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GENETICS AND MOLECULAR BIOLOGY

Histone-like Nucleoid-Structuring Protein (H-NS) Paralogue StpA Activates the Type I-E CRISPR-Cas System against Natural Transformation in *Escherichia coli*

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ABSTRACT Working mechanisms of CRISPR-Cas systems have been intensively studied. However, far less is known about how they are regulated. The histone-like nucleoid-structuring protein H-NS binds the promoter of *cas* genes (P_{cas}) and suppresses the type I-E CRISPR-Cas system in *Escherichia coli*. Although the H-NS paralogue StpA also binds P_{cas}, its role in regulating the CRISPR-Cas system remains unidentified. Our previous work established that *E. coli* is able to take up double-stranded DNA during natural transformation. Here, we investigated the function of StpA in regulating the type I-E CRISPR-Cas system against natural transformation of *E. coli*. We first documented that although the activated type I-E CRISPR-Cas system, due to *hns* deletion, interfered with CRISPR-Cas-targeted plasmid transfer, *stpA* inactivation restored the level of natural transformation. Second, we showed that inactivating *stpA* reduced the transcriptional activity of P_{cas}. Third, by comparing transcriptional activities of the intact P_{cas} and the P_{cas} with a disrupted H-NS binding site in the *hns* and *hns stpA* null deletion mutants, we demonstrated that StpA activated transcription of *cas* genes by binding to the same site as H-NS in P_{cas}. Fourth, by expressing StpA with an arabinose-inducible promoter, we confirmed that StpA expressed at a low level stimulated the activity of P_{cas}. Finally, by quantifying the level of mature CRISPR RNA (crRNA), we demonstrated that StpA was able to promote the amount of crRNA. Taken together, our work establishes that StpA serves as a transcriptional activator in regulating the type I-E CRISPR-Cas system against natural transformation of *E. coli*.

IMPORTANCE StpA is normally considered a molecular backup of the nucleoid-structuring protein H-NS, which was reported as a transcriptional repressor of the type I-E CRISPR-Cas system in *Escherichia coli*. However, the role of StpA in regulating the type I-E CRISPR-Cas system remains elusive. Our previous work uncovered a new route for double-stranded DNA (dsDNA) entry during natural transformation of *E. coli*. In this study, we show that StpA plays a role opposite to that of its paralogue H-NS in regulating the type I-E CRISPR-Cas system against natural transformation of *E. coli*. Our work not only expands our knowledge on CRISPR-Cas-mediated adaptive immunity against extracellular nucleic acids but also sheds new light on understanding the complex regulation mechanism of the CRISPR-Cas system. Moreover, the finding that paralogues StpA and H-NS share a DNA binding site but play opposite roles in transcriptional regulation indicates that higher-order compaction of bacterial chromatin by histone-like proteins could switch prokaryotic transcriptional modes.

KEYWORDS CRISPR-Cas system, *Escherichia coli*, histone-like proteins, natural transformation, transcriptional regulation

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To cope with environmental stresses, bacteria and archaea acquire exogenous genes from genomes and plasmids of other strains via horizontal gene transfer (HGT) (1–3). Simultaneously, prokaryotes are challenged by invasion of harmful genes. To reduce the risk of acquisition of exogenous DNA, prokaryotes have evolved both innate immunity that is mediated by restriction-modification systems (4) and adaptive immunity that is mediated by CRISPR-Cas systems (5–13). The CRISPR-Cas system, consisting of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (*cas*) genes, is widespread in genomes of prokaryotes (5, 6, 14–17). According to the organization of effector modules, CRISPR-Cas systems are grouped into two classes, and each class is further divided into three types (5, 18). Class 1 CRISPR-Cas systems (including types I, III, and IV) are considered the ancestral system, whereas class II CRISPR-Cas systems (including types II, V, and VI) are most widely applied in genome editing of a wide range of organisms (6). In general, the CRISPR-Cas-mediated adaptive immunity involves three processes (adaptation, expression, and interference) (6). Two conserved proteins, Cas1 and Cas2, mediate the adaptation process during which new spacers are formed in the CRISPR region near the leader sequence (19–21). In the expression process, a group of Cas proteins are produced, accompanied by transcription of the CRISPR array into the precursor CRISPR RNA (pre-crRNA), which is subsequently processed into small mature crRNA by CasE or RNase III (10, 22). The interference process involves crRNA-directed cleavage of the targeted DNA by Cas nucleases within the ribonucleoprotein complex composed of Cas proteins and crRNA (12, 14, 23–27).

Escherichia coli K-12 is equipped with a type I-E CRISPR-Cas immune system that consists of eight *cas* genes (*cas1*, *cas2*, *cas3*, and *casABCDE*) and two CRISPR arrays (CRISPR I and CRISPR II) (10, 16, 28). Genes *casABCDE* encode Cas proteins that form the Cascade complex. The Cascade-Cas3 complex and the RecBCD nuclease generate single-stranded DNAs (ssDNAs) that are used for naive and primed adaptation (29–32). Protospacer-adjacent motif (PAM)-containing ssDNA strands are captured by Cas1 and Cas2, which then anneal complementary strands (20, 21, 33, 34). Prespacer precursors are trimmed for integration, with the mature PAM-derived end being integrated at the spacer side of the CRISPR, followed by duplicating repeats as a result of filling of gaps (35, 36). The CRISPR array is transcribed into pre-crRNA and further processed into mature crRNA by CasE (10). Together with crRNA, CasABCDE form a CRISPR ribonucleoprotein (crRNP) complex which can be directed to the targeted DNA region (10, 37, 38). The conformational changes in the Cascade complex lock the R loop, which is formed by hybridization of the crRNA and the target strand of the double-stranded DNA (dsDNA), leading to the nontarget strand being replaced (39–42). Then, the nuclease Cas3, which is stabilized by the heat shock protein 90 homologue HtpG (43), is recruited to the target strand to cleave DNA (39, 44).

The working mechanisms of CRISPR-Cas systems have been intensively studied (6–15, 19–27, 29–44) whereas less is known about the mechanisms regulating them (45). Normally, the type I-E CRISPR-Cas system of *E. coli* is suppressed (28, 46). It can be activated under certain physiological conditions (i.e., in medium supplemented with glucose) and confer immunity to bacteriophage P1 (47). Expression of the type I-E CRISPR-Cas system is regulated by multiple regulators in *E. coli* and *Salmonella enterica* (28, 46–48). H-NS, a histone-like nucleoid-structuring protein that inhibits gene transcription, represses expression of *cas* genes by binding to the upstream region of the promoter of the *casABCDE* operon (P_{cas}) (28). It also reduces the amount of crRNA through inhibiting transcription of the CRISPR array and limiting pre-crRNA processing due to insufficient CasE (28). H-NS also partially suppresses transcription of *cas3* (49), which encodes the nuclease for DNA cleavage (50, 51). Temperature of incubation is important to maintain a sufficient amount of Cas3 for DNA interference. Incubating the *hns* mutant at 30°C improves the immunity conferred by the CRISPR-Cas system against λ phage infection (49). Overexpression of LeuO, the LysR-type regulator, can activate transcription of *casABCDE* through antagonizing H-NS-mediated transcriptional repression and increase the amount of crRNA (46). The carbon catabolism regulator cAMP-

cAMP receptor protein (cAMP-CRP) complex negatively regulates type I-E CRISPR-Cas system by competing with LeuO for the binding site of P_{cas} (47) whereas CRP plays an opposite role in *Pectobacterium atrosepticum* (52). It activates the type I-F CRISPR-Cas system in that species.

The H-NS paralogue StpA, considered a backup for H-NS (53), can fill the role of H-NS in repressing transcription of genes when H-NS is absent (54, 55). At the transcriptional level, H-NS and StpA can suppress expression of each other (54). In some cases, StpA and H-NS can serve as transcriptional activators and stimulate transcription of the maltose regulon and *crp* via the stringent response regulator ppGpp (56, 57). Normally, H-NS and StpA bind to the same region of promoters, and StpA has a 4- to 6-fold-greater affinity for DNA than H-NS (58).

As a main driving force for evolution, natural transformation has been found widespread in prokaryotes (59). In general, during natural bacterial transformation, a single-stranded DNA (ssDNA) from extracellular dsDNA is pulled into the cytoplasm via a highly conserved DNA uptake machinery that is assembled in the cell membrane (59–61). CRISPR-Cas systems limit ssDNA uptake during natural transformation of both *Streptococcus pneumoniae* and *Neisseria meningitidis* (62, 63). Our previous work has shown that stationary-phase *E. coli* is able to develop natural competence and allows dsDNA to enter the cytoplasm (64–67). Natural transformation of *E. coli* is regulated by the transcriptional regulators RpoS and cAMP-CRP complex (66, 68). Although CRISPR-Cas-mediated immunity to bacteriophage infection has been well documented in *E. coli* (10, 47), the potential effect of the CRISPR-Cas system on limiting dsDNA uptake during natural transformation has not yet been investigated. It has been demonstrated that StpA is able to bind the promoter of the *cas* operon in *E. coli* (28). However, the role of StpA in regulating expression of the *cas* genes remains unknown. In this study, we explored the potential role of StpA in regulating the type I-E CRISPR-Cas system of *E. coli* and CRISPR-Cas-mediated immunity to dsDNA transfer during natural transformation. Our data showed that StpA played a role opposite to that of H-NS: it served as an activator in regulating the type I-E CRISPR-Cas system and enhanced immunity against DNA transfer during natural transformation of *E. coli*.

RESULTS

StpA is required for the immunity against plasmids that is conferred by the CRISPR-Cas system in an *hns* null mutant. H-NS suppresses expression of the type I-E CRISPR-Cas system in *E. coli* (28). Although the H-NS paralogue StpA was shown to bind the promoter of the *cas* operon (28), its role in regulating the type I-E CRISPR-Cas system remains unexplored. We attempted to characterize the role of StpA in regulating the type I-E CRISPR-Cas system against natural transformation in *E. coli*. To this end, we constructed *stpA* null mutants in *hns*-deficient and hns^+ *E. coli* strains. By using λ-Red-mediated recombineering, *hns* and *stpA* single- and double-deletion mutants were constructed (see Fig. S1 in the supplemental material). Construction and genotype examination of these mutants are shown in Fig. S1 and S2. A Western blot assay showed that StpA was indeed absent in the *hns stpA* null mutant (Fig. S1D). Morphologies of constructed strains were observed under a phase-contrast microscope. Similar to a previous observation (69), both *hns* and *hns stpA* null mutants were obviously longer than their wild-type (WT) parent and the *stpA* null mutant (Fig. S2). The *hns stpA* null mutant grew remarkably more slowly than the *hns* null mutant (Fig. S8A), in line with previous reports (54, 70).

To evaluate the DNA interference by the type I-E CRISPR-Cas system, we constructed the plasmid pCR1, which can be targeted by the CRISPR-Cas system of *E. coli*. To ensure the sensitivity of pCR1 to the CRISPR-Cas system, the DNA fragment (CR1) with four protospacer-adjacent motif (PAM)-containing DNA regions was chemically synthesized and cloned into pUC57 (Fig. S3). DNA interference by the type I-E CRISPR-Cas system was evaluated by levels of natural transformation (Fig. 1) and by the viability of transformants after transformation (Fig. S9). Natural transformation was performed by spreading the mixture of plasmid and the cell culture onto an LB plate containing 5%

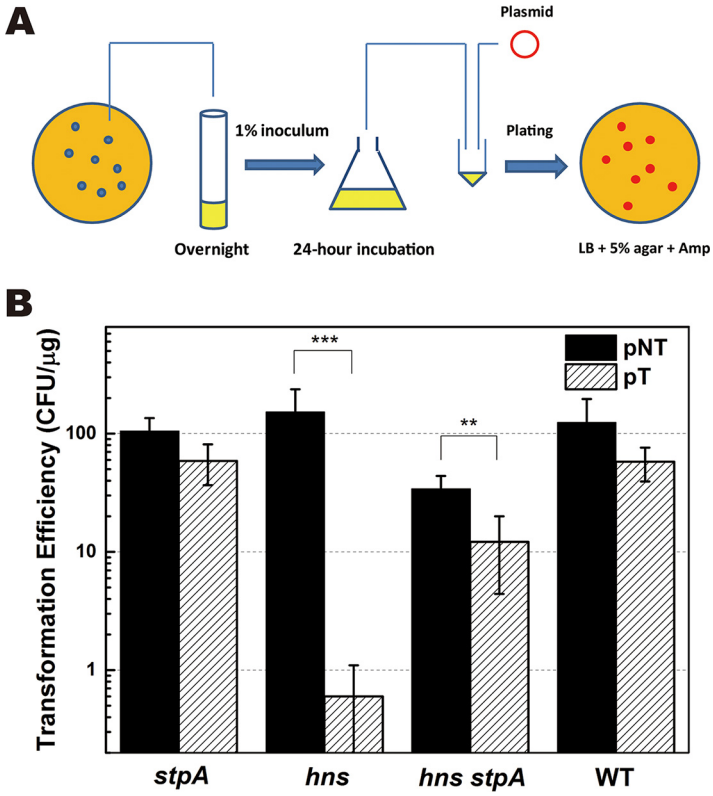

FIG 1 Effect of *stpA* inactivation on natural transformation with CRISPR-Cas-targeted plasmid. (A) Schematic representation of natural transformation of *E. coli* for evaluating the CRISPR-Cas-mediated immunity against extracellular DNA. (B) Natural transformation was performed by spreading the mixture of the 24-h-incubated *E. coli* culture and plasmid onto LB plates containing 5% (wt/vol) agar supplemented with ampicillin (100 μg ml^{-1}). Levels of natural transformation with the CRISPR-Cas-targeted plasmid pCR1 (pT) or nontargeted plasmid pDsRED (pNT) were measured in Δ*hns*, Δ*stpA*, Δ*hns* Δ*stpA*, and WT strains. Cell viability of each transformant was measured (see Fig. S9 in the supplemental material). Data are shown as means ± standard deviations ($n = 3$). Statistical significance was determined using a two-tailed Student's *t* test (**, $P \leq 0.01$; ***, $P \leq 0.005$).

(wt/vol) agar (Fig. 1A). Levels of natural transformation were compared with those of the CRISPR-Cas-targeted plasmid (pCR1) and nontargeted plasmid (pDsRED) carrying a red fluorescence gene whose sequence cannot be recognized by the CRISPR-Cas system in *E. coli*. Both pCR1 (GenBank accession number MT437282 and Addgene plasmid repository catalog no. 154270 [https://www.addgene.org/154270/]) and pDsRED are derivatives of pUC19 (Table 1), and their sequences are available in the supplemental material. With pDsRED as the donor plasmid, transformation efficiencies of the *stpA* and *hns* null mutants and their WT parent were similar (~100 CFU/μg) (Fig. 1B). In contrast, with pCR1 as the donor plasmid, although the transformation efficiency was not significantly reduced in the *stpA* null mutant with respect to that of the WT strain, it was reduced by more than 200-fold in the *hns* null mutant (Fig. 1B). The results demonstrate that activation of the type I-E CRISPR-Cas system in the *hns* null mutant strongly inhibits plasmid transfer during natural transformation. We observed that transformation of the *hns stpA* null mutant with pCR1 as the donor plasmid reduced the level of transformation by less than 3-fold (Fig. 1B). Although *stpA* inactivation restored transformation in the *hns* null mutant, the level of transformation in the *hns stpA* null mutant was still lower than that in the WT strain, indicating that StpA is partially responsible for activating CRISPR-Cas-mediated DNA interference in the *hns* mutant.

We analyzed DNA interference in transformants of the *hns* and *hns stpA* null mutants after transformation. Growth rates of transformants of mutants and their wild-type parent were evaluated. In liquid LB broth, no significant difference in cell growth was observed in WT and the *hns* null mutant carrying either the CRISPR-Cas-targeted

TABLE 1 Strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
MC4100	F$^-$ λ$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA deoC1 ptsF25 rbsR flbB5301*	66
ZJUTCBB0015	MC4100 Δ*stpA*::*cat*, Cm^r	This study
ZJUTCBB0016	MC4100 Δ*hns*::*kan*, Kan^r	This study
ZJUTCBB0017	MC4100 Δ*stpA*::*cat* Δ*hns*::*kan*, Kan^r Cm^r	This study
ZJUTCBB0018	MC4100 Δ*stpA* pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0019	MC4100 Δ*hns* pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0020	MC4100 Δ*stpA* Δ*hns* pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0021	MC4100 pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0022	MC4100 Δ*hns* pSUStpA pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0023	MC4100 Δ*hns* pSUHNS pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0024	MC4100 Δ*hns* pSU19 pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0025	MC4100 Δ*stpA* Δ*hns* pSUStpA pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0026	MC4100 Δ*stpA* Δ*hns* pSUHNS pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0027	MC4100 Δ*stpA* Δ*hns* pSU19 pGLO-P$_{cas}$-GFP	This study
ZJUTCBB0028	MC4100 Δ*hns* pSUStpA pGLO-P$_{cas}$*-GFP	This study
ZJUTCBB0029	MC4100 Δ*hns* pSUHNS pGLO-P$_{cas}$*-GFP	This study
ZJUTCBB0030	MC4100 Δ*hns* pSU19 pGLO-P$_{cas}$*-GFP	This study
ZJUTCBB0031	MC4100 Δ*stpA* Δ*hns* pSU-P$_{BAD}$-*stpA*	This study
ZJUTCBB0032	MC4100 Δ*stpA* Δ*hns* pSU-P$_{BAD}$-*hns*	This study
ZJUTCBB0033	MC4100 Δ*stpA* pSU-P$_{J23119}$-GFP	This study
ZJUTCBB0034	MC4100 Δ*hns* pSU-P$_{J23119}$-GFP	This study
ZJUTCBB0035	MC4100 Δ*stpA* Δ*hns* pSU-P$_{J23119}$-GFP	This study
ZJUTCBB0036	MC4100 pSU-P$_{J23119}$-GFP	This study
Plasmids		
pKD46	Expressing Red recombinase, *repA101*(Ts) *oriR101*, Amp^r	94
pCP20	Expressing FLP recombinase, *repA101*(Ts) pSC101 *ori*, Cm^r, Amp^r	94
pKD3	R6K γ *ori*, Ts replicon, Cm^r	94
pKD4	R6K γ *ori*, Ts replicon, Kan^r	94
pSU19	p15A replicon, Cm^r	Lab reserve
pSUStpA	pSU19 derivative, *stpA* expressed with the original promoter, Cm^r	This study
pSUHNS	pSU19 derivative, *hns* expressed with the original promoter, Cm^r	This study
pGLO-P$_{cas}$-*gfp*	pGLO derivative, *gfp* expressed with P$_{cas}$, Amp^r	This study
pGLO-P$_{rscA}$-*gfp*	pGLO derivative, *gfp* expressed with P$_{rscA}$, Amp^r	This study
pGLO-P$_{cas}$*-*gfp*	pGLO-P$_{cas}$-*gfp* with mutated P$_{cas}$	This study
pSU-P$_{BAD}$-*gfp*	pSU19 derivative, *gfp* expressed with P$_{BAD}$, Cm^r	This study
pSU-P$_{BAD\text{-}s}$*tpA*	pSU19 derivative, *stpA* expressed with P$_{BAD}$, Cm^r	This study
pSU-P$_{BAD\text{-}h}$*ns*	pSU19 derivative, *hns* expressed with P$_{BAD}$, Cm^r	This study
pSU-P$_{J23119}$-*gfp*	pSU19 derivative, *gfp* expressed with P$_{J23119}$ which is a constitutive promoter, Cm^r	Lab reserve
pUC57	pUC19 derivative, Amp^r	Lab reserve
pT/pCR1	pUC19 derivative, carrying a DNA fragment targeted by the type I-E CRISPR-Cas system of *E. coli*, Amp^r	This study
pNT/pDsRED	pUC19 derivative, expressing red fluorescence protein, Amp^r	Lab reserve
pET28a-StpA-His	pET28a derivative, StpA expressed by T7/*lac* promoter, Kan^r	This study
pET28a-HNS-His	pET28a derivative, HNS expressed by T7/*lac* promoter, Kan^r	This study

[a]Ts, temperature sensitive.

plasmid pCR1 or the nontargeted plasmid pDsRED (Fig. S9A) whereas a significant growth defect in LB broth was observed in the *hns* and *hns stpA* null mutants carrying pCR1 compared with those carrying pDsRED (Fig. S9A). A growth defect was also observed in the *hns* and *hns stpA* null mutants carrying pCR1 and grown on LB-agar plates. By using drop plating and plate streaking methods, we showed that both the *hns* and *hns stpA* null mutants carrying pDsRED grew well on plates, but the mutants carrying pCR1 grew poorly under the same conditions (Fig. S9B and C). To quantify DNA interference after natural transformation, plasmid loss was evaluated by counting the number of viable cells having (ampicillin positive [Amp$^+$]) or losing (Amp$^-$) the targeted plasmid. In both the *hns* and *hns stpA* null mutants carrying pCR1, the number of Amp$^+$ viable counts in the overnight-grown culture was ~1,000-fold smaller than that of Amp$^-$ viable counts (Fig. S9D), suggesting that the targeted plasmid could have suffered significant damage in these mutants. Together, these results clearly demonstrate that StpA is required for CRISPR-Cas-mediated DNA interference in plasmid transformation of *E. coli*.

To evaluate the potential effect of StpA on DNA interference during chemical transformation, we compared transformation efficiencies in the *hns* and the *hns stpA* null mutants. The CRISPR-Cas-targeted plasmid pCR1 transformed both the *stpA* null mutant and its wild-type parent with a high efficiency (1×10^5 to 2×10^5 CFU/μg). In contrast, it transformed the *hns* null mutant with an efficiency of ~4 CFU/μg. Inactivating *stpA* in the *hns* null mutant increased transformation efficiency by more than 10-fold, reaching ~50 CFU/μg. Nevertheless, the efficiency of transformation with pCR1 was more than 1,000-fold lower than that with the nontargeted plasmid pDsRED in the *hns stpA* mutant, reflecting that StpA weakly stimulated CRISPR-Cas-mediated DNA interference during chemical transformation of the *hns* mutant.

StpA is required for transcription of *cas* genes in an *hns* null mutant. H-NS was reported to be able to both suppress and activate gene expression (55, 56, 71) while StpA serves as a backup for H-NS, fulfilling the role of H-NS when it is absent (54, 55). Since deleting *stpA* remarkably reduced the activity of the CRISPR-Cas system against natural transformation in the *hns* mutant (Fig. 1B), we suspected that H-NS and StpA played opposite roles in regulating the type I-E system in *E. coli*. To test this, we quantified transcription of the *cas* operon in *E. coli* strains with single or double deletions of *hns* and *stpA*. We observed that inactivating *stpA* in an *hns* null mutant further increased transcription of an H-NS-suppressed gene, *bglG*, by more than 5-fold (Fig. 2A), and further decreased transcription of an H-NS-activated gene, *malE*, by about 7-fold (Fig. 2B). These results are in good accordance with previous reports (55, 56, 71). Transcription of the *cas* operon was reported to be increased by inactivating *hns* (55, 56, 71). We also observed that transcription of a gene in the *cas* operon (*casB*) was increased by more than 6-fold in an *hns* null mutant with respect to the level in the wild type (Fig. 2C). However, transcription of *casB* was increased by only about 3-fold in the *hns stpA* null mutant, about 2-fold lower than that in the *hns* null mutant (Fig. 2C). The data clearly show that StpA is required for high-level transcription of the *cas* operon.

To further explore the role of StpA in regulating the type I-E CRISPR-Cas system, we monitored transcription of the *cas* operon during cell growth by fusing the promoter of the *cas* operon with the green fluorescent protein (GFP) gene (P_{cas}-*gfp*) (Fig. 3A and Fig. S4A). Inactivation of *hns* was reported to relieve transcriptional repression of *cas* genes (28). As expected, strong green fluorescence was observed in an *hns* null mutant whereas it was much weaker in the WT parent (Fig. 3B). The H-NS-suppressed promoter P_{rcsA} was reported to be activated by mutating *hns* (72). We also observed that relative intensities of green fluorescence with the transcriptional fusion construct of P_{rcsA}-*gfp* were remarkably higher in the *hns* and *hns stpA* null mutants than those in the *stpA* null mutant and the WT strain (Fig. S11). We noticed that transcription of the *cas* operon was low in the *stpA* null mutant (Fig. 3B), in line with the previous observation that inactivation of *stpA* did not significantly affect transcription of *cas* genes (28). After 12 h of incubation in LB medium, green fluorescence was ~3-fold lower in the *hns stpA* null mutant than that in the *hns* null mutant (Fig. 3B), indicating that StpA played a positive role in regulating the *cas* operon. In contrast, expression levels of GFP with a constitutive promoter in the *hns* and *hns stpA* null mutant were not higher than those in the *stpA* null mutant and WT (Fig. S12). A stimulation effect of StpA on transcription of *cas* genes was more evident when cells were grown in M9 minimal medium. Throughout incubation, relative intensities of green fluorescence were ~3-fold lower in the *hns stpA* null mutant than in the *hns* null mutant (Fig. 3C). Taken together, these results clearly show that StpA activates transcription of *cas* genes in the absence of *hns*.

StpA activates transcription of *cas* genes by binding to the same site as H-NS in the promoter of the *cas* operon. StpA often shares the same DNA binding site (DBS) as H-NS in the wild-type cell. But DNA binding profiles of StpA were different in wild-type and *hns* mutant cells (73). *In vitro* experiments showed that both H-NS and StpA bound P_{cas}, and the corresponding DBS of H-NS in P_{cas} was predicted (28). We asked whether H-NS and StpA bound to the same site in regulating the *cas* operon. To confirm that H-NS suppressed the *cas* operon by binding to the predicted DBS, we

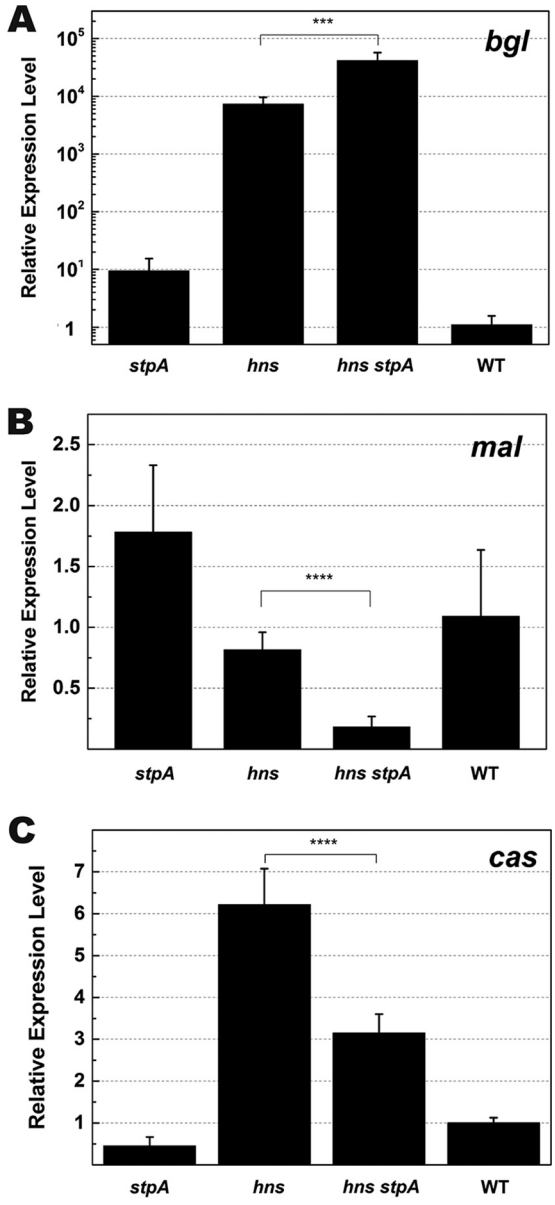

FIG 2 Quantification of transcriptional levels of the *cas* operon in the absence of H-NS and/or StpA. To quantify gene transcription, the cDNA was synthesized with RNA isolated from mutants (Δ*stpA*, Δ*hns*, and Δ*hns* Δ*stpA* strains) and their wild-type parent. Relative expression levels of *bgl* (A), *mal* (B), and *cas* (C) operons in these strains were measured with qPCR using 16S RNA as the reference. Primers used for qPCR are listed in Table 2. Each column represents results from an average of 4 samples. Error bars denote standard deviation. Statistical significance was determined using a two-tailed Student's *t* test (***, $P \leq 0.005$; ****, $P \leq 0.001$).

replaced the four conserved A/T residues with G/C in the putative DBS and constructed the promoter P_{cas}* (Fig. 4A and Fig. S5). Transcriptional activities of P_{cas} and P_{cas}* were compared when H-NS was expressed from a multicopy-number plasmid (Fig. S6). By using GFP as the reporter, we observed that activities of both P_{cas} and P_{cas}* were strong in an *hns* null mutant carrying the empty vector pSU19 (Fig. 4B). Although expressing H-NS on pSU19 reduced the activity of P_{cas} by 4.41-fold in the *hns* null mutant, it reduced the activity of P_{cas}* by only 1.84-fold (Fig. 4B). Moreover, the activity of P_{cas}* was higher than that of P_{cas} in both wild-type and *stpA* mutant cells (Fig. S15). These results demonstrated that mutations in P_{cas}* relieved transcriptional suppression by H-NS, indicating that H-NS indeed inhibited transcription of the *cas* operon by binding to the predicted DBS.

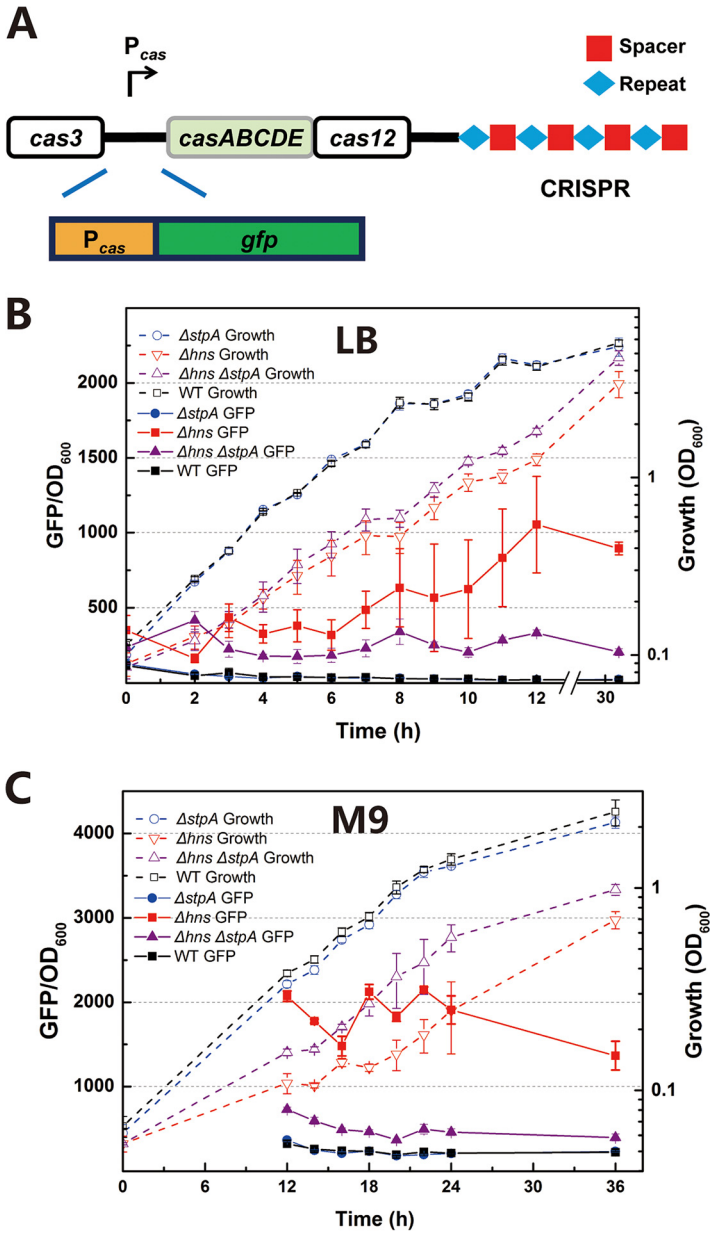

FIG 3 Effect of *stpA* inactivation on transcription of the *cas* operon. (A) The reporter plasmid pGLO-P_{cas}-*gfp* was constructed by fusing the promoter of the *cas* operon (P_{cas}) with *gfp* to quantify the transcriptional level of *cas* genes. (B) Expression levels of P_{cas}-*gfp* were measured in Δ*hns*, Δ*stpA*, Δ*hns* Δ*stpA*, and WT strains that were grown in LB broth. (C) Expression levels of P_{cas}-*gfp* were measured in Δ*hns*, Δ*stpA*, Δ*hns* Δ*stpA*, and WT strains that were grown in M9 minimal medium supplemented with 0.32% (wt/vol) fructose as the carbon source. Dashed lines represent optical growth, and solid lines represent activity of P_{cas}. All experiments were performed at 30°C with shaking at 180 rpm. Refer to supplemental material for details about construction of mutants (Δ*hns*, Δ*stpA*, and Δ*hns* Δ*stpA* strains) (see Fig. S1 and Table S1 in the supplemental material) and the plasmid pGLO-P_{cas}-*gfp* (Fig. S4A). Data are shown as means ± standard deviations ($n = 3$).

Although expressing H-NS on pSU19 increased the natural transformability of the *hns stpA* mutant to the level of the wild type, expressing StpA on pSU19 partially restored the natural transformability of the *hns stpA* mutant (Fig. S13). The results indicated that ectopic expression of H-NS fully, but overexpression of StpA slightly, suppressed the DNA interference activity of the CRISPR-Cas system. In accordance with the above observation, overexpression of StpA slightly suppressed the activity of P_{cas} after 36 h of incubation. This result contradicts our previous observation that StpA

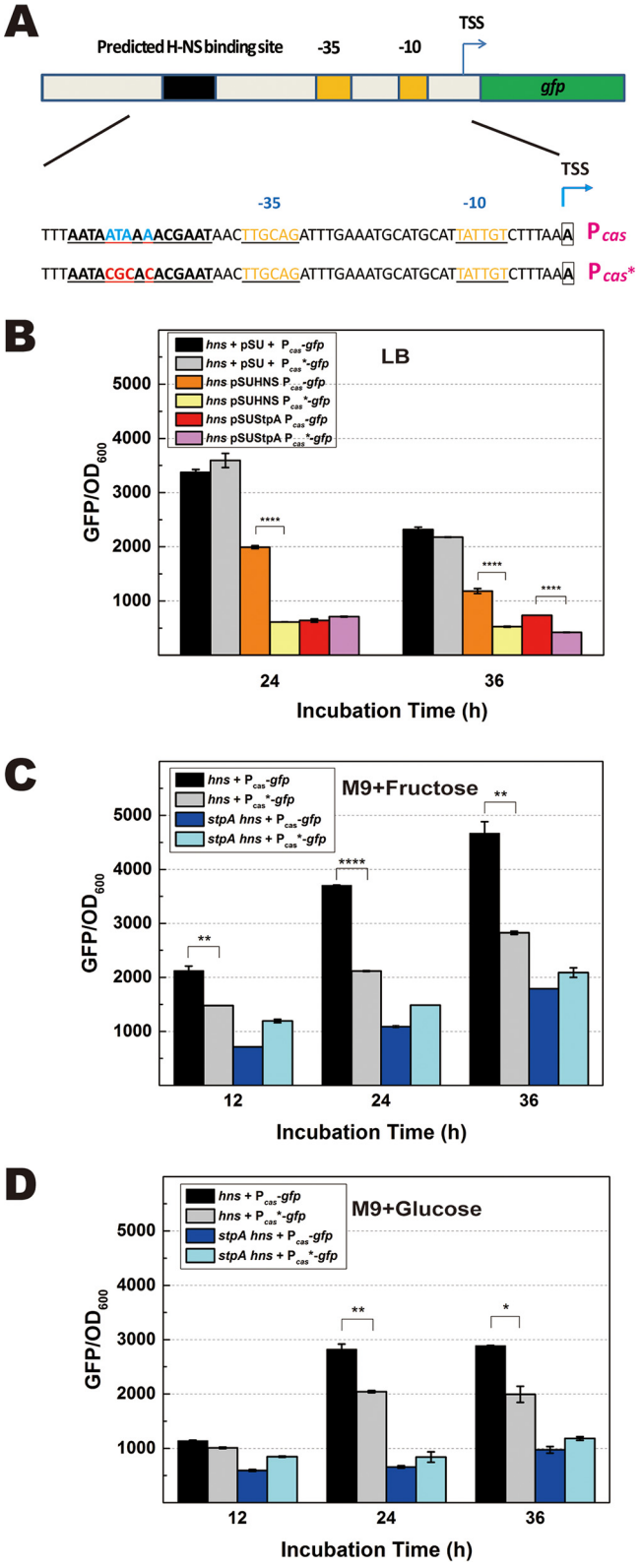

FIG 4 DNA binding site for transcriptional regulation by StpA and H-NS. Previous work has shown that H-NS and StpA can bind to P_{cas}, and the DNA binding site (DBS) for H-NS was predicted. (A) The putative DBS of H-NS/StpA was mutated by replacing the conserved sequence ATAAA to CGCAC. P_{cas} with a disrupted DBS was named P_{cas}*. Refer to Fig. S5 in the supplemental material for details about plasmid construction. (B) With the reporter GFP as an indicator, activities of P_{cas} and P_{cas}* were compared in the *hns* null mutant that expressed StpA or H-NS from the multicopy-number plasmid pSU19 in LB broth.

(Continued on next page)

positively regulated the *cas* operon in the *hns* null mutant. A high concentration of StpA was reported to block the accessibility of DNA and interfere with gene transcription (74). We reasoned that the level of StpA expressed from both the genome and the multicopy-number plasmid pSUStpA in the *hns* null mutant should be very high and thus affect transcription of P_{cas}. To test whether StpA expressed at the physiological level promoted transcription of the *cas* operon by binding to the DBS of P_{cas}, we compared activities of P_{cas} and P_{cas}* in *hns* and *hns stpA* null mutants. In M9 medium supplemented with fructose, we observed that after 24 h of incubation the activity of P_{cas} was about 2-fold higher than that of P_{cas}* in the *hns* null mutant (Fig. 4C). Similar differential activities of P_{cas} and P_{cas}* in the *hns* null mutant were observed in M9 medium supplemented with glucose (Fig. 4D) whereas activities of both P_{cas} and P_{cas}* were similarly low in the *hns stpA* null mutant grown in M9 medium supplemented with either fructose or glucose (Fig. 4C and D). These results reflect that StpA indeed promotes the activity of P_{cas} by binding to the DBS of H-NS when it is expressed by a single chromosomal copy of the *stpA* in the *hns* null mutant.

Expression of StpA at a low level activates transcription of the *cas* operon. Low expression of StpA on the chromosome promoted the activity of P_{cas} (Fig. 2, 3, and 4C) whereas high expression of StpA on a multicopy-number plasmid failed to stimulate P_{cas} (Fig. 4B). This led us to consider whether expressing StpA at different levels yielded different effects on P_{cas}. To test this, we controlled expression of StpA by using an arabinose-inducible promoter (P_{BAD}-*stpA*) on the plasmid (Fig. S7). The activity of P_{cas} was evaluated when StpA was expressed at different levels in the *hns stpA* null mutant grown in LB and M9 media (Fig. 5). We indeed observed that low concentrations of arabinose (0.1 mM to 3 mM) induced weak expression of StpA (Fig. 5B and C), which stimulated P_{cas} in LB medium (Fig. 5A). When 1 mM arabinose was added to the cell culture, the activity of P_{cas} in the *hns stpA* null mutant expressing StpA by P_{BAD} reached the highest level, which was more than 2-fold higher than that in a strain carrying an empty vector (Fig. 5A). We also observed that even when no arabinose was added into the LB medium, the activity of P_{cas} in the strain containing P_{BAD}-*stpA* on the vector was still obviously higher than that in the strain carrying the empty vector (Fig. 5A), indicating that leaky expression of StpA was sufficient for activating transcription of the *cas* operon in LB medium, whereas the activity of P_{cas} was gradually decreased by further increasing the concentration of arabinose (Fig. 5A). In contrast, a high concentration of arabinose (i.e., 10 mM) remarkably increased the amount of StpA expressed by P_{BAD} (Fig. 5B and C) but reduced the activity of P_{cas} to a level similar to that with the empty vector (Fig. 5A). To test the effect of low expression of StpA on the CRISPR-Cas system-mediated DNA interference, levels of natural transformation with the CRISPR-Cas-targeted plasmid pCR1 and nontargeted plasmid pDsRED were evaluated in the *hns stpA* mutant containing P_{BAD}-*stpA*. Considering that transformation occurred exclusively on agar plates (64, 65), expression of StpA was controlled by adding arabinose in both liquid form and on agar plates (see the supplemental material for details about the experimental procedure). When no arabinose was added, pCR1 transformed cells with an efficiency of ~35 CFU/μg (Table S2) whereas no transformants were detected when 1 or 2 mM arabinose was added (Table S2). The result indicated that arabinose-induced expression of StpA activated CRISPR-Cas-mediated DNA interference during natural transformation of the *E. coli hns stpA* mutant.

The effect of StpA on the activity of P_{cas} was also evaluated in *E. coli* strains grown in M9 medium with glucose as the carbon source, which was reported to suppress the activity of P_{BAD} (75, 76). Expressing StpA by P_{BAD} in M9 medium supplemented with

FIG 4 Legend (Continued)
Activities of P_{cas}-*gfp* and P_{cas}*-*gfp* were compared in *hns* and *hns stpA* null mutants that were grown in M9 minimal medium supplemented with 0.32% (wt/vol) fructose (C) or 1% (wt/vol) glucose (D) as the carbon source. TSS, transcriptional start site. Data are shown as means ± standard deviations ($n = 4$). Statistical significance was determined using a two-tailed Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.001$).

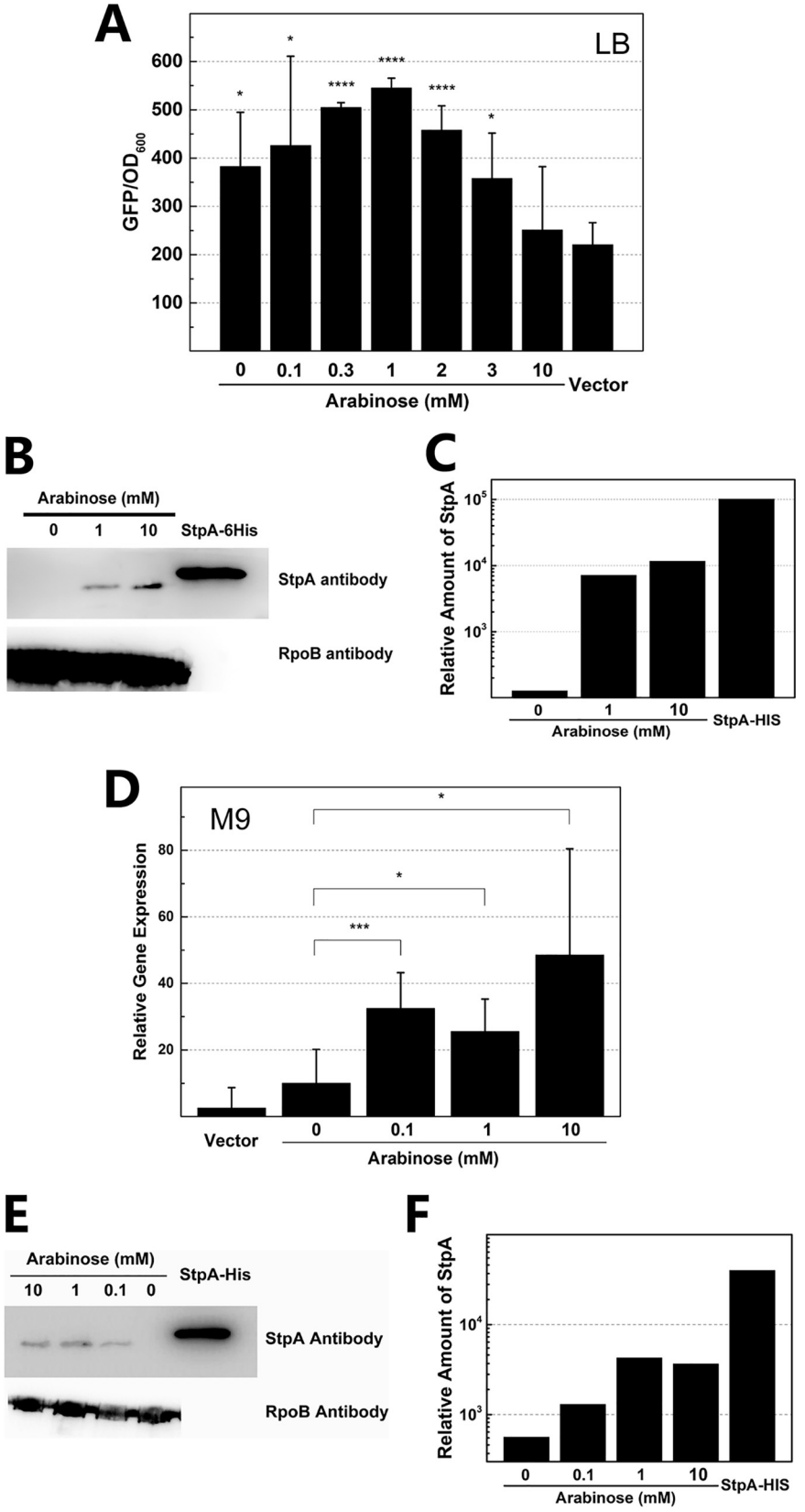

FIG 5 Complementation analysis of the effect of StpA on transcriptional regulation of P_{cas}. To modulate expression of StpA, the *ara*BAD promoter (P_{BAD}) was fused with *stpA*, and the construct was transformed into the *hns stpA* null mutant. The effect of StpA on transcription of the *cas* operon was monitored in cell cultures incubated in LB medium for 10 h (A) and in M9 medium for 24 h (D) supplemented with different

(Continued on next page)

glucose indeed yielded a small amount of StpA (Fig. 5E and F). When no arabinose was added, the activity of P_{cas} in the *hns stpA* null mutant with P_{BAD}-*stpA* on a vector was comparable to that of a strain carrying the empty vector (Fig. 5D), indicating remarkably reduced leaky expression of StpA in M9 medium. Amounts of StpA expressed by P_{BAD} were equally low in cell cultures supplemented with 0.1 mM to 10 mM arabinose (Fig. 5E and F). Correspondingly, activities of P_{cas} in the above cultures were increased by 2- to 5-fold with respect to the level in the culture without arabinose (Fig. 5D). In contrast, expression of H-NS at either a high or a low level failed to stimulate transcription of the *cas* operon in the *hns stpA* null mutant (Fig. S14). Taken together, our results clearly demonstrate that expressing StpA at a low level stimulates transcription of the *cas* operon.

StpA is required for the formation of mature crRNA in an *hns* null mutant. A functional CRISPR-Cas system consists of Cas proteins and mature CRISPR RNA (crRNA), which guides Cas proteins to the target DNA (5, 6, 14, 15). We have shown that StpA activated transcription of the *cas* genes in the *hns* null mutant. Finally, we examined the potential effect of StpA on the amount of mature crRNA. Mature crRNA is formed by processing pre-crRNA with CasE, encoded by a gene in the *cas* operon (10) (Fig. 6A). To remove long RNA (including pre-crRNA), we purified mature crRNA from total RNA by using a microRNA (miRNA) purification kit, in which the spin column contains resins that selectively bind small RNAs (sRNAs) (<200 nucleotides [nt]) with high affinity (Fig. 6B). An adaptor, which consisted of poly(A) and a primer for further quantitative PCR, was attached to the 3′ end of the sRNA fragment (Fig. 6B). Purified sRNA was analyzed by gel electrophoresis, which showed that sizes of most purified sRNAs were approximately 50 bp (Fig. S16A). With tRNA (encoded by *glnU*) as the internal control, quantitative PCR (qPCR) was performed to determine the amount of crRNA. Melt curve analysis showed single peaks for both crRNA and tRNA (Fig. S16B and C), indicating that the target cDNA was specifically amplified. PCR products of expected sizes were detected by gel electrophoresis (Fig. S16D). The qPCR data revealed that the relative amount of mature crRNA in the *hns* null mutant was more than 20-fold greater than that in the *hns stpA* null mutant (Fig. 6C), indicating that *stpA* was required for the formation of a large amount of cRNA in the *hns* mutant. Nevertheless, the amount of crRNA is still significantly higher than that in the wild type. Therefore, StpA is partially responsible for the increase in the amount of mature crRNA in the *hns* mutant whereas in the hns^+ background, the relative amount of mature crRNA in the *stpA* mutant was ~5-fold higher than that in its wild-type parent, showing that the effect of StpA on the amount of crRNA was influenced by H-NS.

DISCUSSION

Previous work established that H-NS suppressed the type I-E CRISPR-Cas system in *E. coli* (28). In this study, we showed that the H-NS paralogue StpA played an opposite role in regulating the type I-E CRISPR-Cas system when H-NS was absent. It is able to promote both the transcriptional level of the *cas* operon and the amount of mature crRNA. We also document that the type I-E CRISPR-Cas system activated by StpA was able to defend against the transfer of DNA mediated by natural transformation in *E. coli*. CRISPR adaptation is mediated by Cas1 and Cas2, whose encoding genes are located in the *cas* operon of *E. coli* (16, 28). Considering that StpA activates transcription of the *cas* operon, we anticipate that it could also stimulate CRISPR adaptation during natural transformation of *E. coli*. The type I CRISR-Cas system is widely distributed in prokaryotes (5, 18). Particularly, homologues of H-NS and StpA have been found in both

FIG 5 Legend (Continued)
concentrations of arabinose. A Western blot assay was performed to determine expression levels of StpA in cell cultures incubated in LB (B) and M9 (E) media. The StpA protein amounts from cell cultures grown in LB (C) and M9 (F) media were analyzed by using Quality One software. All experiments were performed in duplicate or triplicate. Statistical significance was determined using a two-tailed Student's *t* test (*, $P \leq 0.05$; ***, $P \leq 0.005$; ****, $P \leq 0.001$).

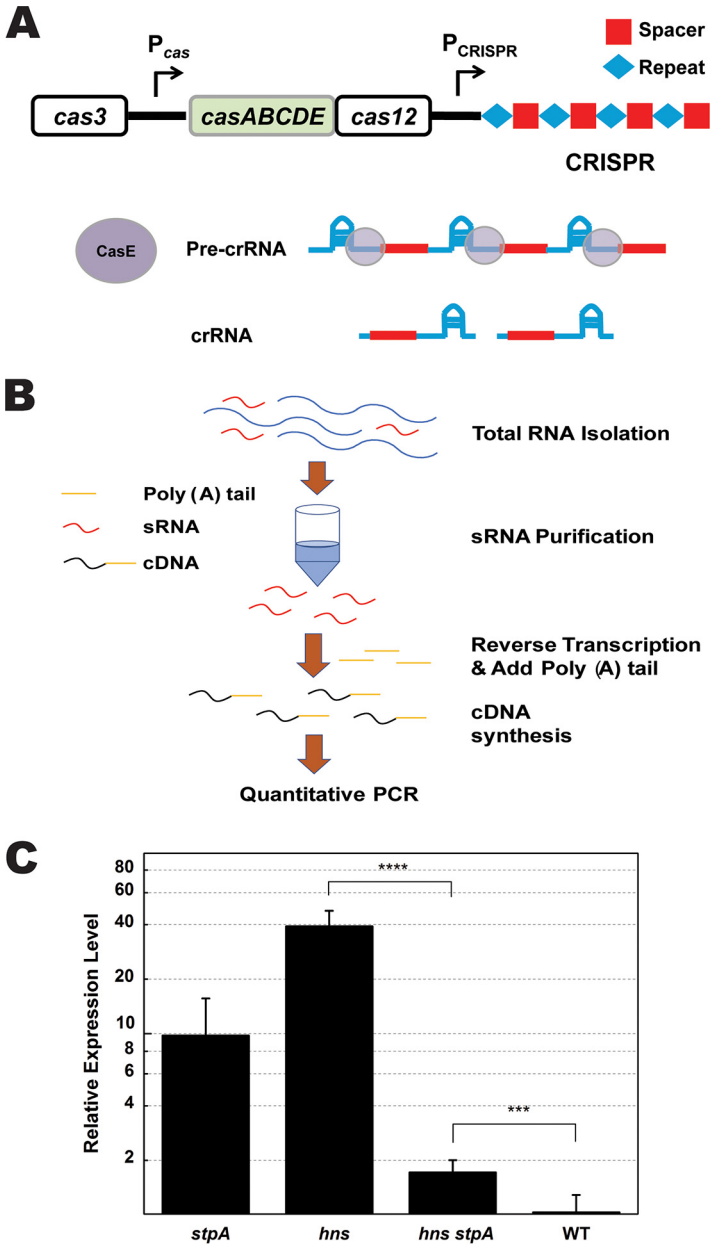

FIG 6 Effect of *stpA* inactivation on the amount of mature crRNA. (A) Schematic representation of the mechanism of crRNA formation. Followed by transcription of the pre-crRNA from CRISPR and expression of the *cas* operon, CasE cleaves the repeat sequence to form mature crRNA. (B) Schematic representation of the procedure of mature crRNA purification (refer to the supplemental material for details). (C) After 12 h of incubation in LB broth, total RNA was isolated, and a small RNA (containing crRNA) was purified for quantitative PCR assay. The relative amounts of mature crRNA from Δ*hns*, Δ*stpA*, Δ*hns* Δ*stpA*, and WT strains were quantified by real-time qPCR assay with tRNA as the reference. Data are shown as means ± standard deviations ($n = 4$). Statistical significance was determined using a two-tailed Student's *t* test (***, $P \leq 0.005$; ****, $P \leq 0.001$).

Salmonella and *Klebsiella*, which are also equipped with the type I-E CRISPR-Cas system (48, 77, 78). MvaT and MvaU play roles similar to those of H-NS and StpA in *Pseudomonas* (79), which also contains an active type I-E CRISPR-Cas system (80, 81). It would be interesting to investigate whether StpA homologues are involved in such regulatory roles in organisms other than *E. coli*.

CRISPR-Cas systems limit not only ssDNA uptake during natural transformation of *S. pneumoniae* and *N. meningitidis* (62, 63) but also dsDNA transferred via bacteriophage

infection in *E. coli* (10, 47). Our previous work uncovered a new type of natural transformation, in which dsDNA was transferred across *E. coli* cell membranes (1). Here, we have shown that the type I-E CRISPR-Cas system in *E. coli* reduced levels of natural transformation with the CRISPR-Cas-targeted plasmid but did not affect transformation with the nontargeted plasmid (Fig. 1). We also observed that an StpA-activated CRISPR-Cas system reduced chemical transformation with the targeted plasmid (see Fig. S10 in the supplemental material). Therefore, transforming dsDNA is subject to CRISPR-Cas-mediated interference. It has been documented that inactivating *cas3*, which encodes the nuclease for DNA cleavage, reduced bacteriophage DNA interference by the type I CRISPR-Cas system in the *hns* mutant (46, 49). The StpA-activated type I-E CRISPR-Cas system could also provide immunity against "infecting" ssDNA (e.g., conjugative DNA and bacteriophage DNA) due to DNA cleavage on the target dsDNA formed by recombination or replication of ssDNA after its entry into the cell. With the *hns stpA* null mutant as the recipient, although we detected a number of transformants carrying the CRISPR-Cas-targeted plasmid, these transformants were unable to grow further (Fig. S9), suggesting that the type I-E CRISPR-Cas system in the *hns stpA* null mutant was unable to cleave all CRISPR-Cas-targeted DNA immediately after plasmid entry. Instead, dsDNA should be gradually degraded over a prolonged period of time in that strain (Fig. S9). Nevertheless, delayed DNA interference in the *hns stpA* null mutant but immediate degradation of the entering plasmid in the *hns* null mutant indicated that the efficiency of DNA interference was enhanced by StpA in the *hns* null mutant (Fig. S9). It is also noticeable that the level of transformation with the CRISPR-Cas-targeted plasmid was slightly lower than that with the nontargeted plasmid in the WT strain (Fig. 1B), indicating that the type I-E CRISPR-Cas system could be moderately activated during natural transformation of *E. coli*.

H-NS and StpA are xenogeneic silencing proteins for suppressing gene transcription from foreign-derived DNAs, which are widespread in the Gram-negative alpha-, beta-, and gammaproteobacteria as well as in the Gram-positive *Actinobacteria* (79). Although interaction between xenogeneic silencers and DNA have recently been intensively studied (74, 82–86), it remains unclear how the H-NS/StpA nucleoprotein structure observed *in vitro* affects gene transcription *in vivo*. It is not unprecedented to discover that StpA can act as a transcriptional activator, given that StpA normally functions as a backup for H-NS, which was shown to act as both activator and repressor, as revealed by transcriptome analysis (73). For example, both H-NS and StpA stimulate transcription of *crp* by changing the DNA topology to a form that favors the use of highly compacted DNA for transcription (57). We also observed that both H-NS and StpA positively regulated transcription of the *mal* operon (Fig. 2B). Nevertheless, data presented here strongly argue that H-NS and StpA play opposite roles in regulating the same promoter (i.e., the *cas* promoter): the former suppresses gene transcription but the latter stimulates gene transcription (Fig. 2 and 5). StpA and H-NS share DNA binding regions in both the *gal* and *cas* promoters (28, 54). In the promoter of the *cas* operon, a footprinting experiment showed that StpA and H-NS bound to the same region (28) in which the H-NS binding motif was predicted (28, 46, 47). In this study, we provided *in vivo* evidence supporting the finding that StpA and H-NS recognized the same DBS in P_{cas} but played different roles in regulating transcription of the *cas* operon (Fig. 4). Overexpressing H-NS strongly suppressed the activity of P_{cas} in the *hns* null mutant (Fig. 4B). In contrast, mutating the DBS derepressed transcription of the *cas* operon that was inhibited by H-NS (Fig. 4B), showing that H-NS suppresses the activity of P_{cas} by binding to the predicted DBS. With respect to the activity of P_{cas} containing an intact DBS, the activity of P_{cas}* containing a mutated DBS was remarkably reduced in the *hns* null mutant (Fig. 4C and D). Nevertheless, P_{cas} and P_{cas}* showed similar activity levels in the *hns stpA* null mutant (Fig. 4C and D). These facts clearly show that certain amounts of StpA promoted the activity of P_{cas} by acting on the DBS of H-NS. The reason for the different behaviors of H-NS and StpA on the activity of P_{cas} needs further exploration. Previous *in vitro* experiments documented that StpA and H-NS compact DNA in

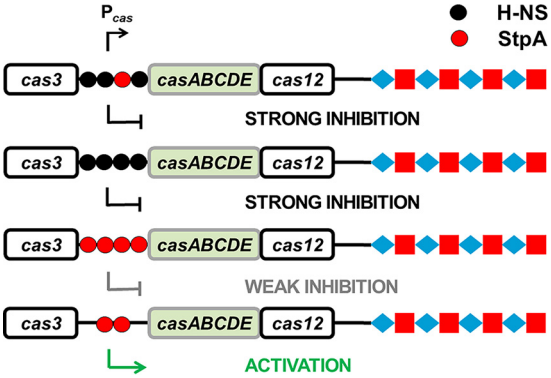

FIG 7 Schematic diagram of transcriptional regulation by StpA and H-NS. Transcription of the *cas* operon was suppressed by H-NS in the presence (first row) or absence of StpA (second row). Although StpA expressed at a high level weakly suppresses transcription of the *cas* operon (third row), a low concentration of StpA stimulated the activity of P_{cas} (fourth row).

different ways (74). It is possible that differential compaction of DNA by H-NS and StpA may lead to different modes of transcriptional regulation *in vivo*.

StpA is more versatile than H-NS in transcriptional regulation. In the presence or absence of StpA, we observed that H-NS inhibited the activity of P_{cas} (Fig. 7). In contrast, a small amount of StpA induced the activity of P_{cas} (Fig. 5A and D and Fig. 7), but a large amount of StpA failed to stimulate P_{cas} (Fig. 5A and 7). Previous *in vitro* experiments revealed that linearized double-stranded DNA incubated with 1 StpA per 1 bp (1:1 StpA/DNA) showed rigid StpA-coated DNA hairpins that blocked DNA accessibility (74) whereas at ratios of both 1:10 and 1:100 StpA/DNA, StpA-induced DNA bridging was evident (74). We calculated the cellular concentration of StpA in cells grown in M9 medium supplemented with 1 mM arabinose and found that P_{cas} was activated when the ratio of StpA/DNA was ~1:200 (see the results and discussion in the supplemental material and Table S3). Possibly, a low cellular concentration of StpA may induce DNA bridging, which could facilitate transcription initiation by altering the configuration of StpA/DNA. Nevertheless, the explanation for transcriptional activation by StpA awaits more direct evidence.

We observed a transcriptional stimulation effect of StpA only in the *hns* null strain. In line with a previous study (28), inactivation of *stpA* did not affect transcription of *cas* genes in the hns^+ strain (Fig. 2 and 3). Considering that transcription of H-NS can be upregulated by inactivating *stpA* (54), the effect of StpA on transcription of the type I-E CRISPR-Cas system could be masked by stronger repression due to the increased expression level of H-NS in the *stpA* null mutant. Similar to previous studies (54), we also observed that inactivating both *hns* and *stpA* caused a more serious growth defect than inactivating only *hns* (Fig. S8A). Interestingly, when a high-copy-number plasmid was introduced into *E. coli* strains, the *hns* null mutant grew remarkably more slowly than the *hns stpA* null mutant (Fig. S8B and C). Providing that H-NS alters plasmid and chromosomal DNA supercoiling (87), as well as chromosome partitioning and replication (69), it is possible that the aberrant topological structure of the plasmid could have an effect on cell growth in the *hns* null mutant.

Although StpA increased the amount of crRNA in the *hns* deletion mutant, it reduced the amount of crRNA in the hns^+ strain (Fig. 6C). The result reflects that H-NS influenced the effect of StpA on the level of crRNA. Considering that StpA can function as both an RNA chaperon (88–90) and a transcriptional regulator of the gene encoding CasE for pre-crRNA processing (Fig. 6A), it could play pleiotropic roles in stabilizing/processing pre-crRNA and/or mature crRNA in the *hns* mutant. The complex interplay between these processes contributes to crRNA abundance in a nonlinear way (91). Alternatively, H-NS and StpA in the WT strain may form a heteromeric complex (92), which could have a stronger suppression effect on expression of crRNA than only H-NS in the *stpA* null mutant.

Of note, a recent report documented that ectopic expression of StpA on a multicopy-number plasmid suppressed transcription of *casA* in an *hns cas1* double mutant of *E. coli*, resulting in a decrease of DNA interference during λ phage infection (93). The result is in accordance with our observation that ectopic expression of StpA at a high level suppressed transcription of *cas* genes in the *hns* null mutant (Fig. 4B) and moderately reduced DNA interference during natural transformation (Fig. S13). It is also documented that although inactivation of *stpA* further increased transcription of *casA* in the *hns cas1* mutant, *stpA* seems to have no additional role to further improve immunity against bacteriophage infection in that strain (93). This finding is different from our observation in that *stpA* deletion in an *hns* null mutant reduced transcription of *cas* genes (Fig. 3) and decreased immunity against plasmid DNA in both natural and chemical transformations (Fig. 1 and Fig. S10).

In summary, we showed that expression of the type I-E CRISPR-Cas system is positively regulated by StpA which activates the promoter of the *cas* operon and augments the amount of crRNA in the *hns* null background. The type I-E CRISPR-Cas system activated by StpA increases DNA interference during natural transformation and thus improves the cellular immune response to horizontally transferred genetic elements. Moreover, we provided evidence showing that DNA-structuring protein paralogues StpA and H-NS bound on the same site in P_{cas} but played opposite roles in transcriptional regulation of the *cas* operon, implying that higher-order compaction of bacterial chromatin by histone-like proteins could switch prokaryotic transcriptional modes.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, growth conditions, and media. Bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. Strains lacking *hns* and/or *stpA* were constructed with pKD46 (see Fig. S1 in the supplemental material), a temperature-sensitive plasmid carrying the λ-derived Red recombination system (94). Mutants were examined through PCR and Western blot assay (Fig. S1 and Table S1). Recombinant plasmids were constructed with a One-Step PCR Cloning kit (Fig. S3 to S7). Details of the construction of mutants and plasmids are described in supplemental material. Plasmids were isolated with a plasmid isolation kit according to the manufacturer's instructions (Axygen Biotech Co., Ltd.). *E. coli* was grown at 30°C or 37°C as follows: in LB broth containing 1% tryptone (wt/vol), 0.5% (wt/vol) yeast extract, and 1% (wt/vol) NaCl; in 1.5× LB broth containing 1.5% tryptone (wt/vol), 0.75% (wt/vol) yeast extract, and 1.5% (wt/vol) NaCl; in M9 minimal medium containing 61 mM $K_2HPO_4{\cdot}3H_2O$, 38.21 mM KH_2PO_4, 4.15 mM $MgSO_4$, 16.70 mM NH_4Cl, 0.1% tryptone (wt/vol), and 0.32% (wt/vol) fructose or 0.8% (wt/vol) glucose as the carbon source, supplemented with an appropriate amount of arabinose for inducing transcription of genes when necessary or not supplemented; or on LB agar plates containing 1.5% (wt/vol) or 5% (wt/vol) agar. When necessary, the medium was supplemented with ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), or chloramphenicol (25 μg ml^{-1}), unless otherwise specified. Cell growth was measured in a Spectrumlab S23A spectrophotometer or in a Tecan Sunrise 96-well plate reader. All experiments were repeated independently at least three times.

Quantification of transcription of *cas* genes with GFP. To monitor transcription of *cas* genes by GFP, the promoter P_{BAD} was replaced by the promoter of the *cas* operon (P_{cas}) in the plasmid pGLO-*gfp* (Fig. 3A and Fig. S4A). The recombinant plasmid was transformed into *E. coli* cells, and transformants were subsequently grown in LB or M9 medium supplemented with appropriate antibiotics. Cell growth was monitored spectrophotometrically at an optical density of 600 nm (OD_{600}). Intensity of the culture fluorescence, as an indicator of transcription of P_{cas}, was measured by a SpectraMax Gemini EM microplate reader with excitation and emission wavelengths at 395 and 509 nm, respectively.

Quantification of gene transcripts and mature crRNA with quantitative PCR. Total RNA was isolated from *E. coli* cells grown in LB broth at 30°C for 12 h. The cell density was adjusted to an OD_{600} of 1.0. To quantify transcription of chromosomal genes, the isolated RNA was reverse transcribed, and quantitative PCR (qPCR) was performed with a Bio-Rad iCycle iQ. Small RNA (sRNA) was purified from the isolated total RNA with an EasyPure miRNA kit (TransGen Biotech Co., Ltd.) which contains columns filled with resin with high affinity to sRNA (<200 bp) fragments (Fig. 2A). The 3′ terminally tagged cDNA was synthesized by reverse transcription (miRNA First-Strand cDNA Synthesis SuperMix; TransGen Biotech Co., Ltd.) and quantified by qPCR. Relative expression levels of genes and sRNAs were calculated according to the formula $2^{-(\Delta CT\ \text{target}\ -\ \Delta CT\ \text{reference})}$ (95), with 16S RNA and tRNA, respectively, as the internal references. Primers used for qPCR are listed in Table 2. Refer to supplemental material for detailed procedures.

Natural transformation of *E. coli*. Natural transformation was performed according to a previously documented method (66, 67). A single *E. coli* colony grown on an LB-agar plate was inoculated into a glass tube containing 5 ml of LB broth and incubated with shaking at 30°C. An overnight-grown *E. coli* culture (14 to 18 h of incubation) was inoculated into a flask containing 1.5× LB broth at a ratio of 1:100 (vol/vol). The culture was incubated with shaking at a speed of 180 rpm at 30°C.

TABLE 2 Primers

Primer no.	Name	Sequence (5′–3′)[a]
P_{01}	ΔstpA H1P1	ATCGCTTACACTACGCGACGAAATACTTTTTTTGTTTTGGCGTT AAAAGG**GTGTAGGCTGGAGCTGCTTC** (−62, −12)
P_{02}	ΔstpA H2P2	ATAAGATGCCGTGGAACCAACGAGCTTGAGAAGCGACGCCG GACGCGCCC**CATATGAATATCCTCCTTAG** (+25, +75)
P_{03}	Δhns H1P1	TCTATTATTACCTCAACAAACCACCCCAATATAAGTTTGAGATT ACTACA**GTGTAGGCTGGAGCTGCTTC** (−51, −1)
P_{04}	Δhns H2P2	TTATTAAATTGTCTTAAACCGGACAATAAAAAATCCCGCCGCT GGCGGG**CATATGAATATCCTCCTTAG** (+443, +493)
P_{05}	ΔstpA-1500F	TATGTATTTACGACCAGAC
P_{06}	ΔstpA-999R	ATTTTAGCGGAGCCTGCC
P_{07}	Δhns-1500F	GCATTGCCCTTCTGGGGCCG
P_{08}	Δhns-999R	AGGAACCAGGATGTTGCCGG
P_{09}	gfp-F	AGGAGAACAATTTAAGAAGGAGATATACAT
P_{10}	gfp-R	AATAATGGTTTCTTAGACGTCAAGCTTGCATGCCTGCAGG
P_{11}	P_{cas} promoter-F	TCCCCGAAAAGTGCCACCTGCTTCGGGAATGATTGTTATC
P_{12}	P_{cas} promoter-R	CCTTCTTAAATTGTTCTCCTTCATATGCTC
P_{13}	gfp-rscA-F	CATTGAGTGAGGGTATGCTACTAGAAAGAGGAGAAATA
P_{14}	gfp-rscA-R	TTACGGAATTATATTAATGGGGTAACGAATCAGACAATTG
P_{15}	P_{rscA} promoter-F	CAATTGTCTGATTCGTTACCGGGTCTGAATGCGACGTTAA
P_{16}	P_{rscA} promoter-R	TATTTCTCCTCTTTCTAGTATTAAATTCTCCTGGACTG
P_{17}	pSU19-stpA-F	CGCTTCTCAAGCTCGTTGGGGTACCGAGCTCGAATTC
P_{18}	pSU19-stpA-R	GAAAACATCCATCACTGGTGCCTGCAGGCATGCAAGCTTG
P_{19}	stpA-pSU19-F	CAAGCTTGCATGCCTGCAGGCACCAGTGATGGATGTTTTC
P_{20}	stpA-pSU19-R	GAATTCGAGCTCGGTACCCCAACGAGCTTGAGAAGCG
P_{21}	hns-pSU19-F	CGTGGATAACACCGATACGGGGGTACCGAGCTCGAATTC
P_{22}	hns-pSU19-R	TACGAGAATTCCCTATCCTGCAGGCATGCAAGCTTG
P_{23}	pSU19-hns-F	CAAGCTTGCATGCCTGCAGGATAGGGAATTCTCGTA
P_{24}	pSU19-hns-R	GAATTCGAGCTCGGTACCCCCGTATCGGTGTTATCCACG
P_{25}	cas-gfp-ΔBS-F	CTTTAATAGCGAGACGAATAAC
P_{26}	cas-gfp-ΔBS-R	GTTATTCGTCTCGCTATTAAAG
P_{27}	tRNA-F	CGGTTTTTGATACCGGCAT
P_{28}	crRNA-F	CTCCCTGTCGGTTGTAATTG
P_{29}	sRNA-R	GATCGCCCTTCTACGTCGTAT
P_{30}	P_{BAD}-gfp-pSU-F	TTATGACAACTTGACGGCTACA
P_{31}	P_{BAD}-gfp-pSU-R	TTATTTGTAGAGCTCATCCATGC
P_{32}	pSU-P_{BAD}-gfp-F	TGGATGAGCTCTACAAATAAGAATTCGAGCTCGGTACCCG
P_{33}	pSU-P_{BAD}-gfp-R	TAGCCGTCAAGTTGTCATAAATTGCGTTGCGCTCACTGCC
P_{34}	pSU-P_{BAD}-F	GAATTCGAGCTCGGTACCCG
P_{35}	pSU-P_{BAD}-R	ATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTC
P_{36}	P_{BAD}-stpA-pSU-F	TTTAAGAAGGAGATATACATATGTCCGTAATGTTACAAAG
P_{37}	P_{BAD}-stpA-pSU-R	GGGTACCGAGCTCGAATTCTTAGATCAGGAAATCGTCGAG
P_{38}	P_{BAD}-hns-pSU-F	TTTAAGAAGGAGATATACATATGAGCGAAGCACTTAAAATTCTG
P_{39}	P_{BAD}-hns-pSU-R	GGGTACCGAGCTCGAATTCTTATTGCTTGATCAGGAAATCGTCG

[a]Boldface letters indicate nucleotide extensions, complementary to antibiotic resistance cassettes, that were introduced in the primers to amplify either the *cat* gene or the *kan* gene from plasmid pKD3 or pKD4, respectively. Following each primer sequence, the corresponding gene and the positions of the first and final nucleotides (in parentheses) are indicated (with respect to the ATG of the gene).

After 24 h of incubation, the cell culture was precipitated by centrifugation at room temperature, and 90% (vol/vol) of the supernatant was discarded. The cell pellet was resuspended in the remaining 10% (vol/vol) of the supernatant and placed in a metal bath at 30°C. Plasmids pDsRED and pCR1 were added to the cell suspension solution to a final concentration of 40 μg ml^{-1}. Fifty microliters of the mixture was spread on LB plates containing 5% (wt/vol) agar (BD Difco) and 200 μg ml^{-1} of ampicillin, which had been placed at room temperature (~30°C) for 1 to 2 days before use. Numbers of transformants were counted on petri plates which were incubated at 30°C for 1 to 2 days. Transformation efficiency was calculated by dividing the number of transformants by the amount of DNA.

Protein purification and Western blot assay. Protein with a His tag was purified from *E. coli* BL21 with a tagged-protein purification kit (soluble protein) (Beijing ComWin Biotech Co., Ltd.). The antibody against StpA, which can interact with both StpA and its paralogue H-NS (Fig. S1C), was used for evaluating the expression level of StpA. RpoB was set as the control, and the corresponding primary antibody was commercially obtained from BioLegend. Refer to the supplemental material for details about procedures for protein purification and Western blot assay.

Data availability. The pCR1 sequence has been deposited in GenBank under accession number MT437282 and the Addgene plasmid repository under catalog no. 154270 (https://www.addgene.org/154270/).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4 MB.

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