Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins provide microbial adaptive immunity against bacteriophages. In type I-F CRISPR-Cas systems, multiple Cas proteins (Cas1–4) compose a surveillance complex (Csy complex) with CRISPR RNA (crRNA) for target recognition. Here, we report the biochemical characterization of the Csy1-Csy2 subcomplex from *Xanthomonas albilineans*, including the analysis of its interaction with crRNA and AcrF2, an anti-CRISPR (Acr) protein from a phage that infects *Pseudomonas aeruginosa*. The *X. albilineans* Csy1 and Csy2 proteins (XaCas1 and XaCas2, respectively) formed a stable heterodimeric complex that specifically bound the 8-nucleotide (nt) 5′-handle of the crRNA. In contrast, the XaCas1-XaCas2 heterodimer exhibited reduced affinity for the 28-nt *X. albilineans* CRISPR repeat RNA containing the 5′-handle sequence. Chromatographic and calorimetric analyses revealed tight binding between the Acr protein from the *P. aeruginosa* phage and the heterodimeric subunit of the *X. albilineans* Csy complex, suggesting that AcrF2 recognizes conserved features of Csy1-Csy2 heterodimers. We found that neither XaCas1 nor XaCas2 alone forms a stable complex with AcrF2 and the 5′-handle RNA, indicating that XaCas1-XaCas2 heterodimerization is required for binding them. We also solved the crystal structure of AcrF2 to a resolution of 1.34 Å, enabling a more detailed structural analysis of the residues involved in the interactions with the Csy1-Csy2 heterodimer. Our results provide information about the order of events during the formation of the multisubunit crRNA-guided surveillance complex and suggest that the Acr protein inactivating type I-F CRISPR-Cas systems has broad specificity.

Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins provide adaptive immunity in bacteria and archaea for defense against invading foreign nucleic acids (1–4). CRISPRs consist of short invariable “repeat” sequences interspaced with similarly sized variable “spacer” sequences derived from phages and plasmids (5–7), and genes encoding Cas proteins lie adjacent to the CRISPR arrays in microbial genomes (8–10). Cas proteins mediate the CRISPR adaptation by integrating short fragments of the foreign genetic material into the host CRISPR locus as new spacers (11–13). The CRISPR array is transcribed as a long precursor CRISPR RNA (pre-crRNA) that is processed in the repeat regions into short CRISPR RNAs (crRNAs) containing single spacers (14, 15). The crRNAs combine with single or multiple Cas proteins to form crRNA-guided surveillance complexes that recognize DNA sequences complementary to the crRNA and direct the degradation of the foreign nucleic acids (14, 16).

The CRISPR-Cas systems are currently classified into two classes and six types with further subdivision into more than 20 subtypes (17–20). Class I CRISPR-Cas systems, including types I, III, and IV, rely on multiprotein surveillance complexes for their crRNA binding and target recognition, and the most common type I systems contain Cas3 proteins as trans-acting nucleases for target cleavage (14, 15, 21). In subtype I-F CRISPR-Cas systems, four different Cas proteins (Cas1–4; also known as Cas8f, Cas5f, Cas7f, and Cas6f, respectively) participate in the crRNA-guided surveillance complex (Csy complex) with a subunit stoichiometry of Csy1:Csy2:Csy3:Csy4 (22–24). Previous studies of the type I-F CRISPR-Cas system from *Pseudomonas aeruginosa* revealed the molecular organization of the Csy complex using electron microscopy (EM) and mass spectrometry (22, 24, 25). *P. aerugi-
Cfsy1 and Cfsy2 proteins, AcrF1 and AcrF2. The overall morphology of the Cfsy1-Cfsy2 subunit (25). Peng et al. (Cfsy3), and tail (Cfsy1 and Cfsy2) (25). The authors suggested that the Cas subunits represent the head (Cfsy4), backbone (Cfsy3), and tail (Cfsy1 and Cfsy2) (25, 26). For target degradation, the Cfsy complex recruits the Cas3 nuclease, which cleaves the 5′-stem of the crRNA (25, 26). Six Cfsy3 proteins are assembled along the Cfsy complex (36, 39). Other Acr proteins inactivating type I-E or I-F CRISPR-Cas systems have also been discovered and characterized (40–46).

To counteract the CRISPR-mediated immunity of bacteria, phages have evolved anti-CRISPR (Acr) proteins that inhibit the host CRISPR-Cas systems (28–32). Several Acr proteins have been identified in phages infecting P. aeruginosa (33–35). Some of them inactivate the immune function of the type I-F CRISPR-Cas system of P. aeruginosa by utilizing distinct mechanisms (36). AcrF1 binds to the Cfsy3 backbone of the P. aeruginosa Cfsy complex and blocks crRNA hybridization to a complementary DNA target (36, 37), whereas AcrF2 interacts with the P. aeruginosa Cfsy1-Cfsy2 (PaCfsy1-Cfsy2) subunit to prevent interactions with target DNA duplex (25, 36, 38). AcrF3 binds to P. aeruginosa Cfsy3 and prevents its recruitment by the Cfsy complex (36, 39). Other Acr proteins inactivating type I-E or I-F CRISPR-Cas systems have also been found, some of which inhibited multiple systems, suggesting their broad specificity (34, 35). Acr proteins inhibiting Cas9 of class 2 CRISPR-Cas systems have also been discovered and characterized (40–46).

Recently, cryo-EM structures of the P. aeruginosa Cfsy complexes have been reported with and without bound Acr inhibitors (25, 35, 37). Chowdhury et al. (25) determined the cryo-EM structure of the Cfsy complex bound simultaneously to two Acr proteins, AcrF1 and AcrF2. The overall morphology of the P. aeruginosa Cfsy complex was consistent with a seahorse shape in which Cas subunits represent the head (Cfsy4), backbone (Cfsy3), and tail (Cfsy1 and Cfsy2) (25). The authors suggested that AcrF1 prevents target DNA hybridization by interacting with Cfsy3, and AcrF2 competes with DNA for binding to the Cfsy1-Cfsy2 subunit (25). Peng et al. (38) also reported the structure of the AcrF1- and AcrF2-bound P. aeruginosa Cfsy complex in which the tail (Cfsy1 and Cfsy2) of the seahorse-shaped complex and the bound AcrF2 were not modeled. Guo et al. (47) determined several cryo-EM structures for the Cfsy complex in distinct functional and inhibited states, including its target DNA-bound state and AcrF1-, AcrF2-, and AcrF10-bound structures.

To study the role of the Cfsy1-Cfsy2 heterodimer in the Cfsy complex of type I-F CRISPR-Cas systems and to characterize the CRISPR inhibition of AcrF2 at the molecular level, we performed biochemical characterization of the Xanthomonas albilineans Cfsy1-Cfsy2 (XaCfsy1-Cfsy2) heterodimer and analyzed its interaction with crRNA and AcrF2. The X. albilineans Cfsy1 and Cfsy2 (XaCfsy1 and XaCfsy2, respectively) formed a stable heterodimer, which recognized the 5′-handle of the crRNA and bound to AcrF2 with a dissociation constant in the nanomolar range. We demonstrated that the heterodimerization of XaCfsy1 and XaCfsy2 is essential for the interactions because neither XaCfsy1 nor XaCfsy2 alone forms a stable complex with the 5′-handle RNA or AcrF2. We also determined the crystal structure of AcrF2 to a resolution of 1.34 Å, enabling a more detailed structural analysis of the surface residues important for interactions with the Cfsy1-Cfsy2 heterodimer. Together, our data provide biochemical information about the Cfsy1-Cfsy2 heterodimer from a previously uncharacterized bacterial species and suggest the molecular basis of the broad specificity of the AcrF2 protein inactivating type I-F CRISPR-Cas systems.

**Results**

**XaCfsy1 and XaCfsy2 form a stable heterodimeric complex**

It has previously been shown in *P. aeruginosa* that Cfsy1 and Cfsy2 form a heterodimeric subunit, which is localized at the periphery of the Cfsy complex (22, 24, 25, 36, 38, 47). The interaction between Cfsy1 and Cfsy2 was also detected in *Pectobacterium atrosepticum* (23). In the present study, we performed biochemical analyses to characterize the assembly of Cfsy1 and Cfsy2 from the type I-F CRISPR-Cas system of *X. albilineans*. First, we tested copurification of XaCfsy1 and XaCfsy2. When N-terminal His₆-maltose-binding protein (MBP)-tagged XaCfsy1 was expressed together with untagged XaCfsy2 in *Escherichia coli* cells, XaCfsy1 and XaCfsy2 were copurified after the removal of the tag and coeluted in size-exclusion chromatography (SEC; Fig. 2A and Table S1), suggesting the formation of a stable complex between XaCfsy1 and XaCfsy2.

We further examined complex formation with individually purified XaCfsy1 and XaCfsy2 proteins. The purification of XaCfsy1 was successful in the absence of XaCfsy2. However, XaCfsy2 precipitated without XaCfsy1, and an N-terminal His₆-MBP tag was required for solubilizing XaCfsy2 under our experimental conditions, indicating that XaCfsy2 is stabilized by the interaction with XaCfsy1. With the separately purified untagged XaCfsy1 and XaCfsy2, we performed isothermal titration calorimetry (ITC) to analyze the interactions between the two Cas proteins quantitatively (Fig. 2C). XaCfsy1 bound to XaCfsy2 with micromolar affinity (*Kₐ = 0.971 ± 0.109 µM*). The binding stoichiometry (*N*) of XaCfsy2 to XaCfsy1 was calculated using a 1:1 binding model (Fig. 2D).
to be 1.00 ± 0.01, which is consistent with 1:1 binding between XaCsy1 and XaCsy2. These results suggest that XaCsy1 and XaCsy2 interact directly to form a heterodimeric complex without the crRNA or other Cas proteins.

XaCsy1-Csy2 heterodimer recognizes the 5′-handle of crRNA

In the cryo-EM structures of the *P. aeruginosa* Csy complex, the *PaCsy1-Csy2* subunit is associated with the 5′-handle of the crRNA (25, 47). To test whether the *XaCsy1-Csy2* heterodimer can bind to the crRNA on its own, we performed electrophoretic mobility shift assays (EMSAs) using 3′-fluorescein-labeled RNA oligonucleotides. The sequence of the 8-nucleotide (nt) 5′-handle part is identical, with the exception of one position, between the *X. albilineans* and *P. aeruginosa* crRNAs (5′-CUCAGAAA-3′ and 5′-CUAAGAAA-3′, respectively; Fig. 1B). In the assays, the mobility of the 5′-handle RNA in the gel was decreased in the presence of the XaCsy1-Csy2 heterodimer, and the shifted RNA band became intensified as more protein complexes were added (Fig. 3A), indicating that the XaCsy1-Csy2 heterodimer can bind to the 5′-handle of the crRNA on its own. The ITC analysis also revealed tight binding (\(K_d = 21.9 \pm 6.0 \text{ nM}\)) between the protein complex and the 5′-handle RNA (Fig. 3B). The stoichiometry (\(N\)) of the 5′-handle of the crRNA was 0.790 ± 0.003 per XaCsy1-Csy2 heterodimer.

We also demonstrated that the RNA binding of the XaCsy1-Csy2 heterodimer was sequence-specific by using RNA oligonucleotides with different sequences: the reverse complement of the 5′-handle of the *X. albilineans* crRNA (5′-UUUCUGAG-3′) and a poly(U) RNA (U8). In the EMSA experiments, the band shift was not evident for these two 8-nt RNAs in the presence of the XaCsy1-Csy2 heterodimer (Fig. 3C, lanes 4 and 6), indicating that the binding to the 5′-handle RNA is specific. Furthermore, we tested the ability of the XaCsy1-Csy2 complex to bind another part of the crRNA, the 20-nt 3′-stem (Fig. 1B). The retardation of the mobility for the 3′-stem RNA was negligible (Fig. 3D, lane 4), implying that the XaCsy1-Csy2 heterodimer binds to the crRNA specifically at the 8-nt 5′-handle part. These results suggest that the interaction between the *Csy1-Csy2* subunit and the 5′-handle part of crRNA is conserved in various species. Interestingly, the binding of the heterodimer to the 28-nt *X. albilineans* repeat RNA,
including both 3′-stem and 5′-handle, was relatively weak compared with that for the 5′-handle alone as the shifted RNA band was present but faint in the gel (Fig. 3D, lane 6, and Fig. S1). This observation was somewhat surprising because the repeat RNA contains the 5′-handle sequence (Fig. 1B). These results suggest that the flanking sequence(s) of the 5′-handle could influence the binding affinity to the XaCsy1-Csy2 heterodimer, although we cannot rule out the possibility that our experimental conditions were not optimal for the strong interaction between the heterodimer and the repeat RNA.

Finally, we analyzed the binding between the 5′-handle of the crRNA and the individual components of the XaCsy1-Csy2 heterodimer by performing EMSAs with the separately purified XaCsy1 and XaCsy2 proteins. Again, we used the N-terminal His$_6$-MBP–tagged XaCsy2 protein because XaCsy2 was not soluble without the tag under our experimental conditions. The band shift was not detectable at all with either of the two proteins (Fig. 3E, lanes 3 and 4). However, when the two individually purified components were added together, a significant mobility shift was observed (Fig. 3E, lane 5), indicating that neither XaCsy1 nor XaCsy2 alone exhibits the binding affinity for crRNA, and the formation of the heterodimeric complex is essential for crRNA binding. It is likely that the assembly of XaCsy1 and XaCsy2 is required for establishment of the binding interface for the 5′-handle of the crRNA. This interpretation is consistent with the structural data of the P. aeruginosa Csy complex, which shows that the 5′-handle part of the crRNA interacts closely with both Csy1 and Csy2 proteins (25, 47). It is
crRNA and AcrF2 binding to Csy1-Csy2 heterodimer

Figure 4. Binding of AcrF2, an anti-CRISPR protein from a P. aeruginosa phage, to Xanthomonas Csy1-Csy2 heterodimers. A, interaction between AcrF2 and XaCsy1-Csy2 heterodimer in analytical size-exclusion chromatography. Elution fractions were analyzed by SDS-PAGE and visualized by Coomassie staining. B, ITC trace for binding of AcrF2 to the XaCsy1-Csy2 heterodimer. AcrF2 was injected consecutively into the chamber containing XaCsy1-Csy2 heterodimer. C, binding of AcrF2 to Csy1-Csy2 heterodimer from another Xanthomonas bacterium, X. citri. Elution fractions of analytical SEC were analyzed by SDS-PAGE and visualized by Coomassie staining. mAU, milliabsorbance units.

also possible that the interaction between XaCsy1 and XaCsy2 may induce proper folding and/or conformational changes required for the 5′-handle RNA binding. In the cryo-EM structures of the P. aeruginosa Csy complex, Csy1 and Csy2 proteins make extensive contacts with each other to form the heterodimeric arrangement (25, 47).

**AcrF2, an Acr protein from a P. aeruginosa phage, binds to Xanthomonas Csy1-Csy2 heterodimers**

AcrF2 is an Acr protein that inhibits the *P. aeruginosa* type I-F CRISPR-Cas system by interacting with the PaCsy1-Csy2 subunit (36). In the present study, we tested whether AcrF2 found in a *P. aeruginosa* phage binds to the Csy1-Csy2 heterodimer from a different bacterial species, *X. albilineans*. In the analytical SEC, AcrF2 and the XaCsy1-Csy2 heterodimer eluted together with a smaller elution volume than those of either AcrF2 or the heterodimer alone (Fig. 4A), indicating that they directly interact with each other to form a larger protein complex. In the ITC experiment, the stoichiometry (N) of AcrF2 to the XaCsy1-Csy2 heterodimer was calculated to be 0.840 ± 0.001, and the dissociation constant (K_d) was determined to be 7.2 ± 1.9 nM (Fig. 4B). The observed binding stoichiometry was consistent with that of a previous study in which AcrF2 bound to the *P. aeruginosa* Csy complex at a ratio of 0.8 ± 0.1 (36). These results suggest that AcrF2 tightly binds the XaCsy1-Csy2 heterodimer in a manner similar to its binding to the PaCsy1-Csy2 subunit in the *P. aeruginosa* Csy complex, indicating broad Acr-Csy interaction in the type I-F CRISPR-Cas systems. We further confirmed the broad specificity of AcrF2 to the Csy1-Csy2 subunit by demonstrating a direct interaction between AcrF2 and a Csy1-Csy2 heterodimer from another *Xanthomonas* bacterium, *Xanthomonas citri* (XcCsy1-Csy2). In the analytical SEC, AcrF2 comigrated with the XcCsy1-Csy2 heterodimer (Fig. 4C) as seen in the experiment with the XaCsy1-Csy2 heterodimer. The sequence identities between the *Pseudomonas* and *Xanthomonas* homologues are ~38 and ~46% for Csy1 and Csy2, respectively (Figs. S2 and S3). XaCsy1 and XaCsy2 share 65 and 63% sequence identity, respectively, with *X. citri* Csy1 and Csy2 (XcCsy1 and XcCsy2, respectively; Figs. S2 and S3).

To analyze the interactions of AcrF2 with the individual components of the XaCsy1-Csy2 heterodimer, we performed analytical SEC with the separately purified XaCsy1 and His6-MBP-tagged XaCsy2 proteins (Fig. 5). In the cryo-EM structure of the AcrF2-bound *P. aeruginosa* Csy complex, AcrF2 is inserted into a “vise-like” structure formed between Csy1 and one of the six Csy3 subunits and is not directly involved in the interaction with the Csy2 protein (25). To our surprise, AcrF2 did not comigrate with either XaCsy1 or XaCsy2 in the analytical SEC (Fig. 5A and B). However, when the individually purified XaCsy1 and XaCsy2 proteins were incubated together with AcrF2, the three proteins coeluted (Fig. 5C), implying the formation of a ternary complex containing XaCsy1, XaCsy2, and AcrF2. Notably, a similar result was also obtained in the EMSAs that tested crRNA binding. The individual components of the XaCsy1-Csy2 heterodimer alone did not bind to the 5′-handle of crRNA, whereas a mixture of the separately purified proteins resulted in a significant RNA band shift (Fig. 3E). Consequently, the heterodimerization of XaCsy1 and XaCsy2 is required not only for the 5′-handle recognition but also for AcrF2 binding, suggesting that XaCsy1 can adopt a structural state capable of binding to AcrF2 only after forming a heterodimeric complex with XaCsy2.

**Crystal structure of AcrF2 reveals densely populated negative charges at the binding interface of the Csy complex**

To better understand how AcrF2 binds to Csy1-Csy2 heterodimers, the crystal structure of AcrF2 was determined to a resolution of 1.34 Å using single-wavelength anomalous diffraction. Data collection and refinement statistics are summa-
rized in Table 1. The asymmetric unit contains two AcrF2 molecules, which form a dimer by swapping their C-terminal regions (residues 56–94; Fig. 6 B). However, the previously determined cryo-EM structures (25, 47) and our SEC with multi-angle light scattering (SEC-MALS) analysis (Fig. 6 C) support the monomeric state of AcrF2. Moreover, the dimerization interface overlaps with that of Csy1 binding. When one of the two AcrF2 monomers in the dimeric structure is docked into its binding site at the cryo-EM structure of the *P. aeruginosa* Csy complex (25), severe steric clashes are observed between the other AcrF2 monomer and PaCsy1 (Fig. S4). These suggest that the dimeric assembly of AcrF2 is a crystallographic artifact and that its monomeric form is biologically relevant. Therefore, we hereafter describe the structure of the physiologically relevant monomeric AcrF2 unit composed of the N-terminal region (residues 1–52) of one AcrF2 molecule and the swapped C-terminal region (residues 56–94) of the other molecule in the asymmetric unit (Fig. 6 D).

The topology and overall crystal structure of AcrF2 are almost identical to those of the previously reported cryo-EM structures (25, 47). The AcrF2 structure contains a four-stranded antiparallel β-sheet (β1–β4) sandwiched between two pairs of antiparallel α-helices (α1–α4) as seen in the cryo-EM structure (Fig. 6, A and D) (25, 47). The root mean square deviation (r.m.s.d.) values of Ca atomic positions between our AcrF2 monomer and the two cryo-EM structures, one of which has been deposited as an ensemble of five atomic models, range from 1.5 to 2.0 Å (Table S2). The largest structural deviations between our crystal structure and the previous cryo-EM structures were observed primarily in the α1 and α2 helices (Fig. S5 and Table S2). The structural differences in the region were also recognized between the individual cryo-EM models (Fig. S5 and Table S2), suggesting its intrinsic structural flexibility. Nevertheless, the high-resolution electron density map allows more precise determination of atomic positions, including those of side chains potentially interacting with Csy1-Csy2 heterodimers.

A search for structural neighbors using the Dali server (48) did not identify any meaningful structural matches to the crystal structure of AcrF2 in the Protein Data Bank (49). In a previous structural study of the AcrF2-bound *P. aeruginosa* Csy complex using cryo-EM, Wiedenheft and co-workers (25) suggested that AcrF2 is a double-stranded DNA (dsDNA) mimic that blocks target recognition by binding to the “Lys-rich, viselike” structure created by Csy1 and Csy3. Several acidic AcrF2 residues were found in close proximity to the surface Lys residues of Csy1 and Csy3, including Asp-30, Glu-36, Asp-75, Asp-76, Glu-85, Glu-87, and Glu-91 (25). The electrostatic potential of our AcrF2 crystal structure was calculated using the Adaptive Poisson-Boltzmann Solver (50). In the AcrF2 structure, neg-
ative charges are densely populated at its binding interfaces with Csyl and Csyl3 formed by residues in or adjacent to the β1 strand and the α4 helix (Fig. 6G). Glu-36, Asp-75, and Asp-76 are located in close proximity to one another near the C terminus of the β1 strand, whereas Asp-30, Glu-85, Glu-87, and Glu-91 are positioned adjacent to the opposite side of the central β-sheet. This arrangement of carboxyl side chains in the crystal structure resembles the negative charge distribution of the dsDNA backbone as suggested previously (25). The two groups of carboxyl side chains, with the exception of Glu-91, are separated by 16–19 Å, which is similar to the distance (∼18 Å) between two phosphates of base-pairing nucleotides in the B-form dsDNA helix. Distances between the nearby carboxyl side chains within the same group ranged from 5 to 10 Å, comparable with the intrastrand phosphate–phosphate distance (∼7 Å).

Discussion

The results obtained in the present study provide information about the order of events during the formation of the multitysusbunit Csy complex in type I-F CRISPR-Cas systems. XaCsy1 and XaCsy2 interact directly with each other to form a stable heterodimeric complex in the absence of crRNA or other Cas proteins (Fig. 2). The XaCsy1-Csy2 heterodimer is responsible for the sequence-specific binding to the 8-nit 5′-handle of the crRNA, but XaCsy1 or XaCsy2 alone did not show binding affinity for the 5′-handle RNA (Fig. 3). These observations suggest that the heterodimer is assembled prior to its binding to the 5′-handle part of the crRNA during the formation of the crRNA-guided surveillance complex. In our EMSA analyses, the XaCsy1-Csy2 heterodimer exhibited reduced affinity to the X. albilineans repeat RNA compared with that of the 5′-handle RNA (Fig. 3D), although the 28-nit repeat RNA includes the 8-nit 5′-handle sequence. The interaction of the XaCsy1-Csy2 heterodimer with the 5′-handle region may be impeded by the proximal hairpin structure formed by the preceding 20-nit sequence in the repeat, which becomes the 3′-stem of another crRNA (Fig. 1B). Alternatively, simply the 5′-end location of the 8-nit sequence is important for the binding. Therefore, the XaCsy1-Csy2 heterodimer may not effectively bind to the long pre-crRNA. In the course of crRNA generation, the CRISPR array is transcribed into a long pre-crRNA, which is processed into small crRNAs containing a 5′-handle, a single spacer, and a 3′-stem by the Csy4 endoribonuclease at the repeat sequences (Fig. 1B) (15, 21, 26). Thus, our results suggest that the cleavage of pre-crRNA occurs ahead of Csyl-Csy2 heterodimer binding.

Based on our experimental data, we concluded that the formation of the XaCsy1-Csy2 heterodimer is required for binding to the 5′-handle RNA and AcrF2 because the separately purified individual components did not form a stable complex with them (Figs. 3E and 5A and B). It is not likely that the N-terminal His6-MBP tag of the separately purified XaCsy2 interfered with the interactions because the binding affinities were recovered when XaCsy1 and the tagged XaCsy2 were added together (Figs. 3E and 5C). The requirement for heterodimerization for crRNA binding may seem reasonable because the cryo-EM structures of the P. aeruginosa Csy complex showed that the 5′-handle of the crRNA is in close proximity to both Csyl and Csyl2 (25, 47). However, it was still not anticipated that the binding to the 5′-handle RNA would be completely lost without residual affinity in the EMSA experiments using the individual components of the XaCsy1-Csy2 heterodimer (Fig. 3E, lanes 3 and 4). Interestingly, AcrF2 did not form a stable complex with XaCsy1 in our experiment (Fig. 5A). The binding interfaces for the 5′-handle RNA and AcrF2 are completely different in the Csyl-Csy2 heterodimer. AcrF2 is associated with PaCsy1, but not with PaCsy2, in the cryo-EM structures of the P. aeruginosa Csy complex (25, 47). These results suggest that XaCsy1 and XaCsy2 mutually stabilize each other’s structural state capable of binding to the 5′-handle RNA and AcrF2. Indeed, the stability of XaCsy2 was enhanced by XaCsy1 as indicated by the fact that, in the presence of XaCsy1, XaCsy2 was soluble without the N-terminal His6-MBP tag (Fig. 2A). Notably, in the structure of the P. aeruginosa Csy complex, the elongated conformation of PaCsy1 makes extensive contact with the more globular PaCsy2 (Fig. S6A) (25, 47). In our analytical SEC studies, individually purified XaCsy1 eluted earlier than the His6-MBP–tagged XaCsy2 (Fig. 2B) whose molecular mass (79 kDa) is greater than that of XaCsy1 (52 kDa). This is consistent with the extended architecture of XaCsy1. XaCsy1 exhibited a circular dichroism spectrum typical of folded α-helical proteins (Fig. S6B), suggesting that the earlier elution of XaCsy1 was not caused by its unfolding but resulted from its intrinsic extended conformation.

In the present study, we demonstrated that AcrF2 from a P. aeruginosa phage binds to the Csyl-Csy2 heterodimers of two Xanthomonas bacteria, X. albilineans and X. citri. In a previous study of the P. aeruginosa Csy complex, charge-swap mutations in PaCsy1 (K28E, K31E, and K247E) resulted in severe binding defects for AcrF2 (25). In the sequence alignment of PaCsy1, XaCsy1, and XcCsy1 (Fig. S5), Lys-28 and Lys-247 are conserved in all three Csy1 proteins, and XaCsy1 and XcCsy1 contain Lys-30 instead of Lys-31. Considering the fact

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**Table 1**

| Data collection, phasing, and refinement statistics |
|---|
| Space group | P4_2_2_1 |
| Unit cell parameters (Å) | a = b = 65.5, c = 101.3 |
| Wavelength (Å) | 0.9793 |
| Resolution range (Å) | 50.00–1.34 (1.39–1.34)* |
| Number of reflections | 1,402,615 (50,291) |
| Completeness (%) | 100.0 (100.0) |
| Rmerge (%) | 0.102 (0.582) |
| Redundancy | 27.9 (25.6) |
| Mean I/σ | 29.8 (6.4) |

**Phasing statistics**

| f, f' used in phasing | ~8.0, 4.5 |
| Figure of merit | 0.540 |

**Refinement statistics**

| Resolution range (Å) | 23.17–1.34 |
| Rmerge/Rfree (%) | 18.0/19.6 |
| r.m.s.d. bonds (Å) | 0.006 |
| r.m.s.d. angles (°) | 0.961 |
| Average B-factor (Å²) | 15.1 |
| Number of water molecules | 364 |
| Ramachandran favored (%) | 98.9 |
| Ramachandran allowed (%) | 1.1 |

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*a* Values in parentheses are for the highest-resolution shell.

*b* \( R_{merge} = \sum |I(h) - \langle I(h) \rangle|/\sum I(h) \) where I(h) is the intensity of an individual measurement of the reflection and \( \langle I(h) \rangle \) is the mean intensity of the reflection.

\( R_{merge} = \sum |F_{calc}(h) - F_{obs}(h)|/\sum |F_{calc}(h)| \) where Fcalc and Fobs are the observed and calculated structure factor amplitudes, respectively.

\( R_{merge} = \sum |F_{calc}(h) - F_{obs}(h)|/\sum |F_{calc}(h)| \) where Fcalc and Fobs are the observed and calculated structure factor amplitudes, respectively.
that Lys-31 of PaCsy1 is located in the middle of the flexible loop connecting two \( \alpha \)-helices (25), it is likely that the Lys-30 residues in XaCsy1 and XcCsy1 can interact with AcrF2 in a similar manner to Lys-31 in PaCsy1. In addition to the Lys residues, most (23 of 25) of the PaCsy1 residues found in close proximity (6 Å) to AcrF2 in the cryo-EM structure are also conserved in XaCsy1 and XcCsy1 (Fig. S2)(25). PaCsy1 shares only \( \sim 38\% \) sequence identity with the two \textit{Xanthomonas} Csy1 homologues (Fig. S2). These observations suggest that AcrF2 recognizes Csy1 residues essential for its folding and/or the target DNA binding because the conserved residues most likely play crucial roles in the structure and function of the protein. Hence, our analyses indicate the possibility that AcrF2 can bind to other Csy1 homologues from different species, implying the broad specificity of AcrF2 for suppressing the adaptive immune function of type I-F CRISPR-Cas systems.

Previous cryo-EM structures for the AcrF2-bound \textit{P. aeruginosa} Csy complex have been determined to moderate resolutions (25, 38, 47). One study reported that the average resolution was 3.4 Å, and the authors stated that the density of acidic residues on AcrF2 was not sufficient to confidently model side chain positions (25). Another study located the Csy1-Csy2-AcrF2 subcomplex but could not model it (38). More recently, Guo et al. (47) reported a cryo-EM structure of the complex bound to AcrF2 at a nominal resolution of 3.2 Å. In the current study, we determined the crystal structure of AcrF2 to a resolution of 1.34 Å, which enabled a more detailed structural analysis of the Acr protein, including the negatively charged residues involved in interactions with the Csy complex. In the crystal lattice, AcrF2 forms a dimer by swapping the C-terminal regions (\( \sim 4 \) and \( \sim 3–5 \)). However, we speculate that this dimerization is a crystallographic artifact because...
other experimental evidence is consistent with a monomeric AcrF2. It is not uncommon to observe swapping between protein monomers in the crystal lattice, especially under non-physiological crystallization conditions, such as low pH (51, 52). In our experiment, AcrF2 was crystallized under acidic conditions (pH 5.5).

**Experimental procedures**

**Cloning, expression, and purification**

To produce Csy1-Csy2 heterodimers, the synthetic genes of Csy1 and Csy2 were cloned into pET28a, which has an N-terminal His<sub>6</sub>-MBP tag and a tobacco etch virus (TEV) protease cleavage site, and pET21a, which does not contain a tag, respectively. *E. coli* BL21 (DE3) cells transformed with these constructs were cultured in LB medium at 37 °C until the optical density at 600 nm reached 0.6. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside followed by incubation at 17 °C for 15 h. Cells were harvested by centrifugation and resuspended in lysis buffer (150 mM NaCl, 5 mM β-mercaptoethanol, 10% (w/v) glycerol, 20 mM Tris-HCl, pH 7.5). After sonication and centrifugation, the supernatant was loaded onto a 5-ml HisTrap HP column (GE Healthcare) pre-equilibrated with affinity chromatography buffer (150 mM NaCl, 5 mM β-mercaptoethanol, 10% (w/v) glycerol, 30 mM imidazole, 20 mM Tris-HCl, pH 7.5). After washing the column with the buffer, we eluted thebound Csy1-Csy2 complex by applying a linear gradient of imidazole (up to 450 mM). The His<sub>6</sub>-MBP tag on the Csy1 protein was cleaved by TEV protease and separated with a HisTrap HP column. The untagged Csy1-Csy2 heterodimer was further purified by SEC using a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with SEC buffer (300 mM NaCl, 2 mM dithiothreitol (DTT), 5% (w/v) glycerol, 20 mM Tris-HCl, pH 7.5).

To purify XaCsy1 and XaCsy2 individually, we used the synthetic genes cloned into pET28a with an N-terminal His<sub>6</sub>-MBP tag and a TEV protease cleavage site. XaCsy1 was expressed and purified as described above. XaCsy2 was purified with the N-terminal His<sub>6</sub>-MBP tag by a two-step procedure involving affinity chromatography using the HisTrap HP column and SEC using the Superdex 200 column as described for the Csy1-Csy2 complexes. The His<sub>6</sub>-MBP tag was required for solubilizing XaCsy2 under our experimental conditions. AcrF2 was generated by using the synthetic gene cloned into pET32a containing an N-terminal thioredoxin-His<sub>6</sub> tag and a TEV protease cleavage site. The protein was expressed and purified as described above for Csy1-Csy2 heterodimers except using a different buffer (sodium phosphate, pH 7.0).

**Analytical SEC**

Analytical SEC was performed on a Superdex 200 10/300 GL column (GE Healthcare). The column was equilibrated with buffer (150 mM NaCl, 2 mM DTT, 20 mM Tris-HCl, pH 7.5). For testing the complex formation, identical amounts (1.4 nmol) of protein samples were incubated together at 4 °C for 1 h and loaded onto the column at a flow rate of 0.5 ml/min. The control SEC runs for XaCsy1, His<sub>6</sub>-MBP-XaCsy2, and AcrF2 shown in various figures represent independent chromatographic experiments. Elution fractions were analyzed by SDS-PAGE and visualized by Coomassie staining. Uncropped gel images are shown in Fig. S7.

**ITC**

ITC experiments were performed at 25 °C using a MicroCal iTC200 system (GE Healthcare). Proteins in a 200-µl sample cell were titrated with 19 consecutive 2-µl injections unless stated otherwise. In the analysis of the formation of the XaCsy1-Csy2 complex, XaCsy1 (20 µM) in buffer (150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine, 5% (w/v) glycerol, 20 mM Tris-HCl, pH 7.5) was titrated with His<sub>6</sub>-MBP–tagged XaCsy2 (200 µM) in the syringe. To examine the crRNA binding of the XaCsy1-Csy2 heterodimer, the synthetic 5′-handle of the crRNA (250 µM; Bioneer, Korea) was injected into the sample cell containing the XaCsy1-Csy2 heterodimer (25 µM) in buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5). For the binding between AcrF2 and the XaCsy1-Csy2 heterodimer, 37 consecutive 1-µl injections of AcrF2 (500 µM) were titrated into the solution containing the XaCsy1-Csy2 heterodimer (70 µM) in buffer (150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine, 5% (w/v) glycerol, 20 mM Tris-HCl, pH 7.5). Origin software (OriginLab) was used for processing and for the analysis of the ITC titration data.

**EMSA**

RNA oligonucleotides with a fluorescein label at the 3′-end were commercially synthesized and purified by high-performance liquid chromatography (Bioneer). They were refolded by heating at 65 °C for 10 min followed by cooling to room temperature. The RNAs (2.0 µM) were incubated with proteins (2.0 µM) in reaction buffer (150 mM NaCl, 20 mM HEPES, pH 7.5) at 20 °C for 1 h. The RNA-protein mixtures were analyzed on 9% polyacrylamide gels.

**Crystallization and structure determination of AcrF2**

The selenomethionyl-AcrF2 protein was expressed in *E. coli* BL21 (DE3) cells grown in M9 medium supplemented with SeMet as described previously (53). The protein was purified as described above for the native AcrF2 protein. AcrF2 crystals were grown at 20 °C by the sitting-drop method from 39 mg/ml protein solution in buffer (300 mM NaCl, 2 mM DTT, 5% (w/v) glycerol, 20 mM sodium phosphate, pH 7.0) mixed with an equal amount of reservoir solution (21% (w/v) PEG 6000, 0.4 M CaCl<sub>2</sub>, 100 mM MES, pH 5.5). The crystals were cryoprotected in the reservoir solution supplemented with additional 12% (w/v) ethylene glycol and flash frozen in liquid nitrogen. Diffraction data were collected at beamline 7A of the Pohang Accelerator Laboratory at 100 K. Diffraction images were processed with HKL2000 (54). Determinations of selenium positions, density modification, and initial model building were made using PHENIX (55). Two of the three selenium sites in each AcrF2 chain (residues 7 and 59) were found and used for the phasing. The structure was completed using alternate cycles of manual fitting in Coot (56) and refinement in PHENIX (55). The stereochemical quality of the final model was assessed using MolProbity (57).
SEC-MAILS

SEC-MAILS analysis was performed on a TSKgel G2000SWxl column (Tosoh Bioscience) coupled with a DAWN HELEOS II 18-angle light scattering detector (Wyatt Technology). The column was equilibrated with buffer (300 mM NaCl, 2 mM DTT, 20 mM sodium phosphate, pH 7.0), and AcrF2 (11 mg/ml) was loaded onto the column at a flow rate of 0.5 ml/min at 25 °C. Data were analyzed using ASTRA 6 software (Wyatt Technology).

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