High-temperature protein G is essential for activity of the *Escherichia coli* clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system

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Prokaryotic DNA arrays arranged as clustered regularly interspaced short palindromic repeats (CRISPR), along with their associated proteins, provide prokaryotes with adaptive immunity by RNA-mediated targeting of alien DNA or RNA matching the sequences between the repeats. Here, we present a thorough screening system for the identification of bacterial proteins participating in immunity conferred by the *Escherichia coli* CRISPR system. We describe the identification of one such protein, high-temperature protein G (HtpG), a homolog of the eukaryotic chaperone heat-shock protein 90. We demonstrate that in the absence of *htpG*, the *E. coli* CRISPR system loses its suicidal activity against λ prophage and its ability to provide immunity from lysogenization. Transcomplementation of *htpG* restores CRISPR activity. We further show that inactivity of the CRISPR system attributable to *htpG* deficiency can be suppressed by expression of Cas3, a protein that is essential for its activity. Accordingly, we also find that the steady-state level of overexpressed Cas3 is significantly enhanced following HtpG expression. We conclude that HtpG is a newly identified positive modulator of the CRISPR system that is essential for maintaining functional levels of Cas3.

defense mechanism | phage-host interactions | positive selection

The clustered regularly interspaced short palindromic repeats (CRISPR) system is a significant defense mechanism of prokaryotes against viruses and horizontally transferred DNA (1–3) and RNA (4). It is found in ∼90% of archaeal genomes and ∼40% of bacterial genomes, and consists of a CRISPR array, usually preceded by a leader DNA sequence, located near a cluster of CRISPR-associated (*cas*) genes (5–7). RNA transcribed from the CRISPR array (crRNA) is processed by Cas proteins and directs interfering proteins to target DNA/RNA matching the sequences between the repeats. These sequences, called spacers, often originate from plasmids and phages; thus, the system adaptively targets these invaders. High variability is found among bacterial species in the number, sequence, and length of the CRISPR arrays; the sequence and length of the leader DNA; and the number and sequence of the associated proteins. The CRISPR arrays are composed of 2 to ∼250 DNA repeats of ∼25 to ∼70 bp that flank similarly sized spacer sequences (8). The leader sequence is usually AT-rich and promotes transcription of the CRISPR array; it may also serve as a unique sequence that confers directional acquisition of new spacers into the arrays (1, 2, 9). The functions of most of the *cas* genes are not yet known, but many motifs, such as DNA-binding, RNA-binding, helicase, and nuclease domains, are present in their encoded proteins (10).

The *cas* genes have been mostly identified by bioinformatics. Systematic analysis of uncharacterized genes identified in various species in close proximity to the CRISPR arrays has led to the identification of many Cas protein families (11). One reported example of the experimental identification of a novel non-*cas* gene, which participates in the activity of the CRISPR system, is the *rnc* gene of *Streptococcus pyogenes*, encoding the enzyme RNase III (12). This enzyme is important for processing crRNA transcripts with the aid of an RNA molecule and the Cas protein, Cas9. H-NS and LeuO have been shown to regulate the CRISPR system in both *Escherichia coli* and *Salmonella typhi*, whereas LRP regulates the *S. typhi* CRISPR system (13–15). Other non-*cas* genes participating in the activity of the CRISPR system have not yet been identified in other bacteria. The identification of RNase III, as well as H-NS, LRP, and LeuO, suggests the existence of other non-*cas* gene products participating in the activity of the CRISPR system. Such gene products may have a direct role in CRISPR activity, such as DNA cleavage, crRNA processing, and mediating the encounter with incoming DNA, or they may have indirect supportive activities, such as proper folding of the involved proteins, energy supply for the system, regulation of the system, and bridging between protein machineries.

In this study, we established a system to search for genes whose products are required for activity of the *E. coli* CRISPR system. No such systematic search has ever been reported for any CRISPR system. The search is based on positive selection of *E. coli* colonies having disruptions in genes essential for activity of the CRISPR system. Our search coverage is thorough, consisting of 50,000 independent mutants covering over 10-fold the *E. coli* genome and identifying *cas* genes numerous times as essential for the CRISPR activity. We therefore believe that we have identified all the nonessential *E. coli* genes that are essential for the CRISPR activity. In particular, we identified high-temperature protein G (HtpG), a protein that is homologous to the eukaryotic chaperone heat-shock protein 90 (Hsp90) (16), as essential for maintaining functional levels of Cas3, a required protein for CRISPR interference with incoming DNA (3). A possible model for these interactions is discussed.

Results

Positive Selection System for *E. coli* Genes Required for a Functional CRISPR System. To identify genes that are essential to the activity of the CRISPR system, we established a genetic screen that positively selects *E. coli* mutants with an inactive CRISPR system. The selection principle is based on the suicidal activity of the CRISPR system when *E. coli* λ lysogens are transformed with a plasmid encoding spacers against the λ phage (17). In such a system, transposon mutagenesis can be applied to identify viable bacterial clones with transposon disruptions of genes essential for CRISPR activity. We selected *E. coli* strain BW25113Δ*hns*, in which the CRISPR system is constitutively active (9, 14, 17, 18), for the genetic screening. This strain was lysogenized with the λ phage,

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making the bacteria incompatible, in a CRISPR-dependent manner, with the plasmid pWUR478 encoding spacers against the λ phage (3, 17). In addition, the strain was genetically engineered to carry an arabinose-inducible T7-RNA polymerase (RNAp) to enhance expression of the CRISPR spacers, encoded downstream of a T7-promoter in the pWUR478 plasmid. When this strain was transformed with plasmid pWUR478 encoding anti-λ spacers, the transformation efficiency, compared with that of plasmid pWUR477 encoding control spacers, was reduced over 10,000-fold (Fig. 1), indicating that the spacers against the λ phage kill the cells in a CRISPR-dependent manner. To demonstrate further that the CRISPR system is responsible for the reduced transformation efficiency, we used an isogenic control strain, with an intact *hns* gene encoding the H-NS protein that represses CRISPR-system activity in vivo (9, 14). This strain showed similar transformation efficiency with pWUR478 and the control plasmid (Fig. 1). We concluded that the apparent decrease in transformation efficiency of strain BW25113Δ*hns* by pWUR478 is attributable to cell death as a result of CRISPR-system activity. This significant difference in transformation efficiency enabled us to establish a robust system for the positive selection procedure by transforming pWUR478 into transposon-mutagenized bacteria and examining gene disruptions in the viable colonies.

Identification of *E. coli* Genes Essential for CRISPR-System Activity. We next genetically engineered BW25113Δ*hns araB*::T7-RNAp λ*cI857-bla*, a strain similar to the above but with a λ prophage

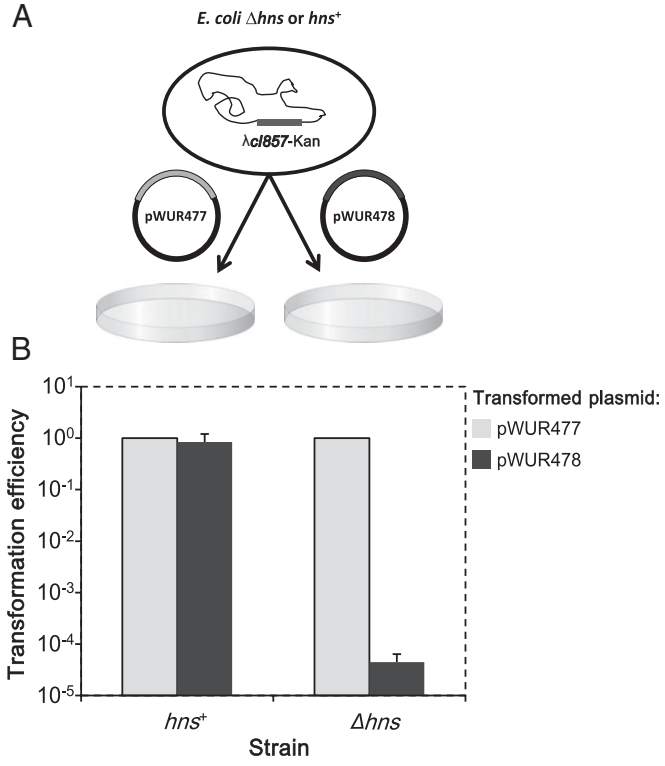

Fig. 1. The *E. coli* CRISPR system specifically prevents establishment of plasmids encoding spacers against an integrated λ prophage. (*A*) Schematic representation of the transformation assay used throughout the study. The indicated bacteria, harboring a λ prophage, were transformed with the indicated plasmids encoding anti-λ (pWUR478) or control (pWUR477) spacers. Transformed bacteria were plated and counted after overnight incubation. (*B*) BW25113 λ*cI857*-kan *araB*::T7-RNAp with (*hns*$^+$) or without (Δ*hns*) the *hns* gene were made competent and transformed with pWUR478 or pWUR477 as described in *Materials and Methods*. The relative efficiency of transformation was calculated by dividing the number of obtained pWUR478 or pWUR477 transformants by the number of pWUR477 transformants. Results are presented as the average ± SD of three independent experiments.

having a different resistance marker, to harbor transposon insertions at various genetic locations, and we followed the selection scheme depicted in Fig. 2*A*. The generated transposon library represented ~50,000 independent events, providing over 10-fold coverage of the *E. coli* genome. This bacterial library was made competent, transformed with pWUR478 encoding anti-λ spacers, and selected on LB-agar plates supplemented with chloramphenicol, resistance to which is conferred by the plasmid. Bacteria able to harbor the plasmid were suspected of having an inactive CRISPR system because, as explained above, the lysogenic bacteria can only coexist with this plasmid in the absence of CRISPR activity. This procedure yielded 127 bacterial candidates that were further tested. We first eliminated transposition events in known CRISPR essential genes, the *cas* genes, from further analyses using multiplex PCR on these colonies. We used a mix of five oligonucleotides, three binding to the *cas* genes at 2- to 3.5-kb intervals, and two primers amplifying outward of the transposon (Fig. 2*A*). This procedure generates a PCR product when a transposon is inserted in one of the *cas* genes. Of the 127 candidates, 113 indeed harbored a transposon insertion in one of these genes, confirming the validity of the selection assay. A typical agarose gel showing PCR-amplified products from nine colonies selected for the ability to harbor pWUR478 is shown in Fig. 2*B*. Because *cas1* and *cas2* have been shown not to be required for CRISPR activity (3), and transposon insertion in *cas1* cannot be excluded by the indicated PCR, we wanted to verify that, indeed, these genes do not contain any transposon insertions. As expected, PCR amplification of *cas1-cas2* did not reveal any transposon insertion in these genes (Fig. 2*B*). These mutants were not examined further. The transposon insertion site was identified in the other 14 candidates by semirandom PCR amplification followed by sequencing, as described previously (19). Redundant candidates were eliminated from further analyses, and P1 transductions generating "clean deletions" were carried out for representatives of each disrupted gene. These reconstructed deletion mutants were then tested for transformation efficiency of pWUR478 compared with pWUR477, and only one showed significant restoration of transformation efficiency, suggesting that the CRISPR system is nonfunctional in the absence of this gene. Thus, the other originally isolated colonies probably carried secondary mutations (perhaps in the *cas* genes), or the transposon somehow modified the gene's activity such that it could not be reconstituted by deletions.

Validating the Essentiality of the Identified Genes to CRISPR-System Functionality. The deleted gene showing consistent restoration of transformation efficiency represented four selected colonies having disruptions in *htpG*, two at position 495,128 and two at position 495,364 (Fig. 2). As described above, to verify that disruption of *htpG* is responsible for the lack of CRISPR activity, and that there are no secondary mutations causing this effect, we reconstructed a deletion of *htpG* in BW25113Δ*hns araB*::T7-RNAp by P1 transduction of the *htpG*::*kan* cassette (20), and after removing the *kan* cassette, we lysogenized these cells with λ-*cI857*-kan, yielding BW25113Δ*hns* Δ*htpG araB*::T7-RNAp λ-*cI857*-kan. The newly constructed strain was tested for transformation efficiency of pWUR478 compared with pWUR477. As shown in Fig. 3*A*, the transformation efficiency of pWUR478, encoding anti-λ spacers, into the *htpG* deletion mutant was comparable to that of pWUR477, encoding control spacers, indicating that the CRISPR system does not function in the absence of *htpG*. To verify further the essentiality of *htpG* to CRISPR interference and to exclude, for example, polar effects of *htpG* deletion on other genes, we complemented the Δ*htpG* mutant with a plasmid encoding *htpG* under an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. As shown in Fig. 3*B*, transformation efficiency of pAC-478, encoding anti-λ spacers, into the Δ*htpG* mutant harboring the HtpG-encoding plasmid was over 1,000-fold lower than that with the control pAC-477, or in comparison to pAC-478 transformation into an Δ*htpG* mutant harboring a control vector. These results indicated that expression of HtpG from a plasmid can restore

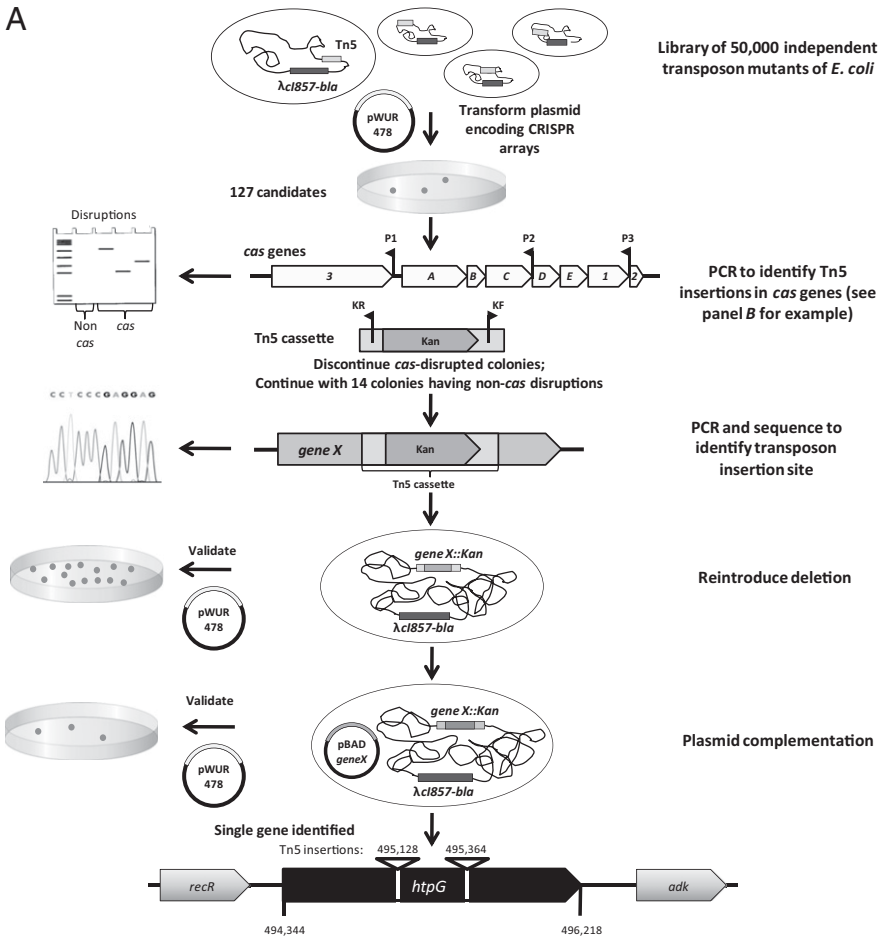

Fig. 2. (*A*) Screening system for identifying *E. coli* genes required for CRISPR activity. A random transposon-insertion library, consisting of ~50,000 cfu, was prepared on the genetic background of BW25113Δ*hns* λ*cI857-bla araB*::T7-RNAp as described in (31) and in *SI Materials and Methods*. Bacterial cells were transformed with pWUR478, encoding spacers against the λ prophage; 127 of the surviving colonies were subjected to colony-PCR assay to rule out transposon insertions in the *cas* genes, as follows: Three oligonucleotides (P1, P2, and P3) at 2- to 3.5-kb intervals in the *cas* genes were used for one direction, and two oligonucleotides, KF and KR, amplifying both ends of the transposon, were used for the other direction. Colonies showing DNA product of this PCR amplification presumably have transposon insertions in one of the *cas* genes and were not studied further. The Tn5-insertion site was identified in 14 colonies having disruptions in non-*cas* genes. The disrupted gene was reintroduced by transduction from the Keio collection, and the newly developed strain was retested for transformation efficiency of pWUR478. Positive clones were complemented by a plasmid encoding the disrupted gene. A single gene, *htpG*, was validated using the above procedure. This gene had two independent Tn5 insertions in 4 of the identified colonies. (*B*) PCR amplification of DNA template from 9 random colonies identified by the screening procedure. (*Upper*) Products from the multiplex PCR assay illustrated in *A*. The presence of amplified product(s) indicates that the transposon inserted at a distance equivalent to the product length from any of the oligonucleotides used. Note similar sizes of several of the products, suggesting either hot spots for transposon insertion, that sibling colonies harboring the same insertion were selected, or both. (*Lower*) PCR amplification of the region encompassing *cas1-cas2*. The expected size when no transposon is inserted is 1,467 bp, and this was the size observed in all the tested colonies, indicating no transposon insertion in these genes. Colonies are numbered 1–9. M, marker; C, control (a random transposon mutant selected for in a control reaction transformed with pWUR477).

CRISPR activity to Δ*htpG* cells, unequivocally confirming that *htpG* is essential for CRISPR-system activity.

We have previously shown that the CRISPR system is active against the establishment of lysogens (17). We wished to demonstrate that deficiency of *htpG* prevents this activity against lysogenization. We therefore transformed pWUR478 or pWUR477 into *E. coli* BW25113Δ*hns* Δ*htpG araB*::T7-RNAp or into BW25113Δ*hns araB*::T7-RNAp as a control. These strains were tested for lysogenization efficiency by infecting them with λ*cI857-bla* phage at 32 °C, conditions favoring the lysogenic cycle, and then determining the number of colonies acquiring antibiotic resistance encoded by the phage (Fig. 3*C*). In this assay as well, lack of *htpG* significantly decreased the CRISPR-dependent protection of the bacteria from lysogenization by over 1,000-fold compared with the isogenic strain carrying *htpG* (Fig. 3*D*). These results indicate that *htpG* is also essential for the *E. coli* CRISPR system's prevention of phage lysogenization.

Expression of Cas3 Restores CRISPR Activity in the Δ*htpG* Mutant. It is important to note that in both the lysogenization and transformation-efficiency assays described above for *E. coli* Δ*htpG*, the size of the colonies harboring pWUR478 was smaller than that of those harboring pWUR477. These results led us to assume that CRISPR activity is not completely silent in the absence of *htpG* and that HtpG somehow modulates the CRISPR system's functionality. HtpG is the bacterial homolog of the eukaryotic Hsp90 protein, which is a well-characterized chaperone (16). Because we established that HtpG is essential for CRISPR activity, we speculated that one or more of the Cas proteins are client proteins of this chaperone. Consequently, if HtpG is indeed essential for the accumulation of an active Cas protein, *htpG* deficiency should be suppressed by expression of this client protein in higher amounts than its endogenous expression. This speculation assumes that *htpG* deficiency results in a limiting amount of an active Cas protein and that this might be overcome by overexpressing this Cas protein from a plasmid. To test this possibility and to identify the

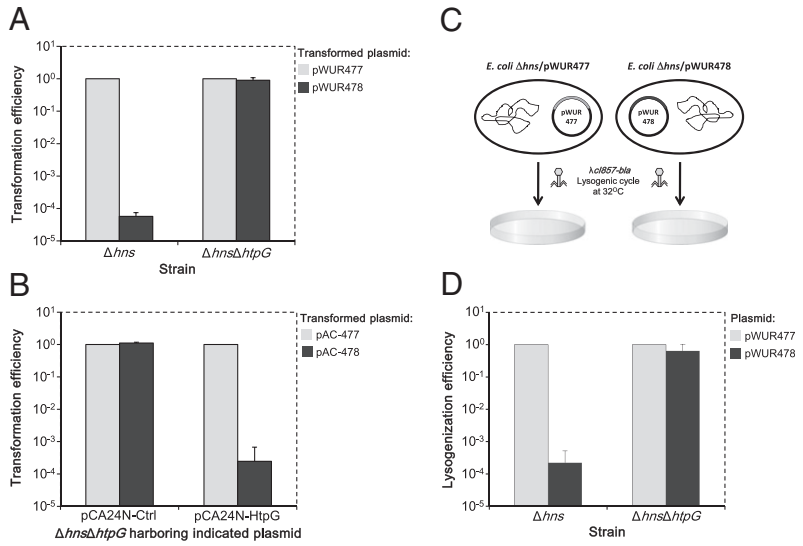

Fig. 3. *htpG* is essential for activity of the CRISPR system. (*A*) "Clean" *htpG* deletion was introduced into BW25113Δ*hns* T7-RNAp, and cells were then lysogenized with λ*cI857*-kan. BW25113Δ*hns* Δ*htpG* λ*cI857*-kan T7-RNAp was made competent and transformed with plasmids encoding spacers against the λ prophage (pWUR478) or control spacers (pWUR477) as described in *Materials and Methods*. The relative efficiency of transformation was calculated by dividing the number of obtained pWUR478 or pWUR477 transformants by the number of pWUR477 transformants. Results are presented as the average ± SD of three independent experiments. (*B*) Plasmid encoding *htpG* (pCA24N-HtpG) or control vector (pCA24N-Ctrl) was transformed into BW25113Δ*hns* Δ*htpG* λ*cI857*-kan T7-RNAp. These cells were then transformed with plasmids encoding spacers against the λ prophage (pAC-478) or control spacers (pAC-477) as described in *Materials and Methods*. The relative efficiency of transformation was calculated by dividing the number of obtained pAC-478 or pAC-477 transformants by the number of pAC-477 transformants. Results are presented as the average ± SD of three independent experiments. (*C*) Schematic diagram of the assay used to test CRISPR protection from lysogenization. (*D*) BW25113Δ*hns* or BW25113Δ*hns* Δ*htpG* transformed with either pWUR477 or pWUR478 was lysogenized at 32 °C with λ*cI857-bla*, conferring ampicillin resistance. Bacteria were then serially diluted and plated on LB-agar plates supplemented with ampicillin. Lysogenization efficiency was calculated by dividing the number of lysogenized bacteria in a given plate by the number of lysogenized bacteria obtained in its respective parental bacteria harboring the pWUR477 plasmid. Results are presented as the average ± SD of three independent experiments.

putative client protein, we transformed plasmids from the ASKA library (21) encoding each of the six proteins known to be essential for CRISPR interference into *E. coli* BW25113Δ*hns* Δ*htpG araB*::T7-RNAp. We then measured transformation efficiencies with a plasmid encoding anti-λ spacers, pAC-478, compared with a control plasmid, pAC-477. As shown in Fig. 4, expression of Cas3 was able to relieve the dependency of CRISPR activity on HtpG. In the absence of HtpG, increased expression of Cas3, but not of any other Cas protein, fully restored the CRISPR system's ability to decrease the transformation efficiency of pAC-478 by over 1,000-fold. These results indicate that sufficient expression of Cas3 can overcome the absence of HtpG, suggesting that under *htpG* deficiency, Cas3 protein is the only Cas component whose level is limiting for CRISPR activity. The results suggest that HtpG either helps in recruiting Cas3 protein to the CRISPR interference stage or is involved in this protein's folding and/or in preventing its aggregation, thereby maintaining functional levels of Cas3.

HtpG Expression Significantly Increases Levels of Overexpressed Cas3. Finally, if HtpG is indeed essential for maintaining functional levels of Cas3, its expression should support higher accumulation of Cas3. We first tested this possibility in vivo by transforming *cas3* fused to GFP into *E. coli* BW25113Δ*htpG* with either a plasmid encoding *htpG* or a control plasmid. As negative controls, we transformed the other *cas* genes required for CRISPR interference, fused to GFP, with either a plasmid encoding HtpG or a control plasmid. These bacteria were grown overnight on LB-agar plates supplemented with appropriate antibiotics, with or without L-arabinose for HtpG induction. To detect the level of fluorescence proportional to the levels of the GFP-fused proteins, plates were imaged and quantified as described in *Materials and Methods*. The measured fluorescence intensities indicated that the level of Cas3 increases over threefold when HtpG is expressed (Fig. 5 *A* and *B*). The increase in Cas3 level was only observed when HtpG expression was induced by L-arabinose, further validating that the increase in Cas3 levels is HtpG-dependent. The levels of the other tested Cas-GFP proteins were unaffected by the expression of HtpG, indicating that, of all the known CRISPR-effector proteins, HtpG specifically increases the steady-state level of Cas3-GFP in the bacterial cells. To confirm this in vivo observation further, we used Cas3 without GFP. Cas3 was overexpressed from a plasmid in BW25113Δ*htpG* with or without HtpG, and its protein level was analyzed. As shown by SDS-gel electrophoresis of total cell extracts, coexpression of HtpG and Cas3 resulted, in this case as well, in greater than a threefold increase in the level of Cas3 compared with its level without HtpG. The identity of Cas3 in the gel was confirmed by Western blot analysis against the His-tag preceding this protein (Fig. 5*C*). These findings confirmed that HtpG increases the steady-state level of Cas3. Taken together, our results show that HtpG's essentiality to CRISPR-system activity is mediated by its effect on Cas3.

Discussion

We carried out a thorough experimental search for non-*cas* genes essential for the activity of the *E. coli* CRISPR system. This search yielded the *htpG* gene, which was confirmed to be essential for the suicidal activity of plasmid-encoded spacers against λ prophage, as well as for protection against lysogenization by λ phage.

For the CRISPR system to interfere efficiently with invading DNA, it must go through most, if not all, of the following steps: (*i*) recognize alien DNA entry, (*ii*) induce transcription of *cas* genes, (*iii*) correctly fold or modify the translated Cas proteins, and (*iv*) process the crRNA and mediate binding of the processed crRNA-Cas proteins to the invading DNA/RNA, resulting in (*v*) cleavage

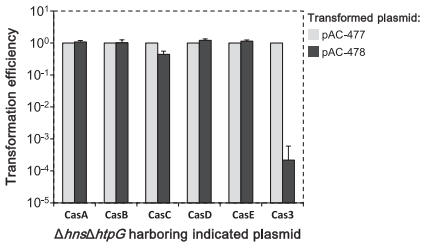

Fig. 4. Expression of Cas3, but not of other Cas proteins, restores CRISPR activity in a Δ*htpG* strain. BW25113Δ*hns* Δ*htpG* λ*cI857*-kan *araB*::T7-RNAp was transformed with the indicated pCA24N-based plasmids, containing the indicated genes under the lac promoter (19). Cells were made competent and transformed with plasmids encoding spacers against the λ prophage (pAC-478) or control spacers (pAC-477) as described in *Materials and Methods*. The relative efficiency of transformation was calculated by dividing the number of obtained pAC-478 or pAC-477 transformants by the number of pAC-477 transformants. Results are presented as the average ± SD of three independent experiments.

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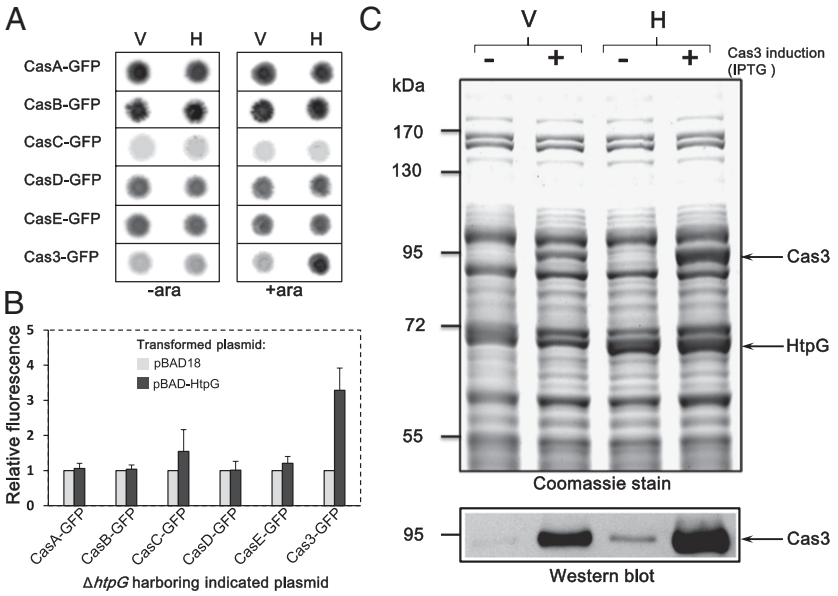

Fig. 5. Cas3 steady-state level increases with coexpression of HtpG. (*A*) BW25113Δ*htpG* was cotransformed with either pBAD18 (V) or pBAD-HtpG (H) along with pCA24N-based plasmids encoding the indicated protein fused to GFP. Bacteria were replicated on agar plates with the appropriate antibiotics, with (+ara) or without (−ara) 0.2% L-arabinose, for induction of HtpG expression as described in *Materials and Methods*. The plates were scanned for GFP fluorescence using a Typhoon 9400. A representative image from three independent experiments with similar results is shown. (*B*) Results of the L-arabinose–induced samples were quantified by ImageJ-based densitometry. Results are the average ± SD of three independent experiments. (*C*) BW25113Δ*htpG* was transformed with either pBAD18 (V) or pBAD-HtpG (H) along with pCA24N-Cas3 (no GFP, from ASKA library). Cell extracts were prepared as described in *Materials and Methods*, and 20 μg of total protein was loaded on a 10% wt/vol polyacrylamide gel. (*Upper*) Protein separation by gel electrophoresis was carried out, followed by Coomassie blue staining and scanning. The gel is representative of three independent experiments showing similar results. (*Lower*) Western blot analysis using an antibody against the His-tag preceding Cas3.

and destruction of the target DNA/RNA. Some of these steps, such as crRNA maturation by the CasABCDE proteins in the *E. coli* system, are fairly well understood (3), whereas others, such as sensing DNA entry, are poorly understood (22). Our screen to identify genes participating in the above steps contributes an additional piece of information to one of the interference steps in *E. coli* by establishing an important role for HtpG in maintaining levels of Cas3, a crucial component in CRISPR interference.

HtpG, a homolog of the ubiquitous eukaryotic Hsp90, is a protein that has been conserved from bacteria to humans (23), suggesting it might play a role in prokaryotic CRISPR systems other than the *E. coli* system as well. The precise role of the *E. coli* HtpG has remained elusive, compared with our detailed knowledge of its eukaryotic homolog. It is ubiquitous in *E. coli*, and its level is elevated following heat stress (hence, its name). HtpG-deletion mutants suffer from slow growth at high temperatures, as well as slight protein aggregation (24). HtpG has been shown to be involved with two client proteins, the ribosomal protein L2 and a linker polypeptide in the phycobilisome of *Synechococcus elongatus*, in an activity that characterizes it as a molecular chaperone (25–27). Recently, HtpG has also been shown to promote reactivation of heat-denatured luciferase in a reaction that requires the activity of another *E. coli* chaperone, DnaK (26).

We demonstrate that overexpression of Cas3 can relieve the dependency of CRISPR-system activity on HtpG, indicating that Cas3 is a limiting factor for this activity in the absence of HtpG. Furthermore, we show that coexpression of HtpG with Cas3 increases steady-state levels of the latter. Our results are not the first to suggest that HtpG is involved in stabilizing large protein complexes, and they are in line with a recent study showing that HtpG is required to stabilize the *S. elongatus* phycobilisome, a large protein complex, by specifically interacting with some of its components (27). Thus, in addition to the unique role of HtpG in the activity of the CRISPR system, we shed further light on its role as a chaperone by identifying its stabilization of another putative client protein.

Analyses of bacterial databases to identify a direct link between co-occurrence of Cas3 and HtpG are complicated by the lack of a straightforward way of identifying functional vs. nonfunctional CRISPR systems, because such co-occurrence is predicted based on the functionality of both proteins together. Nevertheless, basic analysis of bacterial databases using EcoCyc (28) indicates that of 33 *E. coli* strains, only 2 lack an *htpG* ortholog, and these 2 also lack *cas3*. All 7 examined *Salmonella* strains carry *htpG*, and 5 of them also encode *cas3*. Furthermore, all examined *Mycoplasma* and *Staphylococcus* strains and most of the *Streptococcus* strains lack both *htpG* and *cas3*. On the other hand, all the examined *Lactobacillus* strains encode *cas3* but lack *htpG*. In addition, ∼50% of the archaea encode *cas3* (29), but almost all of them lack *htpG* (30). This basic analysis indicates that co-occurrence of HtpG and Cas3 is not easily predictable and that interactions between these two proteins must be experimentally tested in each strain. Lack of co-occurrence between these two proteins in all systems is not surprising because Cas3 has low sequence similarity among different strains, suggesting different modes of action in different systems. It is tempting to speculate that in organisms lacking HtpG, such as the archaea, another non-Cas protein interacts with Cas3. The complexity of interactions of non-Cas proteins with the CRISPR system components is also evidenced by LRP, which regulates the *Salmonella* but not the *E. coli* CRISPR system, whereas other proteins (H-NS and LeuO) have similar regulatory functions in these strains.

Although thorough, genetic searches based on transposon insertions, such as those performed here, have some limitations. For instance, they cannot be relied on to identify genes that are essential for both the function of the CRISPR system and the viability of the transposed *E. coli* strain, because these genes, by definition, cannot be fully disrupted but only partially mutated in nonessential residues. Nevertheless, the experimental details provided herein may serve as a basis for a similar search for essential *E. coli* non-*cas* genes that are required for CRISPR-system activity. Such searches might make use of other mutagenesis methods generating, for example, point mutations. It is reasonable to assume that some essential *E. coli* genes also participate in the activity of the CRISPR system, and such searches may therefore prove valuable.

To summarize, we identified HtpG as a non-Cas protein involved in CRISPR activity using a unique screening method tailored to the CRISPR system. In future studies, we will refine and modify our screening method to identify additional regulatory proteins in this system. We believe that regulatory genes thus identified and characterized will shed more light on the regulation of this intriguing system.

Materials and Methods

Reagents, Strains, Plasmids, and Construction Procedures. Reagents, strains, plasmids, and construction of strains and plasmids are detailed in *SI Materials and Methods*.

CRISPR-Dependent Restriction of Transformation. *E. coli* BW25113Δ*hns araB*::T7-RNAp λ-*cI*857-kan was diluted 1:50 from an overnight culture and aerated at 32 °C in LB medium containing 25 μg/mL kanamycin to an OD_{600} of

0.4–0.6. Bacteria were then centrifuged at 4,000 × *g* at 4 °C, the supernatant was disposed of, and the bacteria were resuspended in 1 mL of ice-cold double-distilled water (DDW) and transferred to a 1.5-mL Eppendorf tube. The cells were spun down for 1 min at 13,000 × *g* at 4 °C. After an additional washing step, the cells were suspended in 150 μL of ice-cold DDW.

Cells (50 μL) were then mixed in an ice-cold 0.2-mm electroporation cuvette (Bio-Rad) with 20 ng of pWUR478 or pWUR477. The mixture was pulsed in a Bio-Rad micropulser at 200 Ω, 25 μF, 1.8 kV. 2YT broth (1.6% wt/vol tryptone, 1.0% yeast extract, 0.5% NaCl) (0.2 mL) containing 0.2% L-arabinose and 0.1 mM IPTG was immediately added, and the cells were aerated for 1 h at 32 °C. Various dilutions were plated on LB-agar plates supplemented with 25 μg/mL kanamycin, 35 μg/mL chloramphenicol, 0.2% L-arabinose, and 0.1 mM IPTG. Plates were incubated overnight at 32 °C. Colonies emerging on selection plates were counted. Efficiency of transformation of pWUR478 plasmid was compared with that of the control pWUR477 plasmid. In several cases, the assay was carried out using specific deletion mutants (e.g., Δ*htpG*) complemented with various plasmids (e.g., a plasmid encoding *htpG* or each of the *cas* genes), as indicated. When resistance was incompatible with the other plasmids, a different set of plasmids, pAC-477 and pAC-478 harboring similar spacers but with an ampicillin rather than chloramphenicol resistance marker, was used as indicated. In experiments in which *cas* genes were supplied on plasmids, no IPTG was added because expression also occurs without the inducer (21).

CRISPR-Dependent Restriction of Lysogenization. Overnight cultures of *E. coli* Δ*hns araB*::T7-RNAp harboring pWUR478 or pWUR477 were diluted 1:25 in LB medium containing 35 μg/mL chloramphenicol, 10 mM $MgSO_4$, and 0.2% maltose. When the culture reached an OD_{600} of 0.6–0.8, 100 μL was mixed with 10 μL of phage λ carrying a kanamycin resistance gene in a 1.5-mL tube and incubated at room temperature for 20 min. Bacteria were then inoculated on LB-agar plates supplemented with 0.2% maltose, 0.1 mM IPTG, 10 μg/mL tetracycline, 35 μg/mL chloramphenicol, and 50 μg/mL kanamycin, and incubated overnight at 32 °C. Lysogenization assays of *E. coli* BW25113Δ*hns* Δ*htpG* were carried out at 10-fold larger cell and phage volumes because of low lysogenization efficiency of this strain compared with BW25113Δ*hns*. Nevertheless, absence of *htpG* does not, by itself, reduce lysogenization efficiency by λ (relative efficiency of lysogenization of BW25113Δ*htpG* compared with BW25113 at a multiplicity of infection of ∼1 is 1.25 ± 0.08), indicating that HtpG does not specifically affect λ lysogenization (because of effects on, e.g., the temperature-sensitive CI857).

Quantification of Cas Proteins Following Coexpression with HtpG. To examine the steady-state level of Cas3 following HtpG overexpression, BW25113Δ*htpG* and either pBAD18 (control) or pBAD-HtpG, along with pCA24N-Cas3-GFP [ASKA library (21)] or other specified control plasmids from the ASKA library, were cotransformed into *E. coli*. Cells were grown overnight in LB supplemented with the appropriate antibiotics at 37 °C. Aliquots of 0.1 mL were transferred into a 96-well microtiter plate, and the bacteria were then replicated on an agar plate with the appropriate antibiotics, with or without 0.2% L-arabinose for induction of HtpG expression. The pCA24N-based plasmids contain a leaky lac promoter, and there is a sufficient amount of GFP for monitoring purposes, even without IPTG induction (21). After overnight growth on plates, the replicated bacteria were scanned for GFP fluorescence using a Typhoon 9400 (GE Healthcare) (488-nm excitation wavelength, 520-nm emission wavelength). The presented image was digitally optimized for background omission and uniform expression levels. Results were quantified using an ImageJ-based (National Institutes of Health) densitometer. To analyze protein quantities by gel electrophoresis, *E. coli* BW25113Δ*htpG* was transformed with either pBAD18 or pBAD-HtpG along with pCA24N-Cas3 (no GFP, from ASKA library). Cells were grown to the logarithmic phase and induced with 0.2% L-arabinose. After 20 min of induction, the cultures were divided and IPTG was added to half of the sample. After an additional 1.5 h of growth, the cells were transferred to ice. Cell pellets (∼5×10^9 cells) were suspended in 1 mL of buffer A [1 mM EDTA, 50 mM Tris·HCl (pH 8), 100 mM NaCl) containing protease inhibitor mixture (Roche). Protein extracts were prepared by three cycles of brief 10-s sonication at 20-s intervals on ice. Equal amounts of total protein (20 μg) were separated on a 10% wt/vol polyacrylamide gel and stained with Coomassie blue. Western blotting was carried out using anti–His-tag antibody (Sigma) according to the manufacturer's instructions.

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