Replacement of All Arginine Residues with Canavanine in MazF-bs mRNA Interferase Changes Its Specificity*

Yojiro Ishida, Jung-Ho Park1, Lili Mao, Yoshihiro Yamaguchi, and Masayori Inouye2
From the Department of Biochemistry, Robert Wood Johnson Medical School and Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey 08854

Background: Canavanine (Can) is a highly toxic arginine (Arg) analogue found in some plant seeds.

Results: Replacement of all Arg residues with Can in MazF-bs(can), an mRNA interferase, resulted in a higher RNA cleavage specificity.

Conclusion: Enzymatic function of a protein can be modulated by Arg-to-Can replacement.

Significance: For the first time, a new functional protein was created by complete Arg-to-Can replacement.

Replacement of a specific amino acid residue in a protein with nonnatural analogues is highly challenging because of their cellular toxicity. We demonstrate for the first time the replacement of all arginine (Arg) residues in a protein with canavanine (Can), a toxic Arg analogue. All Arg residues in the 5-base specific (UACAU) mRNA interferase from Bacillus subtilis (MazF-bs(arg)) were replaced with Can by using the single-protein production system in Escherichia coli. The resulting MazF-bs(can) gained a 6-base recognition sequence, UACAUA, for RNA cleavage instead of the 5-base sequence, UACAU, for MazF-bs(arg). Mass spectrometry analysis confirmed that all Arg residues were replaced with Can. The present system offers a novel approach to create new functional proteins by replacing a specific amino acid in a protein with its analogues.

There are a large number of nonnatural amino acid analogues (1). It is quite intriguing to replace all residues of a specific amino acid in a protein with its analogues, because it may create novel functional proteins with altered structures. However, this is highly challenging because most amino acid analogues are highly toxic to the cells. To circumvent this problem, chemically modified aminoacylated tRNAs have been used in a cell-free system (2, 3). Alternatively, an orthogonal aminoacyl tRNA synthetase/tRNA pair from other species was incorporated into bacteria (4, 5) or eukaryotes (6). One such highly toxic analogue is L-canavanine (Can), a Arg analogue (Fig. 1, A and B) that is found as an insecticide in certain leguminous plants such as jack bean (7). Its incorporation into cellular proteins leads to the production of functionally aberrant proteins, leading to failure of various cellular functions, causing eventual cell death (7). In a previous attempt, 18 of 200 Arg residues in vitellogenin, an egg yolk protein, in locusts were replaced with Can, resulting in the production of an aberrantly structured protein (7), whereas one-third of 3 Arg residues in diptericin A, an antibacterial protein from the fly, Phormia terranovae, was replaced with Can, resulting in loss of the antibacterial activity (8). In another attempt, 21% of Arg residues in the lysisom enzyme molecule from the tobacco hornworm, Manduca sexta, were replaced with Can, resulting in loss of 49.5% of the catalytic activity (9). None of these attempts, however, could achieve the complete replacement of all Arg residues in a protein with Can.

RNA-mediated mRNA interference with the use of antisense RNA, miRNA, and siRNA has been well documented, including their important roles in gene regulation from bacteria to human cells (10, 11) and a possible use for the treatment of human diseases (12). More recently, it has been shown that mRNA interference is also mediated by proteins using sequence-specific endoribonucleases, called mRNA interferases (13). The first such enzyme reported was MazF-ec from Escherichia coli consisting of 111 residues, which cleaves RNA specifically at ACA sequences (14). The x-ray structure of its complex with the cognate antitoxin, MazE, has been determined, consisting of one MazE dimer with two MazF dimers (15). Since then, a number of MazF homologues have been discovered from bacteria and archaea (16) (Fig. 1C). Most recently, a 7-base specific MazF homologue from a superhalophilic archaeon from a hypersaline pool on the Sinai Peninsula (MazF-hw) was found to cleave RNA at UUAUCA, which can be used for regulating specific gene expression in E. coli (17). In the present paper, we attempted to replace all 7 Arg residues in MazF-bs, a 5-base specific RNA interferase from Bacillus subtilis (18) with Can using the Single Protein Production (SPP) system (19, 20).

EXPERIMENTAL PROCEDURES

Strain Construction—E. coli BL21(DE3) (ΔargHΔtrpCΔhisB) was constructed from E. coli BL21(DE3) (ΔtrpCΔhisB) (19) by P1 transduction using the ΔargH strain from the Keio collection (21).

Plasmid Construction—The gene for MazF-bs with a C-terminal His tag (Fig. 2A) was synthesized (Genescript). The gene was designed for the optimal codon usage in E. coli and to have no ACA sequences. The gene was cloned into pColdIII (SP-4) (19, 20).
The BL21(DE3) (ΔargHΔtrpCΔhisB) cells were transformed with pColdII-MazF-bs together with pACYC-mazF(ΔH) and grown in a 1-liter culture of M9-glucose medium in the presence of Arg (20 μg/ml), His (20 μg/ml), and Trp (20 μg/ml) at 37 °C. When the A600 value reached 0.5, the culture was chilled in an ice-water bath for 5 min and incubated at 15 °C for 1 h to acclimate the cells to cold shock conditions. Cells were harvested and washed twice with M9 medium. The cells were resuspended in 50 ml of M9-glucose medium containing Arg (20 μg/ml) and Trp (20 μg/ml) but without His. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM) was added to induce the only expression of MazF(ΔH) followed by an additional 2-h incubation at 15 °C. Cells were harvested and washed twice with M9 medium. The cells were resuspended in 50 ml of M9-glucose medium containing His (20 μg/ml), and Trp (20 μg/ml), Can (100 μg/ml; Sigma) and IPTG (0.5 mM) to incorporate Can into MazF-bs. The cell culture was incubated at 15 °C for additional 24 h to induce MazF-bs(can) (Fig. 2B). Cells were collected by centrifugation and subjected to SDS-PAGE followed by Coomassie Blue staining. MazF-bs(arg) and MazF-bs(can) were purified from BL21(DE3) (ΔargHΔtrpCΔhisB) cells carrying pColdII-MazF-bs with use of nickel-nitrilotriacetic acid resin (Qiagen) following the manufacturer’s protocol. The MazF-bs(can) and MazF-bs(arg) were further purified by ion-exchange chromatography using DEAE-Sepharose (GE Healthcare).

Circular Dichroism (CD) Analysis—CD analysis was carried out using an Aviv model 62DS spectropolarimeter (Aviv Associates, Inc., Lakewood, NJ). Spectra were recorded in 2.0-nm steps between 260 and 200 nm at 4 °C with an integration time of 4 s at each wavelength, and the base line was corrected against buffer alone. Protein melting was examined at 208 nm with increasing temperature, from 0 to 90 °C, in 0.3 °C steps. Protein solutions were equilibrated at each temperature point for 1.5 min, and the temperature was increased with an average rate of 0.1 °C/min. The path length of the cell used was 0.1 cm, and all measurements were carried out in 10 mM Tris-HCl (pH 7.8).

Cleavage of MS2 Phage RNA by MazF-bs(can)—MS2 phage RNA (70 nM; Roche Applied Science) was incubated with 0.5...
Replacing All Arg in a Protein with Canavanine

A DNA sequence of the mazF-bs gene. The gene is designed to be ACA-less and codon-optimized for E. coli. The amino acid sequence of MazF-bs is shown under the DNA sequence. B, dual inducible SPP system. The BL21(DE3) (ΔargHΔtrpCΔhisB) cells were transformed with pColdIII_mazF-bs together with pACYCmazF(ΔH) and grown in a 1-l culture of M9-glucose medium in the presence of Arg (20 μg/ml), His (20 μg/ml), and Trp (20 μg/ml) at 37 °C. When the A600 value reached 0.5, the culture was chilled in an ice-water bath for 5 min and incubated at 15 °C for 1 h to acclimate the cells to cold shock conditions. Cells were harvested and washed twice with M9 medium. The cells were resuspended in 50 ml of M9-glucose medium containing Arg (20 μg/ml) and Trp (20 μg/ml) at 37 °C for 10 min. After denaturation in urea, the products were analyzed by electrophoresis on a 1.2% agarose gel.

**Primer Extension Analysis**—Primer extension analysis of the cleavage sites was carried out as described previously (22). Briefly, 0.7 μM MS2 phage RNA was incubated with 0.5 μM MazF-bs(arg) or MazF-bs(can) in the presence of CspA protein, an RNA chaperone (20 μM) at 37 °C for 10 min in a reaction mixture (10 μl) in 10 mM Tris-HCl (pH 7.8), containing 0.2 μl of the Protector RNase inhibitor (Roche Applied Science). Primer extension was carried out at 47 °C for 1 h. The reactions were stopped by 2× stop solution (90% formamide, 50 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF). The samples were incubated at 90 °C for 5 min prior to electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

**Cleavage of Synthetic RNA by MazF-bs(arg) and MazF-bs(can)**—Four 13-base ribonucleotides (CUCXUACAUAUCA) were synthesized, where the 4th base (X) was A, U, G, or C. Three additional 13-base RNA ribonucleotides (CUCUA-CAYUCAAUAUCA) were synthesized, where the Y position was replaced with U, G, or C. These ribonucleotides were used as substrates. The labeled substrates (0.2 μM) with [γ-32P]ATP (New England Biolabs) were incubated with 1 nM MazF-bs(arg) or 5 nM MazF-bs(can) in a reaction mixture (10 μl) in 10 mM Tris-HCl (pH 7.8) containing 0.2 μl of the Protector RNase inhibitor. The reactions were stopped by the use of 2× stop solution. To analyze the cleavage of the synthetic RNAs, the products were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea with a molecular mass ladder.

**Kinetics Analysis**—A 13-base ribonucleotide (CUCUA-CAYUAAUAUCA) was used as a substrate. The substrate in various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μM) was incubated with 1 nM MazF-bs(arg) or 5 nM MazF-bs(can) in a reaction mixture (10 μl) in 10 mM Tris-HCl (pH 7.8) containing 0.2 μl of the Protector RNase inhibitor. The reaction with MazF-bs(arg) was incubated for 5, 10, 15, and 20 min. The reaction with MazF-bs(can) was incubated for 30, 60, 90, and 120 min. The reaction was stopped by the use of 2× stop solution, and the sample mixtures were incubated at 90 °C for 5 min prior to electrophoresis on a 20% polyacrylamide gel containing 8 M urea with a molecular mass ladder.
Complete Replacement of Arg in a Protein with Canavanine

RESULTS

Production of MazF-bs(can) by the SPP System—To replace all 7 Arg residues in MazF-bs with Can, we applied the SPP system (19, 20) with use of an Arg auxotroph. In the SPP system, E. coli cells are converted into a bioreactor producing only a stable protein. Note that the pI value of MazF-bs changed from 5.68 to 5.47 as a result of the Arg-to-Can replacement.

To examine whether these ribonucleotides are able to inhibit the cleavage of the substrate. The concentration of the substrate analogues was fixed at 1.0 and 4.0 μM. The reaction was stopped with the use of 2× stop solution, and the reaction mixtures were incubated at 90 °C for 5 min prior to electrophoresis on a 20% polyacrylamide gel containing 8 M urea. The cleavage products were analyzed by ImageJ.

Complete Replacement of Arg Residues in MazF-bs with Can—After a 24-h incubation using the SPP system in the presence of Can, a new band was induced at 14 kDa, and purified protein is shown in Fig. 3A. This protein termed (MazF-bs(can)) was subsequently purified by nickel-nitriolactric acid affinity chromatography and DEAE ion-exchange column chromatography (Fig. 3A). If all 7 Arg residues were replaced with Can, the molecular mass of MazF-bs(can) should be larger by 13.8 Da (1.97 Da × 7) than that of MazF-bs(arg). The mass spectrometry analysis revealed that MazF-bs(can) was larger by 13.4 Da than MazF-bs(arg) (Fig. 3B), indicating that 97% of Arg residues were replaced with Can or that in four of five MazF-bs molecules the complete replacement was achieved. In the remaining one molecule, all but 1 Arg residue of 7 were replaced with Can.

Structural Analysis of MazF-bs(can) by Circular Dichroism (CD) Spectroscopy—The secondary structures of purified MazF-bs(arg) and MazF-bs(can) were analyzed by CD spectroscopy. MazF-bs(arg) showed minimum peaks around at 208 and 222 nm, which are characteristic for α-helical structures (23). MazF-bs(can) also showed a minimum peak at 208 nm which is higher than that for MazF-bs(arg), whereas the signal at 222 nm for MazF-bs(can) was lower than that for MazF-bs(arg) (Fig. 3C), indicating that α-helix contents of MazF-bs(can) slightly increased from 27.5 to 29.7%, whereas its β-sheet content decreased from 39.5 to 37.2%. Next, the thermal stability was examined for both proteins between 4 and 90 °C by measuring the change in ellipticity at 222 nm in the CD spectra. Notably, the Tm for MazF-bs(can) was lower by ~4 °C than that for MazF-bs(arg) (Fig. 3D). MazF-bs(can) is likely folded in a manner very similar to MazF-bs(arg); however, the substitution of Arg with Can appears to affect the α-helical structures. The hydrogen bonds between Arg-5 and Ala-112 and a salt bridge between Arg-87 and Glu-20 have been shown to stabilize the dimer formation (24). Although both Arg and Can contain a guanidine group, the replacement of the methyl group with ethyl group in Arg with oxygen in Can results in the reduction of the pKa value from 12.48 to 7.01 (7). Therefore, the salt bridge in MazF-bs(arg) is likely to weaken substantially when all of the Arg residues are replaced with Can, resulting in a less thermostable protein. Note that the pl value of MazF-bs changed from 6.34 to 5.86 as a result of the Arg-to-Can replacement.

Specificity Alteration of a 5′-Base to a 6′-Base Recognition for RNA Cleavage—Next, we analyzed the endoribonucleolytic activity of MazF-bs(can) using 3.5-kb MS2 phage RNA as a substrate (17). Because the RNA cleavage patterns were found to be quite different between the two enzymes (Fig. 4A), in vitro primer extension experiments were carried out to determine the exact cleavage site sequences. As shown in Fig. 4A, after incubation of the RNA with MazF-bs(can) and MazF-bs(arg) at 37 °C for 10 min, MazF-bs(arg) cleaved MS2 phage RNA at all U → ACAU sites as expected († indicates the cleavage site) (18), whereas MazF-bs(can) appears to cleave the MS2 RNA at U → ACAU sites, only when these sites contain one extra A residue at the 3′ end (Fig. 4, B–F), indicating that MazF-bs(can) acquired a higher RNA cleavage specificity from a 5′-base to a 6′-base cutter. To further confirm this notion, we synthesized 13-base RNA substrates covering all of the possible 7-base sequences having an extra base at both sides of UACAU and confirmed
that MazF-bs(can) is specific for U↓ACAUA (Fig. 5). Lanes 2 (MazF-bs(can)) and 7 (MazF-bs(arg)) in Fig. 5A show an extra band corresponding to the product cleaved after the 1st C residue in addition to the cleavage product after the 5th U residue

\[ C_1UCUUAUAUCA \] (1 indicates the cleavage sites), whereas no cleavage products are observed with three other ribonucleotides (CUC\_\_AUAUAUAUCA, CUC\_\_GUAUAUAUCA, and CUC\_\_CUAUAUAUCA) for both MazF-bs(can) and MazF-bs(arg) (bases which are replaced are shown in bold).

Furthermore, lane 2 in Fig. 5B using MazF-bs(can) with CUC-

\[ C_1UUACUAUAUCA \] shows an extra cleavage product (cleaved after the 1st C residue) in addition to the product after the 5th U residue. Lanes 7, 8, and 10 in Fig. 5B using MazF-bs(arg) also show an extra cleavage product corresponding to C↓UCUUACUAUAUCA, C↓UCUUACUAUAUCA, and C↓UCUUACUAUAUCA. These cleavages were not observed with MazF-bs(can).

It is unknown at present why these substrates were cleaved by MazF-bs(arg). It is unknown at present why these substrates were cleaved by MazF-bs(arg) after the 1st C residue.

Kinetic Study—Using UACAUA as a substrate, the \( K_m \) value and the \( K_{cat}/K_m \) value of MazF-bs(arg) were determined to be
Although the $K_{cat}/K_m$ value of MazF-bs(can) is 5% of that of MazF-bs(arg), the $K_m$ value for MazF-bs(can) was almost identical to that of MazF-bs(arg) (see Table 1). The difference in the $K_{cat}/K_m$ values is likely due to the charging status of the guanidino groups of Can residues in MazF-bs(can). Because MazF-bs(can) became 6-base-specific, cleaving at $U\uparrow$ACAUA, but not UACAU, AUAUC, and UACAUU (Fig. 5), we next examined whether the cleavage of $U\uparrow$ACAUA is inhibited by substrate analogues having different bases at the 6th position (UACAU, UACAUC, and UACAUU), which are not cleavable by MazF-bs(can), and we found that there was no inhibition of the cleavage reaction by UACAU, UACAUC, and UACAUU, indicating that the A residue at the 6th position plays a critical role for the substrate binding to the enzyme (Table 2). We found that there was no inhibition of the cleavage reaction by UACAU, UACAUC, and UACAUU, even if the inhibitor-to-substrate ratio increased (Table 2).
To achieve the replacement of all 7 Arg residues in MazF-bs with Can, we used the SPP system for the first requirement so that Can incorporation into cellular proteins but MazF-bs was prevented while maintaining the biosynthetic function of the cells. The second requirement was achieved by using an Arg auxotroph. The use of SPP system for the replacement of all Arg residues in a protein with Can seems to be crucial because Can incorporation into other cellular proteins likely affects their

FIGURE 5. Identification of a change of RNA cleavage specificity in MazF-bs(can). A, 13-base ribonucleotides (CUCUACAUAUCA) synthesized, where the 4th base (X) was U, A, G, or C, were incubated with MazF-bs(can) or MazF-bs(arg) (lanes 2–5 and 7–10, respectively). Lanes 1 and 6 represent control reactions in which no protein was added. Lanes 2 and 7 show an extra band corresponding to the product cleaved after the first C residue in addition to the cleavage product after the 5th U residue C1UC1U1ACAUAUCA (C indicates the cleavage sites), whereas no cleavage products were observed with three other ribonucleotides (CUCUACAUAUCA, CUCUACAUAUCA, and CUCUACAUAUCA) for both MazF-bs(can) and MazF-bs(arg) (lanes 2–5, and 7–10, respectively). Lanes 1 and 6 represent control reactions in which no protein was added. Lane 2 shows an extra cleavage product (cleaved after the 1st C residue) in addition to the product cleaved after the 5th U residue. Lanes 7, 8, and 10 also show an extra cleavage product corresponding to C1UCUUACAU1A1UCA, C1UCUUACAU1G1UCA, and C1UCUUACAU1C1UCA. These cleavages were not observed with MazF-bs(can).

TABLE 1
Kinetic constants for MazF-bs(arg) and MazF-bs(can)

| MazF   | Vmax (μM/min) | K_m (μM) | K_cat (min⁻¹) | K_cat/K_m (μM⁻¹ min⁻¹) |
|--------|--------------|----------|---------------|-------------------------|
| MaxF-bs(arg) | 4.2 ± 1.1 x 10⁻² | 2.0 ± 0.2 | 2.2 ± 0.4 x 10⁻² | 1.0 ± 0.2 x 10⁻² |
| MazF-bs(can) | 8.4 ± 1.2 x 10⁻² | 1.8 ± 0.3 | 8.4 ± 1.2 x 10⁻⁴ | 5.0 ± 1.0 x 10⁻⁴ |

TABLE 2
Relative cleavage activity of MazF-bs(can) using CUCUACAUAUCA as a substrate in the presence and the absence of substrate analogues having different bases at the 6th position (CUCUACAUAUCA, CUCUACAUAUCA, and CUCUACAUAUCA)

| Substrate | CUCUACAUAUCA only | +CUCUACAUAUCA | +CUCUACAUAUCA | +CUCUACAUAUCA |
|-----------|-------------------|---------------|---------------|---------------|
| CUCUACAUAUCA:substrate analogue = 1:1 | 1.0 | 1.1 | 1.0 | 1.1 |
| CUCUACAUAUCA:substrate analogue = 4:1 | 1.0 | 1.1 | 1.1 | 1.2 |
functions, leading to severe inhibitory effects on various bio-
synthetic reactions including protein synthesis.

The present system, therefore, can be used for other toxic
amino acid analogues as far as they can be recognized by E. coli
aminoacyl-tRNA synthetases. The second requirement for the
present system is the use of an amino acid auxotroph to avoid
the incorporation of a natural amino acid into a target protein.
The use of the SPP system in combination with amino acid
auxotroph strains thus opens a new avenue to create proteins of
unprecedented novel structures and functions without genetic
manipulation of tRNAs and aminoacyl tRNA synthetases.

The most intriguing outcome from the present experiments is
that MazF-bs(can) was no more able to cleave RNA at the
original MazF-bs(arg) 5-base sequence, UACAU, requiring one
extra A residue at the 3’ end. The K_m value of MazF-bs(can)
using UACAUA as a substrate is almost identical to that of
MazF-bs(arg) using UACAU as a substrate, suggesting that the
substrate binding affinity in MazF-bs(can) was compensated by
an extra A residue at the 3’ end of the substrate. Notably, how-
ever, the cleavage activity of MazF-bs(can) was reduced to ~5%
of MazF-bs(arg) (Table 1). The MazF-bs functions as a dimer,
and its interphase is predicted to be involved with RNA binding
and catalysis (24). Because of a total of 7 Arg residues in MazF-
bs, Arg-25, Arg-81, and Arg-87 are located in the interphase
between the two monomers in a dimer (Fig. 1D), some or all
these 3 residues may have critical roles in the specific RNA
sequence recognition and the enzymatic activity. At present it is
not known whether the other 4 Arg residues also play roles in
MazF-bs function. It remains to be determined whether some of
these Arg residues can be replaced with Lys without losing
MazF-bs function.

Acknowledgments—We thank Drs. Sangita Phadtare and Vikas
Nanda for critical reading of the manuscript and Drs. Zhuoxin Yu
and Sung-gun Kim for the CD data analysis.

REFERENCES
1. Hendrickson, T. L., de Crécy-Lagard, V., and Schimmel, P. (2004) Incor-
poration of nonnatural amino acids into proteins. *Annu. Rev. Biochem.*
73, 147–176
2. Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., and Schultz, P. G. (1989)
A general method for site-specific incorporation of unnatural amino acids
into proteins. *Science* **244**, 182–188
3. Stigers, D. J., Watts, Z. I., Henriessy, J. E., Kim, H. K., Martini, R., Taylor,
M. C., Ozawa, K., Keilior, J. W., Dixon, N. E., and Easton, C. J. (2011) Incor-
poration of chlorinated analogues of aliphatic amino acids during
cell-free protein synthesis. *Chem. Commun.* **47**, 1839–1841
4. Bae, J. H., Rubini, M., Jung, G., Wiegand, G., Seifert, M. H., Azim, M. K.,
Kim, J. S., Zumbusch, A., Holak, T. A., Moroder, L., Huber, R., and Budisa,
N. (2003) Expansion of the genetic code enables design of a novel "gold"
class of green fluorescent proteins. *J. Mol. Biol.* **328**, 1071–1081
5. Hancock, S. M., Upreti, R., Deiters, A., and Chin, J. W. (2010) Expanding
the genetic code of yeast for incorporation of diverse unnatural amino
acids via a pyrrolysyl-tRNA synthetase/tRNA pair. *J. Am. Chem. Soc.* **132**, 14819–14824
6. Gautier, A., Nguyen, D. P., Rusic, H., An, W., Deiters, A., and Chin, J. W.
(2010) Genetically encoded photocontrol of protein localization in mam-
malian cells. *J. Am. Chem. Soc.* **132**, 4086–4088
7. Rosenthal, G. A., Reichhart, J. M., and Hoffmann, J. A. (1989) i-Canava-
nine incorporation into vitellogenin and macromolecular conformation.
*J. Biol. Chem.* **264**, 13693–13696
8. Rosenthal, G. A., Lambert, J., and Hoffmann, D. (1989) Canavanine incor-
poration into the antibacterial proteins of the fly, *Phormia terranovae*
(Diptera), and its effect on biological activity. *J. Biol. Chem.* **264**, 9768–9771
9. Rosenthal, G. A., and Dahlman, D. L. (1991) Studies of i-canavanine incor-
poration into insectan lysosome. *J. Biol. Chem.* **266**, 15684–15687
10. Davis, B. M., and Waldor, M. K. (2007) RNase E-dependent processing
stabilizes MicX, a *Vibrio cholerae* sRNA. *Mol. Microbiol.* **65**, 373–385
11. Hu, H. Y., Guo, X., Xi, J., Yan, Z., Fu, N., Zhang, X., Menzel, C., Liang, H.,
Yang, H., Zhao, M., Zeng, R., Chen, W., Piabò, S., and Khaitovich, P.
(2011) MicroRNA expression and regulation in human, chimpanzee,
and macaque brains. *PLoS Genet.* **7**, e1002327
12. Angaji, S. A., Hedayati, S. S., Poor, R. H., Madani, S., Poor, S. S., and Panahi,
S. (2010) Application of RNA interference in treating human diseases.
*J. Genet.* **89**, 527–537
13. Yamaguchi, Y., and Inouye, M. (2011) Regulation of growth and death in
*Escherichia coli* by toxin-antitoxin systems. *Nat. Rev. Microbiol.* **9**, 779–790
14. Zhang, J., Zhang, Y., and Inouye, M. (2003) Characterization of the inter-
actions within the mazEF addiction module of *Escherichia coli*. *J. Biol.
Chem.* **278**, 32300–32306
15. Kamada, K., Hanaoka, F., and Burley, S. K. (2003) Crystal structure of the
MazE/MazF complex: molecular bases of antidote-toxin recognition. *Mol.
Cell* **11**, 875–884
16. Yamaguchi, Y., Park, J. H., and Inouye, M. (2011) Toxic-antitoxin systems
in bacteria and archaea. *Annu. Rev. Genet.* **45**, 61–79
17. Yamaguchi, Y., Nariya, H., Park, J. H., and Inouye, M. (2012) Inhibition of
specific gene expressions by protein-mediated mRNA interference. *Nat.
Syst. Biol.* **3**, 687
18. Park, J. H., Yamaguchi, Y., and Inouye, M. (2011) *Bacillus subtilis* MazF-bs
(EndoA) is a UACAU-specific mRNA interferase. *FEBS Lett.* **585**, 2526–2532
19. Vaiphei, S. T., Mao, L., Shimazu, T., Park, J. H., and Inouye, M. (2010) Use
of amino acids as inducers for high-level protein expression in the single-
protein production system. *Appl. Environ. Microbiol.* **76**, 6063–6068
20. Suzuki, M., Roy, R., Zheng, H., Woychik, N., and Inouye, M. (2006) Bacteri-
al bioreactors for high yield production of recombinant protein. *J. Biol.
Chem.* **281**, 37559–37565
21. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M.,
Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construc-
tion of *Escherichia coli* K-12, in-frame single-gene knockout mutants: the
Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008
22. Zhang, Y., Zhang, J., Hara, H., Kato, I., and Inouye, M. (2005) Insights into
the mRNA cleavage mechanism by MazF, an mRNA interferase. *J. Biol.
Chem.* **280**, 3143–3150
23. Greenfield, N. J. (2006) Determination of the folding of proteins as a func-
tion of denaturants, osmolytes or ligands using circular dichroism. *Nat.
Protoc.* **1**, 2733–2741
24. Gogos, A., Mu, H., Bahn, F., Gomez, C. A., and Shapiro, L. (2003) Crystal
structure of YdcE protein from *Bacillus subtilis*. *Proteins* **53**, 320–322