.

We have found that OsmY is remarkably soluble—solutions containing up to ~300 mg/ml OsmY can be isolated without visible precipitation. Previous work has demonstrated that OsmY remains soluble even in the presence of protein precipitation agents such as ethanol (60% for 15 min), extreme acidic pH (0.5 M HCl for 15 min), or high temperatures (100°C for 30 min) [25]. Proteomic studies identified OsmY as present in the extracellular media [26, 27] at levels ~20-fold higher than any other protein tested. Interestingly, OsmY was shown to be able to function very well as a fusion tag allowing extracellular export, increased solubility, and proper folding of several heterologous proteins, many of which are highly unstable unless OsmY is fused to them [26, 28–33]. In view of our results, these previous findings can be reinterpreted to mean that OsmY functions in vivo as a chaperone when fused to proteins, i.e. in *cis*. This is consistent with our in vivo and in vitro evidence that OsmY functions as a chaperone when not fused to proteins, i.e. in *trans*. It is unclear if extracellular export of OsmY is an active process or is instead more passive, such as being due to leakage through the outer membrane. We are currently investigating the possibility that OsmY functions as an extracellular chaperone in addition to its role as a protein folding factor in the periplasm.

Ivy functions as a chaperone in vitro

Ivy was previously assigned the molecular function of an inhibitor of vertebrate lysozyme [34]. We found that purified Ivy effectively inhibits the thermal aggregation of LDH in vitro (Figure 5B), but was unable to inhibit the thermal aggregation of luciferase, or the dithiolthreitol induced aggregation of α-LA (Supplemental Figure 5) suggesting that Ivy's chaperone client specificity is different from OsmY's and possibly more limited. Ivy was originally identified as a gene with no assigned function that was highly expressed in both log and stationary growth phases [35]. Ivy was found to co-purify with hen egg white lysozyme, a protein used to facilitate cell lysis during purification [34]. Further biochemical analysis demonstrated that Ivy acts as a very strong inhibitor of hen egg white lysozyme,

with a reported K_i of ~1 nM [34]. Crystal structures of Ivy alone and in complex with lysozyme have been solved [9, 36].

Deletion of *ivy* from the chromosome of *E. coli* does not lead to sensitivity to hen egg white lysozyme unless the outer membrane of the cell is first made porous [9, 37] to allow for lysozyme entry into the periplasm. This raises the obvious question of whether Ivy may be performing other cellular functions beyond c-type lysozyme binding. One interesting phenotypic observation is that deletion of *ivy* from the chromosome severely inhibits biofilm formation by *E. coli* [38]. Our results demonstrate that in addition to this well-defined property of c-type lysozyme binding, Ivy has the ability to stabilize the poorly folded $MBP_{G32D/I33P}$ in the β-lactamase fusion context in vivo and inhibits the aggregation of LDH in vitro. Consistent with this newly identified role in protein folding, Ivy is highly expressed in response to acid stress [15, 18]. Further, although no chaperone activity has previously been described for Ivy, like for heat shock proteins many of which are chaperones, transcription of *ivy* is also induced by elevated temperature [17] and Ivy has an annotated heat shock sigma factor promoter in addition to a housekeeping sigma factor promoter (ecocyc.org).

DppA and OppA function as chaperones in vitro

DppA and OppA function as the substrate binding subunits of dipeptide and oligopeptide transport systems, respectively. In these well-defined roles, DppA and OppA serve to bind peptides in the periplasm and transfer these nutrients to their cognate ABC transporters for uptake into the cytosol [39]. In addition, it has also been suggested that OppA and DppA might function as chaperones in vitro [12, 40]. However, the in vitro chaperone activity reported for OppA was very weak, a 44-fold excess of OppA inhibit the aggregation of citrate synthase by only 30%. Though DppA was capable of inhibiting the aggregation of acid-unfolded dimethyl sulfoxide reductase, DppA mutants had no effect on the formation of active dimethyl sulfoxide reductase in vivo [41]. Possibly as a result of these considerations, neither protein was mentioned in two recent comprehensive reviews on periplasmic chaperones and protein folding factors in the periplasm [42, 43]. An earlier book chapter on periplasmic chaperones did mention OppA and DppA but raised questions regarding the in vivo relevance of their reported in vitro chaperone activity [44]. Our observations that OppA and DppA are overproduced in folding enhanced strains and that either protein on its own is sufficient to enhance the expression of a poorly folding test protein provides evidence that these proteins may indeed function as chaperones in vivo. It thus led us to reexamine the in vitro chaperone activity of these proteins.

We found that purified *E. coli* DppA and OppA both potently inhibit the dithiolthreitol induced aggregation of α-LA (Supplemental Figure 5B). OppA but not DppA could effectively prevent the thermal aggregation of LDH (Figure 5C). However, neither protein could inhibit thermal aggregation of luciferase (Supplemental Figure 5D). These results confirm that both proteins can function as chaperones in vitro. Chaperones need to be able to interact with proteins and peptides with broad specificity in order to carry out their function. Intriguingly, our results suggest that peptide-binding activity alone may be sufficient for chaperone activity in vivo and in vitro.

HdeB functions as a chaperone at neutral pH

HdeB is homologous to HdeA, a well-studied acid activated chaperone [45–47]. We have recently shown that HdeB is active in both the prevention of aggregation and in facilitating the refolding of proteins at acidic pH [11] consistent with previous suggestions that HdeB functions as an acid activated chaperone [48]. We recently reported that HdeB functions at pH values higher than what is optimal for HdeA [11]; HdeB exhibits maximal in vitro chaperone activity at pH 4, whereas HdeA has optimal chaperone activity at pH 2. Our selections were done on ordinary, not deliberately acidic media, implying that HdeB also has chaperone activity at more neutral pH values. This was confirmed in vitro, as HdeB shows significant in vitro chaperone activity against LDH at pH 7.5, inhibiting LDH aggregation at a level similar to that observed for OsmY (Figure 5A). HdeB was unable to inhibit the dithiolthreitol induced aggregation of α-LA aggregation or thermal of luciferase at neutral pH (Supplemental Figure 5).

Summary

Using our `fold or die' genetic selection, which forces cells to maintain our highly unstable β-lactamase-$MBP_{G32D/I33P}$ biosensor in order to live, we have isolated folding optimized bacteria. We found that our folding optimized strains overexpress five proteins (OsmY, Ivy, DppA OppA, and HdeB) that have chaperone activity in vitro. Interestingly, the proteins found to be overproduced in our folding enhanced strains are very different depending on the unstable protein that was incorporated into the folding biosensor and used to drive the selection. For an unstable mutant of Im7 (L53A/I54A), we exclusively isolated mutations that overproduced Spy [7]. For $MBP_{G32D/I33P}$, a broader range of existing (DppA, OppA, HdeB) and newly discovered (OsmY and Ivy) chaperones were overproduced. This suggests that through selection and mutagenesis, one can generate folding enhanced bacterial strains that are automatically customized dependent on the nature of each individual test protein's expression defect. Because our approach asks the bacteria themselves to solve the expression defect, it does not depend on any prior knowledge of the nature of the defect or the detailed workings of the proteostasis machinery. We show this method is effective in the discovery of previously unknown chaperones.

Materials and methods

Mutagenesis and selection to discover unknown periplasmic folding factors

Ethyl methanesulfonate was used to induce mutations at random positions on chromosomal DNA of *E. coli* lacking the gene encoding MBP (Δ*malE*) and endonuclease R (Δ*hsdR*) that is otherwise a wild type MG1655 strain background (*hsdR* was deleted to improve plasmid transformation efficiency). Ethyl methanesulfonate treated strains were next simultaneously transformed with two plasmids: pCWL2 and pCWL4. pCWL2 contains a tripartite β-lactamase-MBP G32D/I33P fusion gene that is arabinose inducible and is under colE1 plasmid copy number control. pCWL4 carries an unfused $MBP_{G32D/I33P}$ gene that is IPTG inducible and is under CDF replicon plasmid copy number control. The isolation of folding enhanced strains was performed in two complementary ways. In the first method, we induced the tripartite β-lactamase-MBP G32D/I33P fusion gene with 2% arabinose and selected for folding enhanced strains by their increased ampicillin resistance (Amp^R). To

verify that these strains were also able to express more MBP G32D/I33P protein in the absence of the fusion, we then screened the strains for Mal^+ activity on MacConkey-maltose plates under IPTG induction. In the second complementary method, we did the reverse, first screening for Mal^+ activity on MacConkey-maltose plates under IPTG induction and then screening for ampicillin resistance under arabinose induction. This scheme of transformation following mutagenesis utilizing two independent plasmids was designed to reduce the number of plasmid-linked mutations we isolated. For example, the use of plasmids with different copy number control mechanisms decreases the chance that the phenotypes we observe are due to chromosomal mutations that altered plasmid copy number. Additionally, the use of different promoters to drive protein expression decreases the chances that the mutations affect transcriptional control. To determine whether the Amp^R/Mal^+ phenotypes of our folding enhanced strains were due to plasmid-linked mutations, we reisolated pCWL2 and pCWL4 from these strains and tested the Amp^R/Mal^+ activity. All folding enhanced strains found to be Amp^R/Mal^+ as a result of plasmid mutation, rather than chromosomal mutation such as MT708, were excluded from further analysis.

Strains and plasmid construction

The construction of the pMB1-β-lactamase-MBP fusion plasmids, wild type and G32D/I33P, has been previously described [5]. PCR was used to amplify the sequences of *osmY*, *ivy*, *dppA*, *oppA*, and *hdeB* from *E. coli* K-12 (MG1655) chromosomal DNA with flanking restriction enzyme sites using primers as listed in Supplementary Table 2. All plasmids were verified by sequencing and are listed in Supplementary Table 3. All strains used in this study are listed in Supplementary Table 4. *osmY*, *ivy*, *dppA*, *oppA*, and *hdeB* were cloned into the pTrc vector with 5' NdeI and 3' BamHI restriction sites to generate pTrc-OsmY, pTrc-Ivy, pTrc-DppA, pTrc-OppA and pTrc-HdeB. *malE* (wild type and G32D/I33P) was cloned into pCDFTrc with 5' NdeI and 3' BamHI restriction sites to generate pCDFTrc-MBP, and *osmY* was cloned into pCDFTrc with 5' and 3' BamHI restriction sites to generate pCDFTrc-OsmY. *ivy*, *oppA*, and *hdeB* were cloned into pBAD33 using 5' SacI and 3' HindIII restriction sites to generate pBAD33-Ivy, pBAD33-OppA and pBAD33-HdeB; for *dppA*, SacI and XbaI restriction sites were used to generate pBAD33-DppA. For OsmY protein expression, we cloned *osmY* into pET28 with an amino-terminal Sumo tag as was previously done for spy [7]. *osmY* was amplified starting at codon 29 to remove the periplasmic leader sequence with 5' BamHI and 3' XhoI restriction sites to generate pET28b-His_6-Sumo-OsmY 29–201. For DppA, OppA, Ivy, and HdeB protein expression, we cloned each gene (without native stop codon) into pET21 with 5' NdeI and 3' XhoI restriction sites, encoding proteins with a carboxy-terminal hexahistidine tag, generating pET21a-DppA-His_6, pET21a-OppA-His_6, pET21a-Ivy-His_6 and pET21a-HdeB-His_6. Prior to cloning *dppA* into pTrc and pET21 using BamHI, we made a synonymous mutation for glycine at codon 438 (GGG to GGC) to remove a natively encoded restriction site using standard site-directed mutagenesis. Mutagenic primers are listed in Supplementary Table 2.

Protein identification by mass spectrometry

Our folding enhanced variants selected on the basis of high levels of ampicillin resistance and on their ability to ferment maltose were next investigated using proteomic techniques. Most chaperones are needed in stoichiometric quantities to inhibit aggregation or facilitate

protein folding. Thus, we decided to simply look for proteins that were overexpressed to substantial levels to identify candidate chaperones. Proteins in periplasmic extracts observed to be upregulated on either 1- or 2-dimensional SDS-PAGE gels were extracted and identified using mass spectrometry (University of Michigan Core Proteomics and Peptide Synthesis Core; Indiana University Mass Spectrometry Facility). Periplasms were extracted using an improved method developed in our lab that limits cytosolic protein contamination [49].

Western blotting

Rabbit derived OsmY, DppA, OppA, HdeB, and Ivy antisera were made to order by Pacific Immunology (Ramona, CA) starting with ~5 mg of purified proteins. Monoclonal antibody to the β subunit of RNA polymerase (NT63; anti-mouse) was purchased from Neoclone (Madison, WI). All primary antibodies were used at 1:5000 dilutions for western blotting. Fluorescent dye conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE) and used at a 1:25000 dilution. Imaging was performed using the LI-COR Odyssey CLx. Quantitation of relative protein levels of OsmY, DppA, OppA and HdeB between MT426 (non-folding enhanced strain) and presumed folding enhanced strains (MT695-MT803) was done by normalizing to the RNA polymerase β subunit cellular loading control except in the case of Ivy where we normalized to a non-specific background band present on all blots. Samples were prepared by growing cells in LB liquid media with appropriate antibiotics to an optical density at 600 nm of ~0.2–0.8, centrifuging at 2000×*g* rpm for 10 min at 4°C, and extracting whole cell soluble protein using Bacterial Protein Extraction Reagent (Thermo Scientific, Rockford, IL) at an optical density at 600 nm = 10. Equal numbers of cells between strains, as determined by optical density at 600 nm, were analyzed. For determining β-lactamase-MBP levels in folding enhanced strains (Supplementary Figure 1), equal amounts of protein from whole cell soluble extractions were blotted using a mouse derived monoclonal β-lactamase primary antibody (Santa Cruz Biotech Dallas, TX) and goat anti-mouse secondary antibody from LiCOR.

In vivo β-lactamase-MBP$_{G32D/I33P}$ stabilization assay

E. coli MG1655 Δ*malE*, Δ*hsdR* (MT419; unmutagenized, non-folding enhanced strain) was transformed with pCWL2 and either pCDFTrc-OsmY or pCDFTrc, and either pBAD33-OppA, pBAD33-DppA, pBAD33-Ivy, pBAD33-HdeB, or pBAD33. As a positive control for ampicillin resistance, pMB1-β-lactamase MBP (wild type), pCDFTrc, and pBAD33 were transformed into MT419. Cells were cultured at 37°C in LB until reaching an optical density at 600 nm of ~0.5, followed by the addition of 1 mM IPTG and 0.2% L-arabinose for 45 min to allow for protein expression. Next, cells were pelleted at 2000×*g* for 10 min at 4°C, resuspended in 170 mM NaCl to an optical density at 600 nm of 10^1, and serially diluted in 170 mM NaCl to yield optical density at 600 nm cell titers of 10^0 through 10^{-4}. Next, 1.5 μl of each cell titer was spotted on LB agar or on LB agar containing variable amounts of ampicillin and grown for ~36 hr at 30°C. Plates also contained 1 mM IPTG and 0.2% L-arabinose for protein induction.

In vivo $MBP_{G32D/I33P}$ stabilization assay

E. coli MG1655 Δ*malE*, Δ*hsdR* (MT419) was transformed with pCWL4 and either pTrc-OsmY, pTrc-OppA, pTrc-DppA, pTrc-Ivy, pTrc-HdeB, or pTrc-99a. As a positive control for maltose fermentation, pCDF-MBP WT with pTrc-99a was transformed into MT419. We next streaked out single colonies of each strain on MacConkey base agar plates with 1% maltose, 0.1 mM IPTG, and appropriate antibiotics for maintenance of plasmids. Plates were incubated at room temperature for ~48 hr.

Protein expression and purification

For protein expression, BL21(DE3) *E. coli* cells were grown to an optical density at 600 nm of ~1.5 at 37°C in Protein Expression Media (PEM; 12 g tryptone, 24 g yeast extract, 40 ml glycerol, 2 g monobasic potassium phosphate, 12.5 g dibasic potassium phosphate, ddH_2O remaining volume to 1 liter), the temperature was reduced to 20°C, and overexpression of cytosolic (His_6-Sumo-OsmY) or periplasmic (Ivy-His_6, DppA-His_6, OppA-His_6, and HdeB-His_6) proteins was induced by the addition of isopropyl IPTG to a final concentration of 0.3 mM. Protein expression was allowed to continue for ~16 hr at 20°C. We chose to overexpress OsmY in the cytosol rather than in the periplasm prior to purification to avoid loss of OsmY by secretion into the growth media. All proteins were purified using the ÄKTA Pure HPLC and GE Healthcare chromatography columns (GE Healthcare, Amersham, UK) to > 95% homogeneity.

Following overexpression of His_6-Sumo-OsmY, cells were lysed by high-pressure homogenization in Ni^{2+} buffer A (40 mM Tris-HCl, 10 mM NaPi, 400 mM NaCl, 10% glycerol, 10 mM imidazole, pH 8.0) plus “cOmplete” protease inhibitor (Roche, Indianapolis, IN), DNaseI (1 mg/ml), 10 mM $MgCl_2$ and centrifuged to remove insoluble materials. His_6-Sumo-OsmY was next bound to HisTrap immobilized metal affinity chromatography resin, washed with Ni^{2+} buffer A, and eluted with Ni^{2+} buffer B (40 mM Tris-HCl, 10 mM NaPi, 400 mM NaCl, 10% glycerol, 500 mM imidazole, pH 8.0). Next, the His_6-Sumo tag was cleaved from OsmY by His_6-Ulp1 via an engineered protease site (leaving mature OsmY lacking the periplasmic leader sequence and containing single non-native serine on amino-terminus) at 4°C overnight concurrent with dialysis into Ni^{2+} buffer A to remove imidazole. Next, a second round of immobilized metal affinity chromatography was employed, with OsmY not binding to HisTrap resin, to remove His_6-Sumo, His_6-Ulp1, and other immobilized metal affinity chromatography resin binding impurities. As a final step, OsmY was purified using ion-exchange chromatography with HiTrap Q-sepharose. OsmY was exchanged into OsmY Q buffer A (25 mM Tris-HCl, pH 8.9), bound to HiTrap Q resin, and eluted over a gradient from 0%–100% with OsmY Q buffer B (25 mM Tris-HCl, pH 8.9, 1 M NaCl). Finally, OsmY was exchanged into 40 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 100 mM NaCl for storage.

OppA-His_6, DppA-His_6, or Ivy-His_6 were all purified using immobilized metal affinity chromatography under identical conditions. Following over-expression of OppA-His_6, DppA-His_6, or Ivy-His_6, periplasms were extracted using osmotic shock [49] and dialyzed into Ni^{2+} buffer A. Each protein was bound to HisTrap resin and eluted over a gradient from 0%–100% Ni^{2+} buffer B. Fractions containing desired protein were pooled for ion-exchange

chromatography using HiTrap Q sepharose under the same conditions as described for OsmY, except in choice of buffers (Ivy Q buffer A is 20 mM Tris, pH 8.0; Ivy Q buffer B is 20 mM Tris-HCl, 1 M NaCl, pH 8.0; Ivy storage buffer is 50 mM NaPi, 100 mM NaCl, pH 8.0; DppA Q buffer A is 20 mM Tris-HCl, 20 mM NaCl, pH 8.0; DppA Q buffer B is 20 mM Tris-HCl, 1 M NaCl, pH 8.0; DppA storage buffer is the same as Ivy storage buffer; OppA Q and storage buffers are the same as for DppA). HdeB-His_6 was purified as described [11]. Following purification, proteins were flash frozen using liquid nitrogen and stored at −80°C until use except in the case of OsmY, which was stored at −20°C.

Aggregation assays

Recombinant luciferase from the North American firefly was purchased from Promega (Madison, WI; product number E1702) and aliquots were stored in glass vials at −80°C until use. Thermal unfolding was initiated by diluting luciferase to a final concentration of 200 nM in 40 mM HEPES, pH 7.5 at 40.5°C with constant mixing. Aggregation was measured by light scattering at 360 nm using a Hitachi F-4500 fluorimeter (Ex: 360 nm/Em: 360 nm). L-lactate dehydrogenase (LDH) from rabbit muscle was purchased from Roche (Indianapolis, IN; product number 10127230001), dialyzed extensively into 40 mM HEPES, pH 7.5 to remove all possible ammonium sulfate, and stored at −80°C prior to aggregation assays. Thermal unfolding was initiated by diluting LDH to a final concentration of 2 μM in 40 mM HEPES, pH 7.5 at 43°C with constant mixing. Aggregation was measured as described above for luciferase. α-lactalbumin from bovine milk was purchased from Sigma-Aldrich (St. Louis, MO; product number L6010) and resuspended from lyophilized powder into 50 mM sodium phosphate, 100 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid, pH 7.0 (reaction buffer) immediately prior to aggregation. The concentration of α-LA was 50 μM and aggregation was initiated by the addition of dithiolthreitol to a final concentration of 20 mM and incubation at 37°C. Aggregation was monitored using a Biotek Synergy plate reader measuring absorbance at 360 nm every 5 min, shaking for 10 s prior to each measurement. In the case of all substrates, we measured amorphous (nonamyloid) aggregation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MBP	(maltose binding protein)
LDH	(lactate dehydrogenase)
Im7	(immunity protein 7)

α-LA	(α-lactalbumin)
DTT	(dithiothreitol)
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

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Highlights

- Chaperones are usually identified through their upregulation in response to stress
- Using a fold or die genetic selection we have generated folding enhanced bacterial strains
- Folding enhanced strains overproduce OsmY, Ivy, DppA, OppA and HdeB
- OsmY, Ivy, DppA, OppA and HdeB function as chaperones
- Chaperones can thus be identified by selecting for improved protein stability

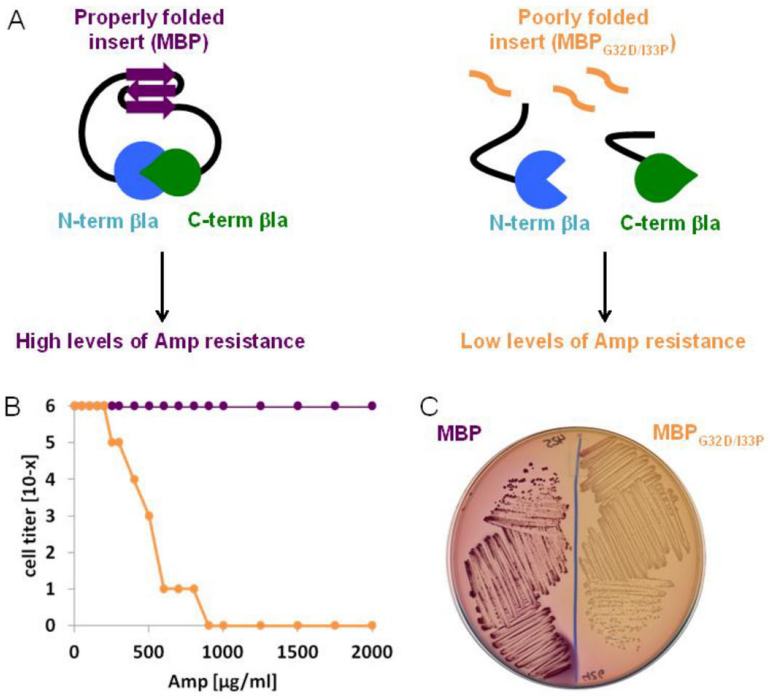

Figure 1.

A protein folding biosensor to quantitatively measure protein stability and directly select for novel chaperones in vivo. (**A**) In this system, a test protein, in this case the maltose binding protein (MBP) (purple or orange) is inserted within the antibiotic resistance gene β-lactamase (blue and green) to form a tripartite fusion. If the test protein is stable, then more of the test protein will fold properly and cells will be able to survive higher levels of antibiotic. This system can be used to select for improved stability of the test protein or, alternatively, to evolve a better cellular folding environment by forcing cells to fold the unstable tripartite fusion. (**B**) β-lactamase-MBP confers higher resistance than β-lactamase-$MBP_{G32D/I33P}$ to ampicillin across a wide range of antibiotic concentrations in our non-folding enhanced genetic background (MT419) (purple line MT425, orange line MT426). (**C**) The test protein MBP provides an additional in vivo measure of protein folding because the activity of MBP can be monitored by maltose fermentation on MacConkey-maltose agar, which requires properly folded MBP. In a strain of *E. coli* lacking the gene for MBP, expression of MBP (MT425) allows for maltose utilization (purple color), whereas expression of $MBP_{G32D/I33P}$ (MT426) does not (yellow color).

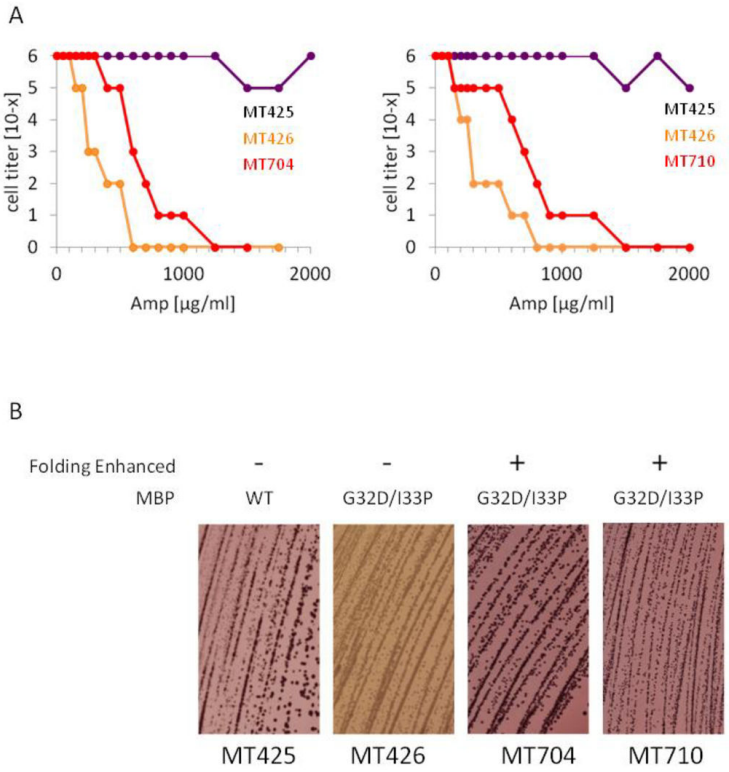

Figure 2.

Presumptive folding enhanced strains e.g. MT704 and MT710 improve the activity of β-lactamase-$MBP_{G32D/I33P}$ and $MBP_{G32D/I33P}$ as determined by (**A**) resistance to ampicillin and (**B**) utilization of maltose. Ampicillin resistance was measured by spot titer assay and maltose utilization was measured by color on MacConkey agar where maltose is the preferred carbon source. MT425 carries plasmids expressing the β-lactamase-MBP fusion and MBP and was not selected/screened for folding enhancement of our test proteins. MT426, MT704 and MT710 all carry plasmids expressing β-lactamase-$MBP_{G32D/I33P}$ and $MBP_{G32D/I33P}$. MT426 was not, while MT704 and MT710, were selected/screened for folding enhancement of our test proteins.

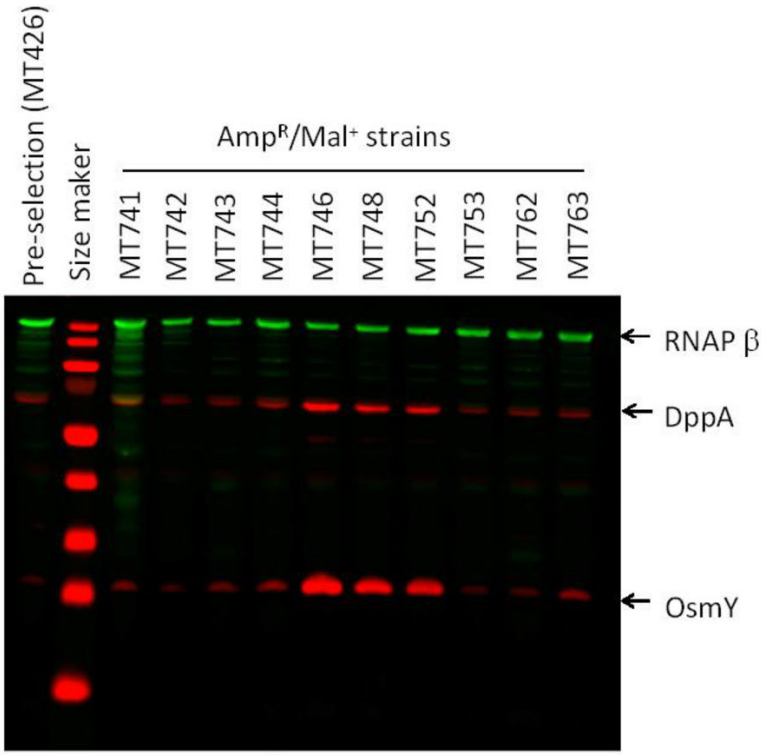

Figure 3.

OsmY and DppA are overexpressed in folding enhanced strains selected/screened for Amp^R/Mal$^+$ activity. Protein levels of OsmY and DppA in strains evolved to stabilize MBP$_{G32D/I33P}$ were measured using quantitative western blotting with infrared dye conjugated secondary antibodies. OsmY (red) and DppA (red) levels were normalized to the β subunit of RNA polymerase (RNAP β) (green). For comparison, baseline levels of OsmY and DppA were also measured in the non-folding enhanced, pre-selected/screened strain (MT426).

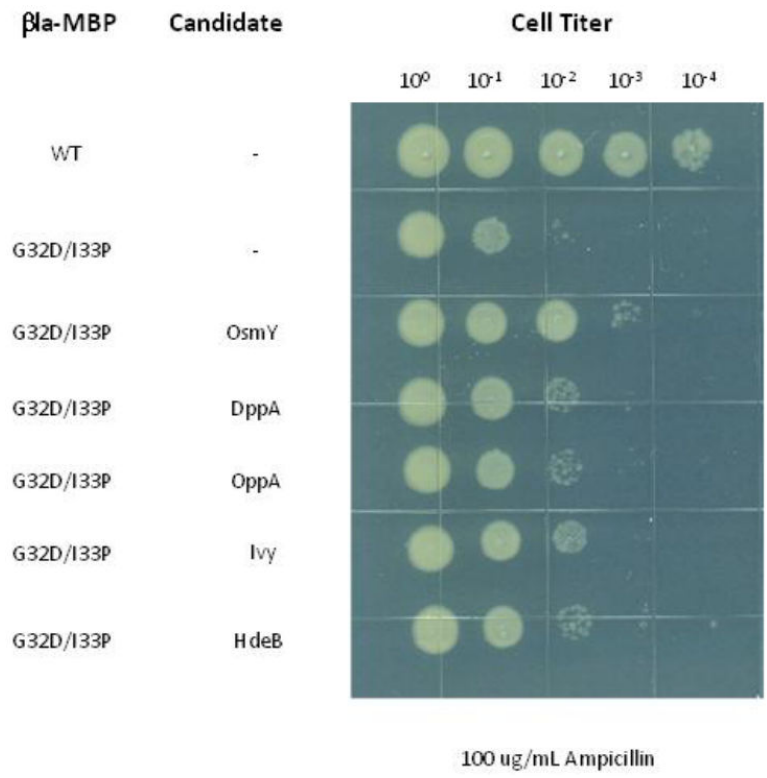

Figure 4.

Overexpressed proteins enhance the ampicillin resistance of β-lactamase-MBP$_{G32D/I33P}$. OsmY, DppA, OppA, Ivy, and HdeB promote the activity of the unstable test protein β-lactamase-MBP$_{G32D/I33P}$ in the pre-selected, non-folding enhanced genetic background of *E. coli* (MT419; K-12, MG1655 Δ*malE* Δ*hsdR*). Resistance to ampicillin determines activity of the β-lactamase-MBP fusion; cells were diluted logarithmically. At the concentration of ampicillin shown (100 ug/ml), plasmid expression of β-lactamase-MBP (WT) provides a 3-log increase in ampicillin resistance compared to that obtained with the expression of the unstable β-lactamase-MBP$_{G32D/I33P}$. Plasmid expression of OsmY (pCDFTrc), DppA (pBAD), OppA (pBAD), Ivy (pBAD), and HdeB (pBAD) all improve the activity of the unstable β-lactamase-MBP$_{G32D/I33P}$ fusion, allowing increased cell survival by 1–2 logs compared to the strain containing only empty vectors. All strains at each titer grow to the same extent on LB plates without ampicillin

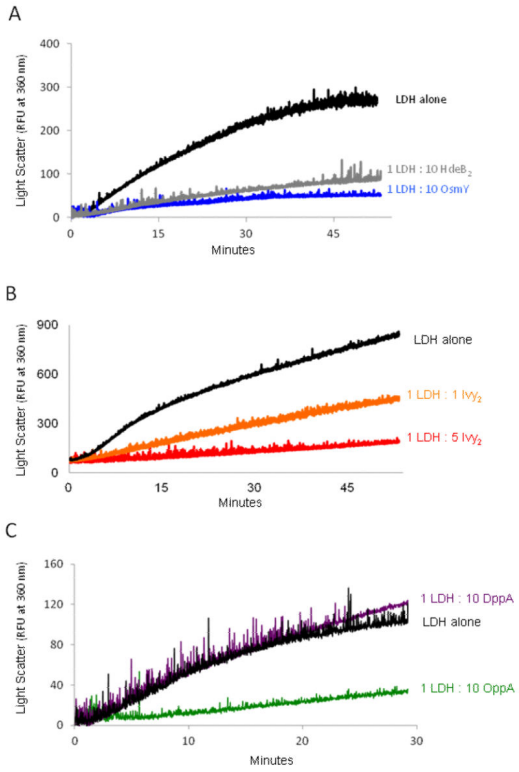

Figure 5.

OsmY, HdeB, Ivy and OppA have chaperone activity in vitro. (**A**) OsmY and HdeB inhibit thermal aggregation of lactate dehydrogenase (LDH). (**B**) Ivy inhibits thermal aggregation of LDH. (**C**) OppA, but not DppA, inhibits the thermal aggregation of LDH. LDH aggregation was measured by light scattering at 360 nm and was induced at 43°C, The concentrationof LDH used was 2 μM for LDH and the concentrations of OsmY (monomer), HdeB (dimer), Ivy (dimer), DppA (monomer) and OppA (monomer) are given relative to the concentration of LDH.