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Synthetic inter-species cooperation of host and virus for targeted genetic evolution

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ABSTRACT

Cooperative inter-species interaction is another way of evolution in nature. Such cooperation leading to mutual benefits provides a new view on the interaction of biological systems, and engineering such inter-species interaction offers an opportunity for diverse potential applications in biotechnology. Here we show a synthetic system with artificially created inter-species cooperation of host and virus. A genetically engineered host (*Escherichia coli*) provides nutrients and energies necessary for virus reproduction, and a genetically engineered virus (phage M13) enhances infected hosts to survive under fatal concentration of antibiotics. We applied the synthetic system to evolve the virus with increasing selective pressure in environment, specifically to evolve a virus-carried heterogeneous gene (*luxR*), which consequently enhances the ability of infected hosts to survive against antibiotics. As a result we obtained evolved *luxR* mutants with improved activity by up to 17 folds from a limited number of viruses randomly isolated and generated over a small number of generations. Our synthetic system would provide the significant sight in the principle of system design with regard to the utilization of inter-species cooperation, and also the targeted evolution technique itself. Advancement of the concept of this system would foster more practical and valuable applications.

1. Introduction

Inter-species cooperation is another way of evolution distinct from the traditional competition-driven evolution, and such cooperation leading to mutual benefits has been commonly discovered in natural ecosystems under diverse environmental conditions and over varying species: probiotic microbes and humans, crocodiles and crocodile birds, ants and aphids, *etc*. Even several viruses have been found to help host organisms survive in harsh conditions (Márquez et al., 2007; Roossinck, 2005). The cooperation-driven evolution theory, complementary to the traditional one, has expanded the understanding on the evolution of living organisms by providing a different view on evolution. As in the understanding of evolution, creating a small ecosystem in laboratory consisting of cooperatively interacting living organisms offers an opportunity for tackling down problems and diverse applications in biotechnology with a different approach.

Recently, the emerging field called synthetic biology has made it possible to create synthetic biological systems with novel functions by utilizing the design principles of engineering (McDaniel and Weiss, 2005): systematical analysis of designed systems in assistance with mathematical models and modularization of each network to build new systems for more complex operation by interfacing with the modularized systems. Synthetic biology allows rational design or redesign of living systems and assists to tackle down biological problems more rationally and systematically. In addition, synthetic biology provides a method to study biological systems and synthesized systems have practical applications in biotechnology as well (Atsumi and Little, 2006; Chuang et al., 2009; Shou et al., 2007).

Here, by utilizing the engineering principle of synthetic biology (Whitehead et al., 2001), we present a synthetic inter-species cooperative system with a genetically engineered virus, phage M13, and a genetically engineered host, *Escherichia coli*, in which the cooperation leads to survival of both species in selective conditions through iterated evolutionary processes. We apply the system for the *in vivo* targeted evolution of a specific heterogeneous target gene carried by the virus. The success in targeted evolution depends on the generation of large sequence diversity and the effectiveness of the selection process, but *in vivo* evolution has been restricted by the difficulty of generating sufficient sequence diversity in living organisms, and the labor-intensive nature of the necessary selection procedures (Das et al., 2004; Jäckel et al.,

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2008). The synthetic system enables us to supply sufficient virus population with mutants by reproducing viruses and hosts, and to simplify the overall evolution process by utilizing the principles of cooperation-driven evolution. The synthetic system offers an opportunity to understand and study the co-evolution of living organisms through inter-species cooperation and the constructed system has practical applications in the targetedevolution of genes as well.

2. Materials and methods

2.1. Cell culture

The utilized *E. coli* strain, XL1-blue MRF' (Stratagene) containing F' episome which mediates M13-derivative carrier phage infection, was incubated in 2 × YT medium (1.6% bacto tryptone, 1% bacto yeast extract, 0.5% NaCl) containing appropriate antibiotics.

2.2. Genetic construction

The oligonucleotide sequences utilized for constructing carrier phage plasmid, evolver plasmid, and packaging plasmid are listed in Table S1 see Supplementary Data. At the end of each constructed gene, we attached a T1/TE transcription terminator (MIT registry, accession no. BBa_B0015). The ribosome binding sites for each gene were designed for optimization of gene expression levels as previously described (Na and Lee, 2010; Na et al., 2010).

The genetic sequences utilized for the construction of carrier phage plasmid as a form of phagemid were PCR-cloned as follows: the M13 replication origin from pBluescript SK+ (Stratagene), the zeocin resistance gene from PCR®-Blunt II-TOPO® (Invitrogen), the *luxR* gene from the *Vibrio fischeri* ES114 genome (ATCC 700601D), and the pUC replication origin from pBluescript. Constitutive promoter sequences were obtained from the MIT registry (accession no. BBa_I14032) and were synthesized.

The DNA sequences used for the evolver plasmid construction were PCR-cloned as follows: the *lux* promoter from *V. fischeri* ES114, the *β-lactamase* gene from pBluescript SK+, the RSF replication origin from pRSF-Duet (Novagen), the *gfp* gene from pEGFP-N1 (Clontech), the *cat-1* gene from the pHS G396 plasmid (Takara), and the *polA* gene from *E. coli* DH5α. The *polA* gene was modified to increase its mutation rate (D424A, A759R, I709N; as previously reported (Camps et al., 2003)) using site-directed mutagenesis, and the resulting sequence was ligated to a weak constitutive promoter (MIT registry, accession no. BBa_J23113) because of the lethality of the *polA* gene.

To construct the packaging plasmid, the M13 replication origin was removed from the M13K07 plasmid (New England Biolabs).

2.3. Evolution

E. coli host cells (OD_{600} < 1.0) were infected with carrier phages for 30 min at 37 °C, and then the host cells were incubated for two hours with shaking to allow expression of LuxR and β-lactamase. The cells were then inoculated to 100× volume of 2 × YT medium containing 25 μg/ml zeocin and the appropriate concentration of aztreonam (AZT). Cultures were incubated until the optical density at 600 nm reached 2.0. The cells were then heated at 60 °C for 30 min and centrifuged for 10 min at 3000 rpm. The carrier phages were harvested from the supernatant and used to infect fresh host cells for another round of evolution.

2.4. LuxR activity measurement

For measuring LuxR activity, the modified *polA* gene was removed from the evolver plasmid and the *gfp* gene was inserted after the *β-lactamase* gene, such that *gfp* was expressed polycistronically under the control of the *lux* promoter where LuxR protein binds and initiates transcription. Mutant *luxR* genes were cut and ligated into the original carrier phage plasmid. Host cells containing the modified evolver plasmid, and carrier phages harboring a mutant *luxR* were grown to stationary phase with zeocin (25 μg/ml) and chloramphenicol (34 μg/ml). The cells were spun down and resuspended in 100 mM sodium phosphate buffer and were observed under a fluorescent microscope equipped with 480 nm (excitation) and 510 nm (emission) filters (Axiovert 25, Carl Zeiss). Average GFP intensity per cell was measured using the SigmaScan Pro 5 image processing software (Systat Software). To get a relative activity, the intensity was divided by the wild type LuxR activity in the absence of N-(3-oxohexanoyl)-L-homoserine lactone ($3OC_6HSL$), since LuxR is a member of a family of acyl-homoserine lactone-responsive quorum-sensing transcription factors and is activated by $3OC_6HSL$ (Urbanowski and Lostroh, 2004).

For measuring wild type LuxR or mutant LuxR activities under various concentrations of $3OC_6HSL$ (Sigma, St. Louis, MO), $3OC_6HSL$ was prepared as a stock solution in ethylacetate and stored at −20 °C. Before cell inoculation, $3OC_6HSL$ was dried well and dissolved in 2 × YT medium.

3. Results and discussion

3.1. Synthetic evolution system

Here we present a synthetic targeted evolution system inspired by inter-species cooperation. A schematic diagram of the host-virus co-survival process, *in vivo* targeted evolution processes, and constructed plasmid genetic structures used in our synthetic system are shown in Fig. 1. We tested our synthetic system for its ability to evolve a transcription factor, *luxR* gene originated from *Vibrio fischeri* ES114, with the aim of increasing its activity in the absence of its cofactor, N-(3-oxohexanoyl)-L-homoserine lactone ($3OC_6HSL$). LuxR protein is activated when bound with $3OC_6HSL$, and activated LuxR protein binds to *lux* promoter and initiates transcription of downstream genes (Urbanowski and Lostroh, 2004). Due to the usefulness of the transcription factor sensing intercellular interactions, LuxR proteins are widely used as a component for developing synthetic systems, and also widely engineered for satisfying diverse objectives (Andersen et al., 2001; Basu et al., 2004; Collins et al., 2005; Egland and Greenberg, 2000; Poellinger et al., 1995; You et al., 2004).

To realize the synthetic system, we constructed three plasmids as shown in Fig. 1a. The carrier phage plasmid carries a target gene to evolve (*luxR*), an M13 replication origin for its reproduction, and a pUC replication origin for high-level duplication inside host cells (Vieira and Messing, 1982; Yanisch-Perron et al., 1985). We used M13 phage as a target-gene carrier. During evolution, host chromosomal genes may be also spontaneously mutated, and which may alter host susceptibility against antibiotics and thereby growth rate. Therefore, genetic rescue of *luxR* mutants from host cells as a form of phage and reinfection into fresh host cells allow us to easily eliminate false selection. Furthermore, M13 phage does not lyse its host cell and therefore we can obtain progeny carrier phages in a large quantity (Marvin and Hohn, 1969). The evolver plasmid contains a β-lactamase gene under the control of the *lux* promoter allowing activation by LuxR proteins for selection with aztreonam (AZT). AZT is an effective β-lactam antibiotics and degraded by β-lactamase like ampicillin, a commonly used β-lactam antibiotics (Brogden and Heel, 1986). Upon carrier phage infection, antibiotic-resistance proteins (β-lactamase) are produced from the β-lactamase gene in the evolver plasmid according to the activity of the target gene (*luxR*) carried by the phages. Treatment with AZT at appropriate

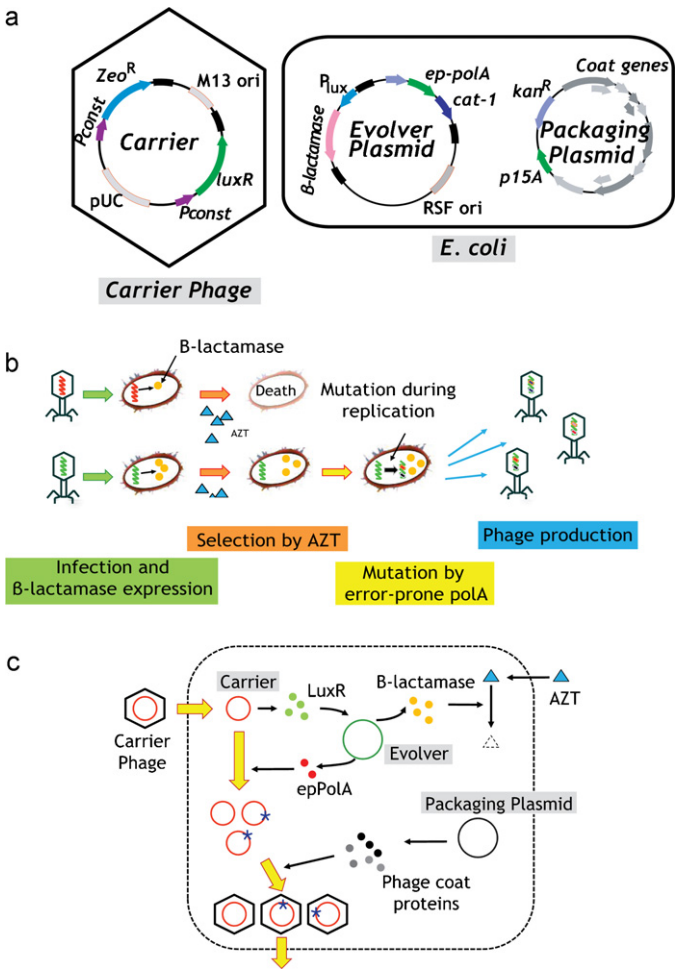

Fig. 1. Schematic diagram of the evolutionary processes and plasmid structures used in our synthetic system. (a) The genetic structures of the plasmids used in the synthetic system are shown. The evolver and packaging plasmids are embedded in the host cells. The phage coat protein-encoding genes in the packaging plasmid are omitted for the sake of simplicity. (b) Upon infection of the host cell, the carrier phage transfers a target gene (*luxR*) that triggers proportional production of the AZT-resistance protein, β-lactamase. AZT treatment kills cells with insufficient resistance proteins, while cells with higher resistance protein production survive. After selection, the host cell population is enriched for cells harboring target genes with enhanced activity. During carrier phage plasmid replication, mutations are introduced by an error-prone polymerase (epPolA) and the plasmid is then packaged as a carrier phage. The procedure is repeated with the application of increasing AZT concentrations, thereby driving evolution of enhanced *luxR* activity. (c) A detailed view of the processes carried out inside the infected host cell is shown. LuxR proteins are constantly produced from the *luxR* gene in the carrier phage plasmid. LuxR initiates the production of AZT-degrading β-lactamase from an evolver plasmid *via* the *lux* promoter. Host cells with sufficient expression of β-lactamase can survive under a high concentration of AZT. The evolver plasmid expresses an error-prone polymerase, which introduces mutations during carrier phage plasmid replication. Replicated carrier phage plasmids are packaged by phage coat proteins produced from a packaging plasmid. Under increasing selection pressure, each subsequent generation of carrier phages should be enriched for those with evolved mutant *luxR* genes having increased activity.

concentration inhibits or retards the growth of host cells with insufficient production of β-lactamase proteins, while host cells with sufficient resistance protein production can be resistant and grow normally. As a result, the population becomes enriched for cells infected with phages that express target gene-encoded proteins with enhanced activity. The evolver plasmid also contains an error-prone polymerase (*polA*) gene for increased mutation of the carrier phage DNA sequences (Camps et al., 2003). The genetic sequences of the carrier phages inside host cells are mutated during their DNA replication by the error-prone polymerase. The packaging plasmid provides all phage genes necessary for carrier phage reproduction (Kazmierczak et al., 1994; Marvin, 1998). Encapsulated carrier phages are secreted from host cells and the new carrier phage population proceeds to the next generation of evolution by infecting new host cells.

The detailed *in vivo* evolution processes are depicted in Fig. 1b and c. We utilized an antibiotics-based genetic selection method which has been reported to be capable of *in vivo* selection (Kambam et al., 2008; Negri et al., 2000). In selection process, upon carrier phage infection LuxR proteins are sufficiently produced in host cells at a constant rate from the carrier phage plasmid and the proteins initiate the transcription of β-lactamase under the control of the *lux* promoter (P_{lux}) in the evolver plasmid. Thus, the activity of the LuxR protein determines the amount of β-lactamase proteins in host cells, and therefore determines antibiotics susceptibility of host cells (Negri et al., 2000). The growth rate of resistant cells is faster than that of susceptible cells and treatment of appropriate concentration of AZT resulting in different growth rates could enrich host cell population with more resistant cells, that is, with higher LuxR activity. After iterated selection the population is enriched for cells harboring *luxR* mutant carrier phages with enhanced activity.

In mutagenesis process, carrier phage plasmids are mutated in aid of an error-prone polymerase during plasmid replication. Carrier phage plasmids are replicated in host cells in the same manner as a conventional plasmid in assistance with a host polymerase (PolA) due to the presence of ColE1-derived pUC replication origin (Itoh and Tomizawa, 1980). The polymerase was engineered as reported previously to increase its mutation rate and was embedded in the evolver plasmid (Camps et al., 2003). During DNA replication, the carrier phage sequences are mutated by the modified error-prone polymerase (epPolA) expressed from the evolver plasmid (Camps et al., 2003), which introduces mutations preferentially in ColE1 plasmids (Itoh and Tomizawa, 1980).

To produce new mutant carrier phages single-stranded phage DNA sequences are produced from carrier phage plasmids and then encapsulated by phage coat proteins produced from the packaging plasmid (Marvin, 1998). The encapsulated phages are secreted from host cells through membrane channels formed by phage proteins expressed from the packaging plasmid as well (Kazmierczak et al., 1994). The newly produced carrier phages are used to infect fresh host cells, thereby beginning the next generation, and higher AZT concentration than the previous generation is supplied to increase selection pressure. These processes continue to drive targeted evolution of *luxR* gene.

3.2. Determination of effective AZT concentration range

For efficient evolution, it is critical to determine appropriate selective concentration range of AZT in order to maximize the growth rate difference of host cells in population, which ensures rapid dominance of host cells harboring more active *luxR* mutants. In order to determine the relative activity, we measured LuxR activities by measuring GFP intensities produced from *lux* promoter-controlled *gfp* gene in various concentrations of $3OC_6HSL$ (0–1000 nM) and then divided the activities by the activity in the absence of $3OC_6HSL$. The relative activity of unactivated LuxR is 1 ($f = 1$) and that of fully activated LuxR is 14 ($f = 14$) as shown in Fig. 2a.

To show that the growth rate difference-based selection by AZT is feasible for selection, we investigated the effects of supplied AZT concentration and LuxR activity on host cell growth rate (Fig. 2b). Infected host cells with carrier phages containing wild type LuxR were incubated with various concentrations of AZT (0–1.0 μM) and $3OC_6HSL$ (0–1000 nM) resulting in different LuxR activities. We calculated the minimal AZT concentration for the complete growth inhibition of host cells at a specific LuxR

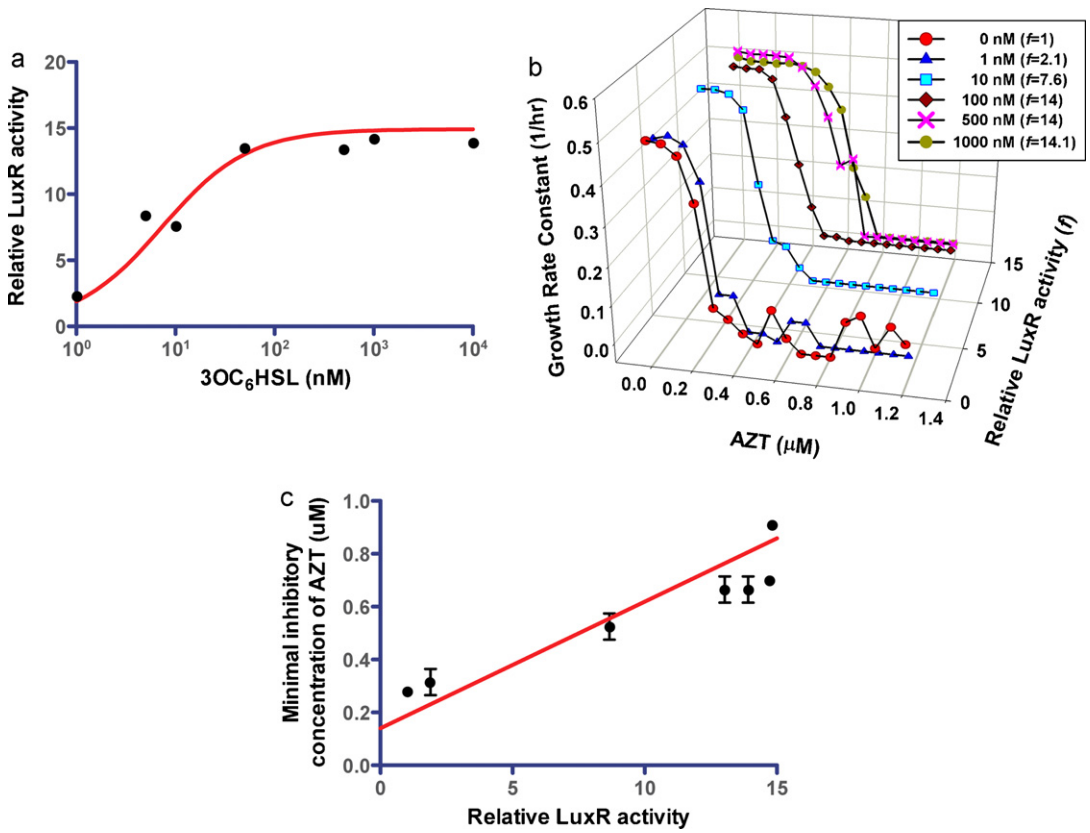

Fig. 2. The experiment results for determining selective AZT concentration for effective selection. (a) The relative LuxR activity in the presence of various $3OC_6HSL$ concentrations ranging from 0 nM to 1000 nM was measured and plotted. First, the GFP intensity produced from *lux* promoter initiated by LuxR protein was normalized by optical density at 600 nm. Then, the normalized intensity under a given concentration of $3OC_6HSL$ is divided by that without $3OC_6HSL$. The relative LuxR activity (f) in the absence of $3OC_6HSL$ is defined as 1 ($f = 1$) and that in the presence of 1000 nM $3OC_6HSL$ is 14 ($f = 14$). (b) The effect of supplied AZT concentrations on host cell growth was measured. Host cells were infected with carrier phages and grown in 2 × YT medium containing zeocin (25 μg/ml), and various concentrations of $3OC_6HSL$ (0–1000 nM) and AZT (0–1.2 μM). The growth rate constants were calculated by measuring OD_{600} at 0 h and 10 h post-incubation. (c) From the results in (b) the minimal AZT concentration to inhibit cell growth completely is plotted with respect to LuxR activity. The triplicate experiment results are also shown as closed circles with standard deviation. A linearly regressed line is also shown in the graph.

activity (Fig. 2c). As shown in Fig. 2c, host cells with different LuxR activities require different minimal supplied AZT concentration for their growth inhibition. For example, for complete growth inhibition of cells with a relative LuxR activity of $f = 5$, at least 0.37 μM AZT should be supplied. For cells with LuxR activity of $f = 10$, more than 0.6 μM AZT should be supplied. For the discrimination of the exemplary cells ($f = 5$ and 10), in other words to enrich the population for cells with $f = 10$, supplying AZT between the concentrations, 0.37–0.6 μM, would give a best selection results. The results indicate that adjusting AZT concentration in medium enables us to inhibit or to retard cell growth enough to discriminate host cells with different LuxR activities. Gradually increasing AZT concentration in medium within 0.1–0.8 μM could enrich population with enhanced LuxR mutants by making a difference on growth rate, and therefore would facilitate LuxR mutant selection process.

3.3. LuxR evolution

Using our synthetic system, we performed *luxR* evolution. To investigate the effect of selection pressure controlled by AZT concentration on evolution rate, we performed three independent evolutions with different selection pressures by supplying different concentrations of AZT within the selective concentration range. The selection and mutagenesis processes were repeated for three generations. At each generation, we increased AZT concentration in medium from 0.115 μM to 0.25 μM for higher selection pressure, from 0.115 to 0.23 μM for intermediate selection pressure, or from 0.115 to 0.17 μM for lower selection pressure (Fig. 3a). To find evolved LuxR mutants we sequenced the *luxR* genes of ten randomly isolated carrier phages from each generation. In this study, the generation represents the number of performed selection and mutagenesis, not the number of carrier phage replications. Found mutants at each generation are also shown in Fig. 3a. From the three evolutions, we found five mutants in total. To measure the activities of the mutants, the mutant *luxR* sequences were ligated to a modified evolver plasmid and their activities on *lux* promoter represented as GFP intensity were examined as described in Section 2. The measured relative activities of the identified mutants in absence or presence of $3OC_6HSL$ are shown in Fig. 3b and c, respectively. The identified mutants K68E, P80S, Y41F, P91S, and A73V, had activities that were 4- to 17-fold higher than the wild type activity in the absence of cofactors. Except for K68E, the increased activities of other four mutants are similar to or higher than the maximal activity of wild type LuxR fully activated by 1000 nM $3OC_6HSL$ (Fig. 3b and c).

As shown in Fig. 3a, as the selection pressure increased within the selective AZT concentration range, so did our rate of identifying evolved mutants. Under high and intermediate selection pressures, we found the first mutant from 2nd generation, but under low selection pressure we found the first mutant at 3rd generation. Those results indicate that higher selection pressure could select out less functional mutants efficiently while enhanced mutants became enriched. Notably, the mutants found under high selection pressure, P91S and A73V, had higher activities compared to those found under lower selection pressures (Fig. 3b).

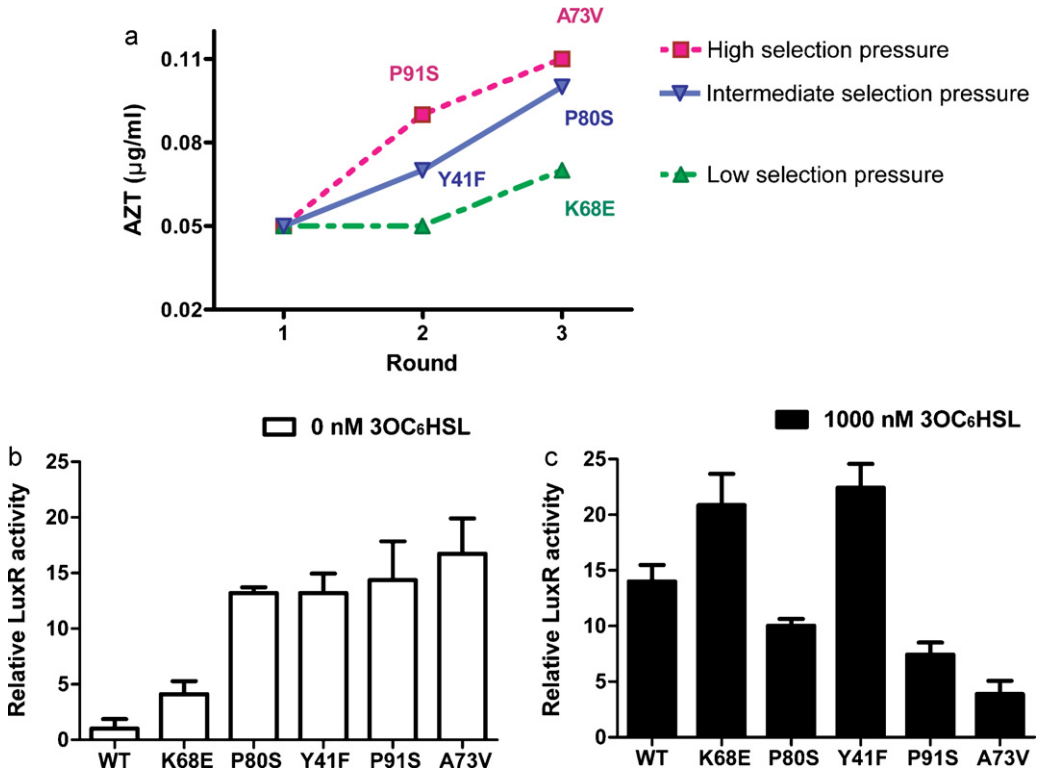

Fig. 3. LuxR evolution results. (a) Different AZT concentrations were supplied for selection in three independent experiments, in order to examine the effects of different selection pressures on evolution. In each generation, carrier phage individuals were randomly isolated and their *luxR* genes were sequenced. The *luxR* mutants identified in each generation are also shown. (b) The activities of mutant LuxR proteins relative to that of wild type LuxR in the absence the cofactor, $3OC_6HSL$, are shown. Activities were measured four times and standard deviations are shown. (c) The relative activities of the mutant LuxR proteins in the presence of 1000 nM $3OC_6HSL$ are shown.

LuxR has four main functional domains (Fig. 4). The five identified mutations were located in a cofactor binding domain (Sitnikov et al., 1996). Therefore, the increased LuxR activities of the mutants in the absence of $3OC_6HSL$ seem to result from structural changes in the cofactor-binding region, rather than from increased affinity of the DNA-binding domain. Since LuxR protein structure has not yet been solved, we investigated the positions of the mutated residues in three-dimensional space by comparison with the known structures of two cofactor-bound LuxR homologs, namely SdiA in *E. coli* and TraR in *Agrobacterium tumefaciens* (Vannini et al., 2002; Yao et al., 2006). The structural information of TraR has been used to investigate the mutational effect of LuxR amino acids in transcriptional activity (Collins et al., 2005). This comparison should provide some initial insight into the structural effects of the obtained LuxR mutations. The three protein sequences were aligned. Though the sequence identity between SdiA and TraR is not high, their secondary structures and tertiary structures are quite similar enough to be comparable (Figs. 4 and 5) (Yao et al., 2006). Furthermore, the predicted secondary structure of LuxR using PSIPRED, which is a reliable protein secondary structure prediction program with high accuracy, was also very similar to the secondary structures of SdiA and TraR (Jones, 1999). Therefore, despite of the relatively low sequence identity among the three proteins, due to the high similarity of secondary structures it was reasonable to compare the sequence and structure alignment results to deduce the role of mutated amino acids of LuxR.

The alignments were used to identify the amino acids in SdiA and TraR that corresponded to the mutated residues in LuxR. Two of five the mutated residues (80th Pro and 41st Tyr) were found to be conserved in the LuxR homologs(Fuqua et al., 1996). As shown in Fig. 5a and b, in the known structures, the estimated distances from the conserved residues to the bound cofactor were less than ~7 Å in both SdiA and TraR, suggesting that these amino acids are likely to

Fig. 4. Alignment of LuxR protein sequence with TraR and SdiA protein sequences. The aligned sequences of LuxR, TraR, and SdiA are shown. The four functional domains of LuxR are indicated with colored bars (Collins et al., 2005; Sitnikov et al., 1996). The positions of mutated residues in LuxR are highlighted in yellow color. Among the five mutations, the 80th Pro and 41th Tyr in the LuxR protein sequence are conserved in LuxR homologous proteins (Fuqua et al., 1996). The five enhancer mutations identified herein are all located in the cofactor-binding domain (region II). Region I comprises of amino acid residues 10–20 and is responsible for autorepression (blue bar). Region II includes residues 33–127 and is responsible for cofactor $3OC_6HSL$ binding (green bar). Region III, including residues 184–230, confers *lux* promoter DNA-binding (violet bar). Region IV, comprising the final 10 residues from the C terminus, functions in the cofactor-dependent activation of the *lux* promoter (red bar) The structurally identified secondary structures of TraR and SdiA are also highlighted: red color for α helix and cyan color for β strand (Vannini et al., 2002; Yao et al., 2006). The secondary structure of DNA-binding domain of SdiA is not yet identified. The secondary structure of LuxR was predicted using PSIPRED (Jones, 1999).

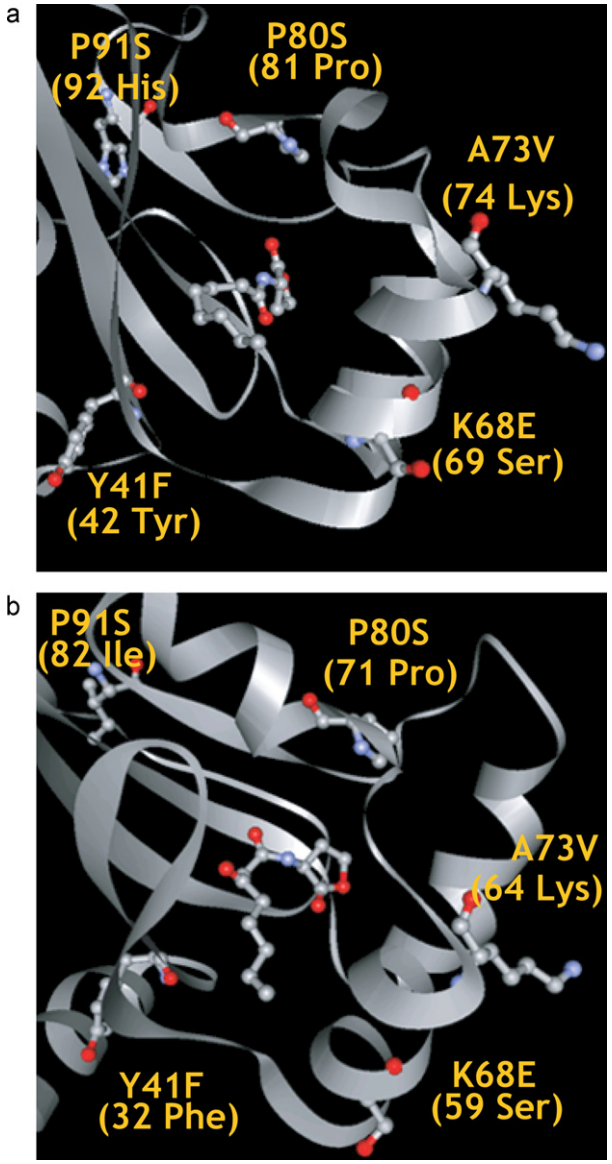

Fig. 5. The identification of mutated LuxR amino acids in three dimensional spaces. The mutated amino acids in LuxR are superimposed over the known structures of the LuxR homologs, the SdiA protein in *Escherichia coli* (a) and the TraR protein in *Agrobacterium tumefaciens* (b). The chemical structures of the amino acids of SdiA or TraR corresponding to the mutated residues of LuxR are shown relative to those of the appropriate cofactors in the center of image.

play a crucial role in cofactor binding and, by extrapolation, in LuxR activation. The estimated distances of the other mutated amino acids to the bound cofactor were around 10 Å, suggesting that these residues may interact with the bound cofactor *via* a water molecule. Therefore, our comparison suggests that all of the mutated amino acids in LuxR may interact with the cofactor.

Interestingly, the activities of the P80S, P91S, and A73 V mutants decreased in the presence of $3OC_6HSL$ while the activities of the K68E and Y41F increased (Fig. 3c). For example, the activity of A73V in the presence of the cofactor decreases by about 4-fold. As shown in Figs. 4 and 5, the sequence and structure alignments revealed that the Ala73 resides within the cofactor-binding domain, and thereby the amino acid Ala73 is important for cofactor binding. In Fig. 3b, the replacement of Ala to Val enhanced LuxR activity up to 17 folds even in the absence of the $3OC_6HSL$. This suggests that the Ala73 plays an important role in cofactor binding and the replacement of Ala with Val induces structural change of LuxR protein similar to that bound with $3OC_6HSL$. Furthermore, the activity of A73V mutant decreased in the presence of $3OC_6HSL$, and which implies that the incorporation of $3OC_6HSL$ into the cofactor-binding site changes again the LuxR structure far from the activated structure. This activity decrease likely results from structural distortions caused by $3OC_6HSL$ binding, providing further evidence that the identified mutations directly affect the structure of the cofactor-binding region.

In our synthetic system, though carrier phage plasmid harboring *luxR* target gene is preferentially mutated than *E. coli* genome by introducing the mutant error-prone polymerase, other functional nucleotide sequences in the carrier phage, M13 replication origin, zeocin resistance gene, and pUC replication origin, could be also mutated by the epPolA. Since mutations introduced in sequences other than that of the *luxR* gene would disrupt the selection process in our system, we sequenced the full plasmids of the randomly isolated carrier phages obtained in each generation, to see if other mutations were present. We found mutations only in transcriptional terminators, but those did not affect the secondary structures of the transcriptional terminators enough to disrupt their termination function when predicted using *mfold* (Zuker, 2003). Consequently, no significant mutated nucleotidewas found outside the *luxR* gene sequence. This is reasonable because carrier phages with a mutant zeocin resistance gene would be eliminated during the infection process which includes zeocin treatment. Carrier phages with a mutant pUC replication origin would replicate fewer carrier plasmids inside a host cell, leading to decreased LuxR production and lower resistance to AZT (Vieira and Messing, 1982; Yanisch-Perron et al., 1985). Carrier phages with a mutant M13 replication origin would fail to be replicated and encapsulated. Thus, mutations in any of these functional sequences would be eliminated during our evolution process.

4. Conclusion

We herein describe a synthetic system of mutually cooperating host and virus, and its application to *in vivo* targeted gene evolution inspired by the co-evolution through inter-species cooperation in nature. To systematically analyze our system we developed a mathematical model. Using the model we determined the selective AZT concentration to be supplied in medium for effective selection, and the results were confirmed again experimentally. Our synthetic system successfully evolved the target gene, *luxR*, to increase its activity with a small number of *in vivo* processes of selection and mutagenesis and allowed us to identify evolved mutants through the evaluation of only a minimal number of isolated virus individuals over a small number of generations.

This novel synthetic system offers an *in vivo* targeted evolution strategy capable of facilitating evolution through artificially created inter-species cooperation. As the integration of an *in vivo* selection and mutagenesis processes simplifies the processes of targeted evolution to virus cultivation and harvest, this new synthetic evolution system should have practical applications in high-throughput and automated targeted evolution of proteins. In addition, the system offers an opportunity to understand and study the cooperation-driven evolution of living organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.03.004.

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