



# Species-Specific Quorum Sensing Represses the Chitobiose Utilization Locus in *Vibrio cholerae*

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**ABSTRACT** The marine facultative pathogen *Vibrio cholerae* forms complex multicellular communities on the chitinous shells of crustacean zooplankton in its aquatic reservoir. *V. cholerae*-chitin interactions are critical for the growth, evolution, and waterborne transmission of cholera. This is due, in part, to chitin-induced changes in gene expression in this pathogen. Here, we sought to identify factors that influence chitin-induced expression of one locus, the chitobiose utilization operon (*chb*), which is required for the uptake and catabolism of the chitin disaccharide. Through a series of genetic screens, we identified that the master regulator of quorum sensing, HapR, is a direct repressor of the *chb* operon. We also found that the levels of HapR in *V. cholerae* are regulated by the ClpAP protease. Furthermore, we show that the canonical quorum sensing cascade in *V. cholerae* regulates *chb* expression in an HapR-dependent manner. Through this analysis, we found that signaling via the species-specific autoinducer CAI-1, but not the interspecies autoinducer AI-2, influences *chb* expression. This phenomenon of species-specific regulation may enhance the fitness of this pathogen in its environmental niche.

**IMPORTANCE** In nature, bacteria live in multicellular and multispecies communities. Microbial species can sense the density and composition of their community through chemical cues using a process called quorum sensing (QS). The marine pathogen *Vibrio cholerae* is found in communities on the chitinous shells of crustaceans in its aquatic reservoir. *V. cholerae* interactions with chitin are critical for the survival, evolution, and waterborne transmission of this pathogen. Here, we show that *V. cholerae* uses QS to regulate the expression of one locus required for *V. cholerae*-chitin interactions.

# **KEYWORDS** quorum sensing, cholera, protease

The facultative bacterial pathogen *Vibrio cholerae*, the causative agent of the diarrheal disease cholera, natively resides in the aquatic environment. In this niche, *V. cholerae* forms multicellular communities on biotic and abiotic chitinous surfaces, like the shells of crustaceans or marine snow (1, 2). Chitin is a polysaccharide made up of  $\beta$ -1,4-linked *N*-acetylglucosamine (GlcNAc) and serves as a major nutrient source for *V. cholerae* in the marine environment (1, 3, 4). The ability of *V. cholerae* to form chitin biofilms is critical for the waterborne transmission of cholera (5, 6). As chitin is the most abundant biopolymer in the ocean, the ability of *Vibrio* species to break down and utilize this highly insoluble polysaccharide also serves an important role in global nitrogen and carbon recycling (1, 4).

When *V. cholerae* is associated with a chitinous surface, chitin induces the expression of a subset of genes in *V. cholerae*. The genes induced by chitin include those required for chitin degradation, uptake, and catabolism (termed the chitin utilization program), as well as the genes required for natural transformation (7, 8). Transcriptional responses resulting from *Vibrio*-chitin interactions are highly regulated. One major chitin-responsive regulator is the orphan hybrid sensor kinase ChiS (7, 9). ChiS senses chitin

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Accepted manuscript posted online 10 July 2020 Published 1 September 2020 indirectly through the periplasmic <u>chitin binding protein (CBP)</u> (9, 10). In the absence of chitin, CBP represses ChiS through interactions with its periplasmic domain (9, 10). In the presence of chitin, the CBP-chitin complex stimulates ChiS activity (9, 10). Thus, in the presence of chitin, ChiS is active and can facilitate expression of the chitin utilization program. Alternatively, ChiS can be genetically activated in the absence of chitin by deleting *cbp* (10, 11).

In the marine environment, *V. cholerae* not only senses chitin to modulate gene expression but also the presence of other bacteria through a process termed "quorum sensing" (QS) (12). This is a process by which bacteria indirectly sense other microbes in their community via small diffusible molecules called autoinducers (AIs). Als are sensed by cognate sensor proteins. *V. cholerae* has four AI sensors, although the autoinducer molecules that serve as inducing cues are only known for two of them (13). AI sensing allows for cell-density-specific gene expression programs, which regulate "group" or "individual" behaviors (14). *V. cholerae* senses both chitin and AIs to regulate natural transformation on chitinous surfaces (15). Though a link between chitin utilization and quorum sensing has previously been suggested, it has not been directly studied (16).

To investigate regulation of the chitin utilization program in *V. cholerae*, most studies employ the chitobiose utilization operon (*chb*) (10, 11, 17, 18). The *chb* operon encodes the genes required for uptake and catabolism of the chitin disaccharide chitobiose and is highly induced in the presence of chitin oligosaccharides (7). Several mechanisms of *chb* regulation have already been identified. ChiS is the master regulator of the chitin utilization program in *V. cholerae*, and we have recently shown that this protein is a direct transcriptional activator required for induction of the *chb* locus (9, 10). Previous work has shown that carbon catabolite repression (CCR) can also play a role in regulating chitin responsive phenotypes, including ChiS-dependent induction of *chb* and natural transformation (18, 19). In addition, our group has previously found that the cell division licensing factor SImA plays an essential role in activating *chb* expression (11). Tight regulation via these diverse signaling systems may act to ensure that the chitin utilization program is only expressed under conditions in which it will provide a competitive advantage.

Here, we sought to identify additional regulators of *chb*. Through a number of genetic screens and complementary molecular methods, we show that quorum sensing is an additional regulatory system that tunes expression of a chitin utilization locus in *V. cholerae*.

# RESULTS

**ClpA is identified in an unbiased screen for activators of**  $P_{chb}$ **.** To identify additional genes required for activation of the *chb* locus, we conducted a transposon mutant screen. This was carried out in a strain containing a chromosomally integrated  $P_{chb}$ -lacZ transcriptional reporter. As shown previously, induction of  $P_{chb}$  is dependent on the activity of the master regulator ChiS (7, 10, 11). In the absence of chitin, ChiS activity is repressed by CBP. In the presence of chitin, CBP repression of ChiS is relieved, which allows for ChiS-dependent activation of  $P_{chb}$ . In addition to being induced by chitin, ChiS can be activated genetically in the absence of chitin by deleting *cbp* (10, 11). As chitin oligomers are prohibitively expensive, a  $\Delta cbp$  mutation was used to induce ChiS-dependent  $P_{chb}$ -lacZ expression in our genetic screen exactly as previously described (11). So, the starting genotype for our screen was a strain containing  $P_{chb}$ -lacZ and a  $\Delta cbp$  mutation. This strain formed blue colonies on 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal)-containing plates, and we screened for white colonies to identify putative activators that contribute to  $P_{chb}$  induction.

Of approximately 60,000 transposon mutants visually screened for loss of  $P_{chb}$ -lacZ expression, one gene identified was *clpA* (2 unique transposon insertions). Other hits identified in this screen are listed in Table S1 in the supplemental material. To study the effect of *clpA* on  $P_{chb}$  activity moving forward, we utilized a previously described chromosomally integrated  $P_{chb}$ -green fluorescent protein (GFP) reporter (10, 11). Using



**FIG 1** HapR is a repressor of P<sub>chb</sub> that is degraded by the ClpAP protease. Expression of a P<sub>chb</sub>-GFP reporter and HapR protein levels were determined in the indicated mutant strains. The parent strain contained a P<sub>chb</sub>-GFP reporter and a  $\Delta cbp$  mutation. A representative Western blot is shown below bars to indicate the protein levels for HapR and RpoA (a loading control) in the corresponding strains. An antibody against LuxR, which has 72% identity and 86% similarity to HapR, is cross-reactive with HapR and so was used to detect HapR protein levels. Fluorescence of cultures was determined on a plate reader from at least six independent biological replicates and is shown as the mean ± standard deviation (SD). Statistical comparisons were made by one-way ANOVA with Tukey's posttest. NS, not significant. \*\*\*, P < 0.001.

this reporter, we found that a  $\Delta clpA$  mutation resulted in an ~3-fold decrease in P<sub>chb</sub> expression relative to the parent (Fig. 1). Importantly, complementation of this strain with an ectopic copy of *clpA* in *trans* restored P<sub>chb</sub> expression to parent levels (see Fig. S1 in the supplemental material). ClpA is a AAA+ unfoldase that recognizes protein substrates, unfolds them, and feeds them into the ClpP protease where they are degraded (20). If ClpA was exhibiting its effect on P<sub>chb</sub> expression as a part of the ClpAP machine, we hypothesized that a  $\Delta clpP$  mutation should phenocopy a  $\Delta clpA$  mutation. Indeed,  $\Delta clpP$  and  $\Delta clpA$  degrades that loss of the ClpAP protease decreases P<sub>chb</sub> expression (Fig. 1). These results suggest that loss of the ClpAP protease decreases P<sub>chb</sub> expression. As ClpAP degrades proteins, we hypothesized that ClpAP may indirectly promote activation of P<sub>chb</sub> by degrading a repressor of the *chb* locus.

HapR is a repressor of P<sub>chb</sub> that is degraded by ClpAP. To identify a putative repressor of P<sub>chb</sub> that is targeted by ClpAP for degradation, we conducted a counterscreen using the P<sub>chb</sub>-lacZ reporter. For the  $\Delta clpA$  strain counterscreen, the parent strain had both  $\Delta cbp$  and  $\Delta clpA$  mutations. This mutant is white on X-Gal plates because P<sub>chb</sub>-lacZ is poorly expressed; inactivation of the putative repressor in this strain should result in restoration of P<sub>chb</sub>-lacZ expression and yield a blue colony phenotype. In the  $\Delta clpA$  strain counterscreen, we visually screened approximately 30,000 transposon mutants for reactivation of P<sub>chb</sub>-lacZ expression and identified hapR (9 unique transposon insertions). Other hits identified in this screen are listed in Table S1. A  $\Delta hapR$ mutation restored  $P_{chb}$  expression in the  $\Delta clpA$  mutant background (Fig. 1). In fact, a  $\Delta hapR$  mutation allowed for higher P<sub>chb</sub> expression than that of the parent strain. This suggests that HapR represses  $\mathsf{P}_{chb}$  expression when ClpAP is intact and that ClpAP does not degrade the entire pool of HapR in the cell (Fig. 1). Importantly, the level of P<sub>chb</sub> expression observed in the  $\Delta hapR \Delta clpA$  mutant phenocopied the  $\Delta hapR$  strain (Fig. 1). This epistasis between clpA and hapR suggests that they are involved in the same pathway for regulating  $P_{chb}$  expression. In addition, complementation of the  $\Delta hapR$ strain with an ectopic copy of hapR in trans decreased P<sub>chb</sub> expression almost to the level in the parent (see Fig. S1). Further, complementation of the  $\Delta hapR \Delta clpA$  strain with hapR in trans brought  $P_{chb}$  expression down to the level observed in the  $\Delta clpA$ parent (Fig. S1).

We hypothesized that the reason  $P_{chb}$  expression was decreased in  $\Delta clpAP$  mutants was due to increased HapR protein levels. Western blotting in these backgrounds

revealed that HapR levels were, indeed, increased in strains containing mutations to clpA and/or clpP (Fig. 1). ClpAP-dependent degradation of HapR is not unique to V. cholerae but has previously been observed in Vibrio vulnificus where CIpAP degrades the HapR homolog SmcR (21). In addition to the ClpAP machine, it was shown that the Lon protease also plays a role in SmcR degradation. So, we next sought to investigate the role of other protease machines on induction of P<sub>chb</sub> and HapR protein levels. Mutations to other Clp components (the ClpS adaptor protein or the ClpX unfoldase) did not have a marked impact on HapR protein levels. Also, a  $\Delta clpS$  mutation did not affect P<sub>chb</sub> expression levels, while a  $\Delta clpX$  mutation slightly decreased P<sub>chb</sub> expression (Fig. 1). Because HapR expression was not affected by the  $\Delta clpX$  mutation, the observed decrease in P<sub>chb</sub> expression may be attributed to a pleiotropic effect (i.e., an effect of clpX that is independent of HapR-dependent P<sub>chb</sub> repression). In contrast to the effect of the Lon protease on SmcR levels in V. vulnificus, we did not observe an impact of the  $\Delta lonA$  mutation on HapR levels in V. cholerae; the  $\Delta lonA$  mutation, correspondingly, did not affect  $P_{chb}$  expression (Fig. 1). Together, these results establish that HapR is a repressor of P<sub>chb</sub> and that HapR levels are controlled specifically by the ClpAP protease in V. cholerae.

**HapR-mediated repression of**  $P_{chb}$  **occurs on chitinous surfaces.** Thus far, we have studied  $P_{chb}$  regulation using a  $\Delta cbp$  mutation to induce ChiS activity. Natural induction of this locus, however, occurs in the presence of chitin oligosaccharides. So, we next wanted to test whether the repression of  $P_{chb}$  by HapR was observed in a more physiologically relevant setting. To test this, we cultured *V. cholerae* strains with CBP intact, a  $P_{chb}$ -mCherry reporter, and a construct that constitutively expresses GFP ( $P_{const2}$ -GFP) on chitin beads (Fig. 2A). The  $P_{const2}$ -GFP construct (derived from the insulated *proD* promoter [22]) served as an internal control for noise in gene expression and was used to normalize  $P_{chb}$ -mCherry expression in single cells (see Materials and Methods for details).

When cells were cultured on chitin beads, both the parent strain and the  $\Delta hapR$ strain exhibited a bimodal distribution of P<sub>chb</sub> expression (Fig. 2A to C). There were at least two possible explanations for the observed bimodality in P<sub>chb</sub> gene expression in this experiment as follows: (i) the signaling pathway that leads to activation of *chb* has switch-like behavior that results in a population that exhibits bimodality in chb expression, or (ii) the environment during growth on chitin beads is heterogeneous and only some cells within the population have access to the chitin oligosaccharides necessary for activation of *chb*. Those cells that do not have access to chitin do not activate P<sub>chb</sub> expression (P<sub>chb</sub> off), while those that do have access to chitin do induce P<sub>chb</sub> expression (P<sub>chb</sub> on). To differentiate between these two possibilities, we induced expression of P<sub>cbb</sub> using the native inducer, chitin oligosaccharides, or genetically by deleting cbp. If the signaling circuit responsible for P<sub>chb</sub> activation has switch-like behavior, we would predict that bimodality in gene expression would be maintained in both of these conditions; however, if bimodality is the result of heterogeneous access to chitin oligosaccharides when cells are cultured on chitin beads, we would expect bimodality to be lost. When P<sub>chb</sub> was induced by chitin oligosaccharides or via deletion of cbp, all cells in the population are uniformly "P<sub>chb</sub> on" (Fig. 2D and F), and the population becomes unimodal (Fig. 2E and G). Thus, these results showed that only the "P<sub>chb</sub> on" cells have access to chitin oligosaccharides when grown on chitin beads. These results are consistent with chitin induction of natural transformation on chitin beads, where cells display bimodality in gene expression that is also likely due to heterogeneous access to inducing chitin oligosaccharides (23).

Next, we tested whether HapR influenced activation of  $P_{chb}$  on chitin beads by just assessing the expression level among " $P_{chb}$  on" cells. As observed in bulk populations, the  $\Delta hapR$  strain exhibited an ~1.7-fold increase in  $P_{chb}$  expression when cultured on chitin beads (Fig. 2C) and an ~2.5-fold increase when cultured with chitin oligosaccharides (Fig. 2E). These values were consistent with the ~2-fold increase in  $P_{chb}$ expression observed in single cells when the population was induced via deletion of



FIG 2 HapR-mediated repression of  $P_{chb}$  occurs on chitinous surfaces. (A) Representative images of the indicated V. cholerae strains grown on chitin beads. The parent strain background contains both a P<sub>chb</sub>-mCherry reporter and a P<sub>const2</sub>-GFP construct, which exhibits constitutive GFP expression. Scale bar = 10  $\mu$ m. (B and C) P<sub>chb</sub> expression in the indicated V. cholerae cbp<sup>+</sup> strains grown on chitin beads. (B) Representative images of cells that were vortexed off chitin beads. Arrows demarcate cells where P<sub>chb</sub> expression is induced ("P<sub>chb</sub> on" cells). Scale bar =  $2 \mu m$ . (C) Scatterplot and bar graph showing the relative expression of the P<sub>chb</sub>-mCherry reporter in cells cultured on chitin beads. Scatterplots (left y axis) represent the entire population, whereas the bars (right y axis) represent only the P<sub>chb</sub> on cells (bracketed in black on scatterplots). The percentage of cells in the  $P_{chb}$  on population is indicated. n = 2,120 for parent; n = 2,191 for  $\Delta hapR$  strain; n = 333 for parent P<sub>chb</sub> on; n = 1,139 for  $\Delta hapR$  strain P<sub>chb</sub> on. Data shown are from two independent biological replicates. (D and E) P<sub>chb</sub> expression of the indicated V. cholerae strains where P<sub>chb</sub> expression is induced by chitin oligosaccharides. (D) Representative images of cells grown with chitin oligosaccharides. Scale bar =  $2 \mu m$ . (E) Scatterplot showing the relative expression of a P<sub>chb</sub>-mCherry reporter in the indicated V. cholerae strains. n = 2,735 for parent; n = 2,384for  $\Delta hapR$  strain. Data shown are from two independent biological replicates. (F and G) P<sub>chb</sub> expression of the indicated V. cholerae strains where P<sub>chb</sub> expression is induced via deletion of cbp. (F) Representative images of cells grown in the absence of chitin. Scale bar =  $2 \mu m$ . (G) Scatterplot showing the relative expression of a P<sub>chb</sub>-mCherry reporter in the indicated V. cholerae strains. n = 2,407 for  $\Delta cbp$  strain; n = 2,313 for  $\Delta hap R \Delta cbp$  strain. Data shown are from two independent biological replicates. Statistical comparisons in panels C, E, and G were made using Student's t test. \*\*\*, P < 0.001.

*cbp* (Fig. 2G). Finally, native *chb* transcripts (measured via reverse transcriptionquantitative PCR [qRT-PCR]) were also induced ~2-fold higher in a  $\Delta hapR$  mutant compared to that of the parent when strains were induced with chitin oligosaccharides (see Fig. S2 in the supplemental material). Together, these results indicate that HapR is a bona fide repressor of P<sub>chb</sub> under physiologically relevant inducing conditions.

HapR repression of  $P_{chb}$  is conserved in other V. cholerae El Tor isolates. Previously, it was suggested that HapR was an activator of  $P_{chb}$  expression in the V. cholerae El Tor isolate A1552 (16). Specifically, the authors showed that deletion of hapR resulted in a decrease in  $P_{chb}$  expression when cells were cultured on chitin flakes. In the present study, we use the V. cholerae El Tor isolate E7946 to study  $P_{chb}$  regulation. To assess if HapR exhibits different effects on  $P_{chb}$  expression depending on the strain background, we assessed expression of a  $P_{chb}$ -mCherry reporter in hapR<sup>+</sup> and  $\Delta hapR$  derivatives of both E7946 and A1552. Consistent with our previous results, we observed that  $P_{chb}$  expression is elevated ~2-fold in the  $\Delta hapR$  derivative of both strain backgrounds when induced with chitin oligosaccharides or via deletion of *cbp*, which is consistent with HapR acting as a repressor of this locus (see Fig. S3 in the supplemental material). It is unclear what explains the discrepancy between our findings and those that were previously reported; however, these data suggest that they cannot be attributed to differences between strain backgrounds.

Deletion of HapR does not confer a growth advantage during growth on chitobiose. Our data indicate that in the absence of HapR,  $P_{chb}$  expression is elevated. The *chb* locus encodes the genes required for uptake and degradation of the chitin disaccharide, chitobiose. Thus, we wanted to assess if the increased expression of  $P_{chb}$  confers a fitness advantage to  $\Delta hapR$  mutant cells during growth on chitobiose. To test this, we conducted competitive growth assays with a 1:1 mixture of a parent and a  $\Delta hapR$  mutant strain on minimal medium with chitobiose as the sole carbon source. We hypothesized that if the  $\Delta hapR$  mutant had a competitive growth advantage due to increased expression of *chb*, then it should outcompete the parent strain in this assay. Even after ~48 generations of growth on chitobiose, however, we did not observe a competitive advantage for the  $\Delta hapR$  mutant (see Fig. S4 in the supplemental material). HapR is a global regulator that controls the expression of dozens of genes (24). Thus, even though a  $\Delta hapR$  mutant has increased  $P_{chb}$  expression, there may be negative pleiotropic effects associated with the  $\Delta hapR$  mutant during growth on chitobiose.

**HapR binds**  $P_{chb}$  *in vitro* and *in vivo*. Thus far, our data suggest that HapR is a repressor of  $P_{chb'}$ , but it does not distinguish whether HapR is a direct or indirect regulator of this locus. To assess if HapR could be regulating  $P_{chb}$  directly, we used an *in silico* approach to identify putative HapR binding sites (BSs) in  $P_{chb}$  based on consensus binding sequences generated for the HapR homologs LuxR (from *Vibrio harveyi*) and SmcR (from *V. vulnificus*) via chromatin immunoprecipitation sequencing (ChIP-seq) (25, 26). Using the Motif Alignment and Search Tool (MAST) in the Multiple Em for Motif Elicitation (MEME) suite (27), we identified two potential HapR binding sites in  $P_{chb}$  (Fig. 3A). Interestingly, these binding sites overlap with other elements in the *chb* promoter required for transcriptional activation. HapR BS 1 overlaps with the SImA binding site, which is a critical activator of  $P_{chb}$  expression (11). HapR BS 2 overlaps with the -35 signal, which is required for RNA polymerase to bind to the promoter and initiate transcription. This sequence analysis suggests that the repressive effect of HapR may be due to HapR binding directly antagonizing SImA and/or RNA polymerase (RNAP) at this locus.

We next sought to determine whether HapR could directly bind to  $P_{chb}$  using both *in vitro* and *in vivo* approaches. First, we tested whether HapR could bind to  $P_{chb}$  in vitro using electrophoretic mobility shift assays (EMSAs). Consistent with the presence of two HapR binding sites in  $P_{chb}$ , we observed two shifts by EMSA when using a DNA probe of the *chb* promoter (Fig. 3B). HapR does not regulate the ClpP promoter and thus did not bind  $P_{clpP}$  (Fig. 3B), which is consistent with previous studies (28). EMSAs were also done using 32-bp probes that encompassed each putative HapR BS (probe sequences



**FIG 3** HapR binds  $P_{chb}$  in vitro and in vivo. (A) Promoter map of  $P_{chb}$  highlighting putative HapR binding sites (BSs). Other sites required for  $P_{chb}$  activation (the SIMA BS and the -35 and -10 signals) are highlighted. The exact sequences of the region containing the HapR BSs (bolded), the SIMA BS, and the -35 signal are shown. The transcriptional start site (+1) and the translational start site (+137) are also shown. (B) A representative EMSA using HapR and Cy5-labeled DNA probes of the indicated promoters. The  $P_{chb}$  probe was incubated with (from left to right) 0 nM (-), 12.5 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, or 800 nM purified HapR. The  $P_{clpP}$  probe was incubated with 0 nM (-) or 800 nM HapR (+). (C) A representative EMSA using HapR and 32-bp Cy5-labeled probes encompassing each of the putative HapR binding sites within the *chb* promoter (exact probe sequences are shown in panel A). The 32-bp probes were incubated with (from left to right) 0 nM (-), 100 nM, 200 nM, 400 nM, 800 nM, 1.6  $\mu$ M, or 3.2  $\mu$ M purified HapR. (D) ChIP-qPCR assays showing enrichment of the indicated promoters relative to *rpoB*, a reference locus that HapR does not bind to. Data are from five independent biological replicates and are shown as the mean  $\pm$  SD. Statistical comparisons were made by Student's *t* test. \*, P = 0.0240.

can be found in Fig. 3A). We observed that HapR was able to bind to both probes, suggesting that HapR binds to both HapR BS 1 and HapR BS 2 (Fig. 3C). Next, we wanted to assess if HapR bound to  $P_{chb}$  in vivo via ChIP assays under physiologically relevant conditions. To that end, we first generated a FLAG-HapR strain that was functional for regulating  $P_{chb}$  expression (see Fig. S5 in the supplemental material). Using this strain in ChIP-quantitative PCR (qPCR) assays, we found that  $P_{chb}$  was, indeed, bound by HapR *in vivo*, while the negative control  $P_{clpP}$  locus was not bound by HapR (Fig. 3D). Together, these data demonstrate that HapR binds to  $P_{chb'}$ , which suggests that it is a direct repressor of this locus.

Quorum sensing regulates expression of  $P_{chb}$  through the cholera-specific autoinducer CAI-1. HapR is the master regulator of quorum sensing (QS) in *V. cholerae* and is highly expressed at high cell density (HCD) (29). Thus far, we have established that HapR is a repressor of  $P_{chb}$  expression. Next, we wanted to probe the role of QS in regulating  $P_{chb}$ . To that end, we sought to test the effect of mutations in genes upstream of HapR in the *V. cholerae* QS cascade on  $P_{chb}$  induction and HapR protein levels.

QS in *Vibrio* species is controlled by autoinducer-responsive sensor proteins that indirectly modulate phosphorylation of the response regulator LuxO, which in turn indirectly regulates production of HapR (14). When autoinducer concentrations are low (i.e., at low cell density [LCD]), multiple histidine kinase sensors act as kinases (30–32). This results in high levels of phosphorylated LuxO, which prevents HapR production (see Fig. S6A in the supplemental material) (33). By contrast, at high autoinducer concentrations (i.e., at HCD), the sensors act as phosphatases, which ultimately leads to dephosphorylation of LuxO and allows for HapR production (Fig. S6B) (14, 34).



**FIG 4** Quorum sensing regulates expression of  $P_{chb}$  through the cholera-specific autoinducer CAI-1. Expression of a  $P_{chb}$ -GFP reporter and HapR protein levels were determined in the indicated strains. The parent strain contains a  $P_{chb}$ -GFP reporter and a  $\Delta cbp$  mutation. A representative Western blot is shown below the bars to indicate the protein levels for HapR and RpoA (a loading control) in the corresponding strains. An antibody against LuxR, which has 72% identity and 86% similarity to HapR, is cross-reactive with HapR and so was used to detect HapR protein levels. Statistical comparisons were made by one-way ANOVA with Tukey's posttest. NS, not significant. \*\*\*, P < 0.001. The  $P_{chb}$ -GFP fluorescence data for the "parent" and " $\Delta clpA$ " strains are identical to the data presented in Fig. 1 and were included here to ease comparisons.

First, we assessed the impact of HCD on  $P_{chb}$  induction and HapR levels by deleting *luxO*, which genetically locks cells in an HCD state. Induction of the parent strain is tested under HCD conditions; thus, as expected, HapR levels were similar between the parent and the  $\Delta luxO$  mutant; accordingly, expression of  $P_{chb}$  was also similar between the parent and  $\Delta luxO$  mutant (Fig. 4). Next, we tested the effect of LCD on  $P_{chb}$  and HapR expression by generating a  $luxO^{D47E}$  (D-to-E change at position 47 encoded by *luxO*) mutant, which mimics phosphorylated LuxO and genetically locks cells in an LCD state. In this strain, we saw that  $P_{chb}$  expression increased and was correlated with a decrease in HapR protein levels (Fig. 4). Together, these data suggest that HapR repression of  $P_{chb}$  is mediated through the canonical QS circuit.

Next, we wanted to move further upstream in the QS regulatory cascade to address whether distinct autoinducers differentially affected expression of P<sub>chb</sub> and HapR. There are four parallel histidine kinase sensors that coordinate QS-dependent control of HapR expression (13); however, the autoinducer signal is only known for two of these systems. The sensor LuxPQ is responsive to the interspecies autoinducer AI-2, and the sensor CqsS is responsive to the V. cholerae-specific autoinducer CAI-1 (Fig. S6) (35). To assess the role of each autoinducer in regulation of  $P_{chb'}$  we made mutations to the synthase genes responsible for production of each autoinducer. LuxS makes AI-2 (36) and CqsA makes CAI-1 (35) (Fig. S6). In a strain that no longer produces AI-2 (ΔIuxS strain), expression of  $\mathsf{P}_{chb}$  was similar to that observed in the parent, and HapR levels remained similar in these two strains (Fig. 4). By contrast, a strain that is unable to produce CAI-1 ( $\Delta cqsA$  strain) had increased expression of P<sub>chb</sub> likely due to the low level of HapR produced (Fig. 4). The observed decrease in  $P_{chb}$  expression in the  $\Delta cqsA$ strain background was, indeed, due to a lack of CAI-1 production, as exogenously adding back synthetic CAI-1 restored HapR levels and repression of  $P_{chb}$  to the parent level (Fig. 4). In addition, a strain that does not make CAI-1 induced P<sub>chb</sub> to the same level as that of a strain that does not produce both CAI-1 or HapR (Fig. 4). This epistasis indicates that CAI-1 production and HapR are involved in the same regulatory pathway for modulating expression of P<sub>chb</sub>. These data support previous results, which indicate that CqsS signaling plays a dominant role in regulating HapR (23, 35, 37, 38). Together, these results indicate that P<sub>chb</sub> expression is strongly influenced by QS signaling mediated by the V. cholerae-specific autoinducer CAI-1 and less via the interspecies autoinducer AI-2.



**FIG 5** Model of quorum sensing regulation of chitobiose utilization genes in *V. cholerae*. When *V. cholerae* grows on a chitinous surface, the periplasmic chitin binding protein (CBP) binds to chitin oligosaccharides. This derepresses ChiS, which can then activate expression of the chitobiose utilization operon (*chb*) through recruitment of RNA polymerase (RNAP) (10). Binding of SImA to  $P_{chb}$  is also required for transcriptional activation (11). (A) At low CAI-1 concentrations, the CqsS sensor indirectly (dashed line) promotes LuxO phosphorylation, which indirectly blocks HapR expression. In this state,  $P_{chb}$  is maximally expressed. (B) At high CAI-1 concentrations, the CqsS sensor indirectly dephosphorylates LuxO, which results in indirect activation of HapR expression. HapR then exerts a repressive effect on  $P_{chb}$  by decreasing expression of this locus ~2-fold in the presence of the ClpAP protease machine. If HapR is not degraded by ClpAP, its repressive effect can result in an ~7-fold decrease in  $P_{chb}$  expression. The mechanism of HapR repression may be through occluding binding of SImA or RNAP to  $P_{chb}$ .

## DISCUSSION

Here, we show that HapR acts as a repressor of chitobiose utilization genes in *V. cholerae.* When this organism forms communities on a chitinous surface, chitin induces expression of  $P_{chb}$  through activation of the chitin sensor ChiS (Fig. 5). *V. cholerae* then modulates  $P_{chb}$  expression depending on the presence of other *V. cholerae* cells, which it senses via the *V. cholerae*-specific autoinducer CAI-1. At low CAI-1 concentrations,  $P_{chb}$  expression is high because the repressor HapR is produced at a low level (Fig. 5A). At high CAI-1 concentrations, HapR is produced at higher levels and can repress  $P_{chb}$  expression (Fig. 5B). As the HapR binding sites in  $P_{chb}$  overlap with the SIMA binding site and the -35 signal, it is possible that HapR competes with these activators for binding at  $P_{chb}$ . Thus, HapR-mediated repression may occur through antagonism of the binding activity of SIMA and/or RNAP at  $P_{chb}$ .

When HapR is produced, it gets proteolyzed by the ClpAP machine (Fig. 5B). While the dynamic range of HapR-dependent  $P_{chb}$  repression is only  $\sim$ 2-fold when ClpAP is intact, the dynamic range of repression increases to  $\sim$ 7-fold in the absence of ClpAP. Thus, it is tempting to speculate that regulation of ClpAP may modulate  $P_{chb}$  expression under some conditions. Currently, however, little is known about the regulation of ClpAP in V. cholerae. ClpAP is induced during heat shock in V. vulnificus (21). Also, in Escherichia coli, ClpP expression is induced during heat shock (39-41). Thus, it is believed that the CIpP protease plays a role in the heat shock response. In Bacillus subtilis, ClpP is upregulated under various stress conditions, suggesting that this protease may also be induced by a general stress response (42). Thus, one possibility is that stress-dependent regulation of CIpAP indirectly regulates P<sub>chb</sub>. It has also been hypothesized that V. cholerae makes use of proteolysis machines to rapidly respond to changes in their environment; namely, the transition from the human gut to the aquatic environment after infection (43). ClpAP acting as an activator of P<sub>chb</sub> may be a way for cells to rapidly induce expression of a locus that is not important in the human gut but is useful for survival in its aquatic reservoir. Consistent with this idea, chb is induced late

in infection, suggesting that this is a critical locus for preparing to reenter the aquatic environment after infection of a host (44).

The mechanisms underlying distinct responses to AI-2 or CAI-1 remain a topic of interest in QS. It has been shown that the CAI-1 sensor CqsS plays a dominant role over LuxPQ in modulating HapR levels. Thus, it remains possible that the presence of CAI-1 allows for more robust regulation of HapR-regulated genes. CAI-1 has been shown to be critical for expression of the virulence factor hapA (38), natural transformation (15), and for repressing chitobiose utilization as shown in this study. HapA is a protease that has been implicated in mediating V. cholerae detachment from host epithelial cells, thereby promoting dissemination of cells back into the aquatic environment (45, 46); natural transformation and chitin utilization aid in V. cholerae fitness in the marine environment. By contrast, expression of tcpA, a protein that contributes to V. cholerae pathogenesis in the human gut, was shown to be primarily regulated by AI-2 (37). It is tempting to speculate that CAI-1 controls V. cholerae behaviors important for marine survival, whereas AI-2 controls behaviors involved in infection. It has been hypothesized that sensing both of these autoinducers plays a critical role in biofilm dispersal and that only when both are sensed are V. cholerae cells induced to leave a surface (i.e., QS works as a coincidence detector for both signals) (37). Thus, signaling via distinct autoinducers may allow V. cholerae to modulate responses depending on the context of the environment that they inhabit.

The data that we present here suggests that at HCD, V. cholerae dampens its expression of a chitin utilization locus. Below, we speculate on a few reasons why this regulation may be advantageous. Chitin polymers in the shells of crustacean zooplankton are long-chain polysaccharides in a crystalline insoluble lattice. In order to be used as a nutrient source, the long-chain chitin must be broken down into smaller, soluble chitin oligosaccharides. V. cholerae secretes chitinases that enzymatically degrade insoluble chitin into soluble chitin oligosaccharides for uptake and catabolism. The production and secretion of chitinases is an energetically costly process; thus, liberated chitin is a valuable "public good" in the context of a chitin biofilm (47). So, it is possible that QS regulation of chitin utilization allows V. cholerae to modulate chitin uptake based on the composition of its microbial community. When the level of V. cholerae cells (and the corresponding concentration of cholera-specific autoinducer CAI-1) in the community is high, chitin uptake may decrease among individual cells within the population in an effort to "share" liberated chitin oligosaccharides. By contrast, when the level of V. cholerae in the community is low, sensing of only the interspecies signal Al-2 (which is produced by many bacterial species) does not suppress chitin uptake and utilization, thus, allowing the V. cholerae within this population to maximally compete for liberated chitin oligosaccharides.

Another possibility is that this regulation allows *V. cholerae* to control its production of toxic metabolites. In a previous study, it was shown that cells in an HCD state alter their metabolic flux to produce neutral by-products as opposed to organic acids when grown in LB supplemented with a fermentable sugar like glucose (48). This regulation allows for a more stable community; by contrast, cells locked in an LCD state will excrete harmful metabolic by-products, which leads to the demise of the community (48). It has been shown that *V. cholerae* excretes ammonium as a potentially toxic by-product when grown on chitin (16). Also, because chitin oligosaccharides likely feed into glycolysis, it is possible that catabolism of chitin results in the production of potentially toxic organic acids. Thus, repressing chitin uptake and utilization may also help slow the rate of metabolism to prevent the accumulation of toxic intermediates in a dense community setting.

#### **MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *V. cholerae* strains were routinely grown at 30°C in LB medium and on LB agar supplemented when necessary with carbenicillin ( $20 \mu g/ml$  or  $50 \mu g/ml$ ), kanamycin ( $50 \mu g/ml$ ), spectinomycin ( $200 \mu g/ml$ ), trimethoprim ( $10 \mu g/ml$ ), and/or chloramphenicol ( $2 \mu g/ml$ ). Strains were grown in defined artificial salt water (DASW) medium for microscopy (see below

for details), Instant Ocean for generating mutant strains (see below for details), or M9 minimal medium for competition experiments (see below for details).

**Transposon mutagenesis.** Transposon mutant libraries were generated with a Carb<sup>r</sup> mini-Tn10 transposon exactly as previously described (49). Briefly, the transposon mutagenesis plasmid pDL1086 was first mated into parent strains containing chromosomally integrated  $P_{chb}$ -lacZ and a  $\Delta cbp$  mutation (activator screen) or  $\Delta clpA \Delta cbp$  mutations ( $\Delta clpA$  strain counterscreen). The parent strains also carried a chromosomally integrated  $P_{chb}$ -mCherry reporter at an ectopic site to ensure that candidate transposon mutants affected expression of  $P_{chb}$  and did not simply disrupt the  $P_{chb}$ -lacZ reporter. The activator screen parent also carried or of  $P_{chb}$ . Transposition was induced by plating cultures on LB agar supplemented with 50  $\mu$ g/ml carbenicillin (Carb) at 42°C. To screen colonies, plates also contained 40  $\mu$ g/ml X-Gal and 5 mM isopropyl- $\beta$ -o-thiogalactopyranoside (IPTG). IPTG was added to competitively inhibit the basal activity of the  $P_{chb}$ -lacZ reporter.

The sequences of transposon-genomic junctions in transposon mutants were determined by inverse PCR followed by Sanger sequencing. Briefly, genomic DNA was purified from mutants of interest and digested with the Fatl restriction enzyme per the manufacturer's instructions (NEB). Digested genomic DNAs were then incubated with T4 DNA ligase per manufacturer's instructions (NEB) to generate self-ligated circles. The transposon-genomic junction was then amplified by PCR using the primers specified in Table 1 and subsequently Sanger sequenced (Eurofins Genomics).

Generating mutant strains. V. cholerae E7946 served as the parent for all strains generated in this study, except for those used in Fig. S3 in the supplemental material where we compare E7946 to A1552 (50, 51). Mutant constructs were generated by splicing by overlap extension PCR (SOE PCR) exactly as previously described with the primers indicated in Table 1 (52). PCRs were performed to generate up (F1/R1), middle, and down (F2/R2) arms. Up and down arms were designed to have 3-kb arms of homology to the genome at the site targeted for mutagenesis. All three arms were then mixed and used as a template for SOE PCRs with the F1 and R2 primers to generate the full-length mutant construct SOE product. Mutant constructs were introduced into V. cholerae cells by chitin-induced natural transformation and/or cotransformation exactly as previously described (53, 54) or by chitin-independent transformations using a plasmid that ectopically expresses tfoX and qstR as previously described (55). For chitin-dependent natural transformation, V. cholerae was grown to mid-log in LB medium, washed with Instant Ocean medium (7 g/liter; Aquarium Systems), and incubated with chitin flakes (Alfa Aesar) at a final optical density at 600 nm (OD<sub>600</sub>) of 0.1 overnight at 30°C. The next day, SOE PCR products were added to chitin-induced cells, incubated at 30°C for 5 h, and then outgrown and plated on selective media. For chitin-independent transformation, cells harboring pMMB67EH-tfoX-qstR were grown overnight in LB medium supplemented with 100  $\mu$ M IPTG and 1  $\mu$ g/ml chloramphenicol. The next day, 7  $\mu$ l of the overnight culture was diluted into 350  $\mu$ l of Instant Ocean medium. Then, SOE products were added as described above for chitin-dependent transformations. Mutant strains were confirmed by colony PCR, mismatch amplification mutation assay (MAMA) PCR (56), and/or sequencing. Complete lists of strains and primers used in this study are outlined in Table 2 and Table 1, respectively.

**Measuring GFP and mCherry fluorescence in bulk populations.** GFP and mCherry fluorescence in reporter strains were determined exactly as previously described (57). Briefly, single colonies were grown in LB overnight at 30°C. Where indicated, medium was supplemented with 10  $\mu$ M CAI-1. CAI-1 was synthesized exactly as previously described (58). The next day, cells were washed and resuspended to an OD<sub>600</sub> of 1.0 in Instant Ocean medium (7 g/liter; Aquarium Systems). Fluorescence was determined on a BioTek H1M plate reader. For GFP measurements, excitation was set to 500 nm and emission was set to 540 nm; for mCherry measurements, excitation was set to 580 nm and emission was set to 610 nm.

**Antibody generation.** Purified *Vibrio harveyi* LuxR protein (300  $\mu$ g; purified as previously described [59]) was sent to Cocalico Biologicals, Inc. for serial injection into a rabbit host for antibody generation. Serum obtained from the third bleed has been used for Western analyses.

**Western blotting.** From overnight cultures, cells were concentrated to an OD<sub>600</sub> of 20 in Instant Ocean medium. Cells were lysed on a FastPrep-24 classic instrument at 4°C, and then lysates were clarified by centrifugation. Lysates were then boiled with an equal volume of 2× SDS-PAGE sample buffer (220 mM Tris, pH 6.8, 25% glycerol, 1.2% SDS, 0.02% bromophenol blue, and 5%  $\beta$ -mercaptoethanol). Proteins were separated on a 15% SDS polyacrylamide gel by SDS electrophoresis, electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with rabbit polyclonal  $\alpha$ -LuxR serum or mouse monoclonal  $\alpha$ -RpoA (BioLegend) primary antibodies. LuxR is the HapR homolog in *Vibrio harveyi* and has 72% identity and 86% similarity to *V. cholerae* HapR. The LuxR antibody was empirically found to be cross-reactive with *V. cholerae* HapR, and so it was used to detect HapR protein levels. Blots were then incubated with  $\alpha$ -rabbit or  $\alpha$ -mouse horseradish peroxidase (HRP)-conjugated secondary antibodies, developed using Pierce ECL Western blotting substrate (Thermo Fisher), and exposed to film.

**Chitin bead culturing.** Chitin beads (200  $\mu$ l of a 50% slurry; NEB) and overnight cultures of the indicated strains were washed with defined artificial salt water (DASW) medium, which was prepared exactly as previously described (8). Chitin beads were inoculated with *V. cholerae* cells to an OD<sub>600</sub> of 0.1 in a final volume of 5 ml in a Costar 6-well plate (Corning). Chitin mixtures were incubated statically at 30°C for 7 days before imaging.

**Microscopy data collection and analysis.** To image chitin beads, cultured beads were gently transferred to a coverslip using wide-bore pipette tips. To image chitin-grown cells, chitin bead reaction mixtures were vortexed and then centrifuged at  $250 \times g$  for 1 min. Cells found in the supernatant were transferred to a coverslip. To image  $\Delta cbp$  strains, overnight cultures grown in LB were washed and resuspended to an OD<sub>600</sub> of 0.2 in DASW and then transferred to a coverslip. Samples on the coverslip

# TABLE 1 Primers used in this study

Primer	Sequence <sup>a</sup>	Description	
Used for inverse PCR	-		
BBC 244	CCCGGGATCCTGTGTGAAATTGTTATCCGC	Tn10-specific primer for inverse PCR	
BBC 434	GTGTGGGCACTCGACATATGACAAG	Tn10-specific primer for inverse PCR	
Used for reporter constructs			
ABD 332	GGCTGAACGTGGTTGTCGAAAATGAC	$\Delta lacZ$ F1 (up arm)	
BBC 219	GTTTATTTTGTCGACTGTACAGCGTTTAAATAGAGGTCGATATTGACCC	$\Delta lac Z B1$ (up arm)	
		$\Delta V C 1907 E1 (up arm)$	
BBC 719			
BBC 218	CGCIGIACAGICGACAAAAAIAAAC	Kan' F (middle arm)	
BBC 262	TACCGAGGACGCGAAGCTG	Kan <sup>r</sup> R (middle arm)	
BBC 266	CAGCTTCGCGTCCTCGGTAGAATAAAGCAATCCGCAAGCG	P <sub>chb</sub> F (middle arm)	
BBC 267	CCCGGGATCCTGTGTGAAATTGAGTTGCTTTCATTTCACTAATGG	P <sub>chb</sub> R (middle arm)	
BBC 732	ctcaagccgaggagtaaagaagAGTTGCTTTCATTTCACTAATGG	P <sub>chb</sub> fuse to <i>lacZ</i> R (middle arm)	
BBC 252	CAATTTCACACAGGATCCCGGGAGGAGGTAACGTAATGCGTAAAGGAGAAGAAC	GFP F (middle arm)	
BBC 254	tgtaggctggagctgcttcTTAGTTGTATAGTTCATCCATGCC	GFP R (middle arm)	
BBC 1356	TTGTTTCGGCGTGGGTATGGTGCGCTGTACAGTCGACAAAAATAAAC	mCherry F (middle arm)	
BBC 206	tatagactagagctacttcttacttatacagctcgtccatg	mCherry R (middle arm)	
ABD 785		P $_{ij}$ stitch to $lacZ \in (down arm)$	
ABD 255		$\Lambda lacZ E2$ (down arm)	
ABD 256	CCCAAATACGCCAACTTCGCC	$\Delta lacZ B2$ (down arm)	
ABD 241		$\Delta V(C1807 E2 (down arm))$	
		$\Delta V C 1807 T 2 (down arm)$	
BBC 3083	tggataactttacggcatgcataaggctcgtataatatattcagggagaccacaacggtttccctctac	$P_{const2}$ F	
PPC 2002			
BBC 3082	tatacgagccttatgCatgCccgtaaagttatccagcaaccactatagacctagggcagcagtagatagg gacgacgtggtgttagctgtgCTCATTAGGCACCCCAGGC	P <sub>const2</sub> K	
Used for SOE deletion, FLAG fusions,			
and point mutants			
BBC 742	attccggggatccgtcgacCTGCAGTTCagaagcagctccagcctaca	MiniFRI F	
BBC 743	tgtaggctggagctgcttctGAACTGCAGgtcgacggatccccggaat	MiniFRT R Kapi Speci Carbi Cmi or Tmi	
ADD 123		cassette F	
ABD 124		cassette R	
ABD 796	TTAGAATCTGCGCCAGAAGCG	$\Delta cbp$ F1 (up arm)	
ABD 797	gtcgacggatccccggaatCATAGCTGTTCCTTACTAGTTGC	Δ <i>cbp</i> R1 (up arm)	
BBC 920	gtcgacggatccccggaatGCTCATCAGGTCGTCAGCC	Δ3' <i>cbp</i> R1 (up arm)	
ABD 798	gaagcagctccagcctacaGTACTGGATCTGAAACCAGTTAAG	Δ <i>cbp</i> F2 (down arm)	
ABD 799	GTATTGCGGAATGACCAGCATG	Δ <i>cbp</i> R2 (down arm)	
BBC 2755	TTACCCCTAAGTCGGCGAGC	ΔVC0995 F1 (up arm)	
BBC 2756	gtcgacggatccccggaatAATATTCACCTTAAGTTCCCCC	ΔVC0995 R1 (up arm)	
BBC 2757	gaagcagctccagcctacaCTGCCTTAATCGAGTTTAAACCC	ΔVC0995 F2 (down arm)	
BBC 2758	GCACCACGATAGCAATAAGC	ΔVC0995 R2 (down arm)	
CAK 407	GCTACGCACTGCCAAATTACC	Δ <i>clpA</i> or Δ <i>clpS</i> F1 (up arm)	
CAK 408	gtcgacggatccccggaatAAGCATAAGGCCTCCTTAAGGAAC	$\Delta clpA$ R1 (up arm)	
CAK 409	gaagcagctccagcctacaCACTAGACATCTCACAATACGC	$\Delta clpA$ F2 (down arm)	
CAK 410	CCGCTAACATCTCAGGACTG	$\Delta clpA$ or $\Delta clpS$ R2 (down arm)	
CKP 449	TGCTCGGTTTTGATCCGTTC	$\Delta clpP$ or $\Delta clpX$ F1 (up arm)	
CKP 450	gtcgacggatccccggaatGGGCGACATTGCATTTTTTC	$\Delta clpP$ R1 (up arm)	
CKP 451	gaagcagctccagcctacaGGCGAGTAAGCTCGTAATTG	$\Delta clpP$ F2 (down arm)	
CKP 454	GTCAAACTAGAAACCAGCGC	$\Delta clpP$ or $\Delta clpX$ B2 (down arm)	
CKP 447		$\Delta clnS B1$ (up arm)	
CKP 448	gaageageteeegguuttinketernenenenen and a	$\Delta c \ln S E2$ (down arm)	
CKD 452	atcascagatccccagatGTCTGTCATTCGCTAACCTC	$\Delta clp X R1$ (up arm)	
CKD 452		$\Delta clp X = R^2 (down arm)$	
CKD 180	σααγταγτιταγτιαταστουλοιλαιτιταποταλα ΤΤΓΩΔΓΩΓΤΓΓΩΔΔΔΩΓΩΔΩΔΩ	$\Delta (\mu \pi + 2 (\mu \mu \pi m))$	
		$\Delta lon \Lambda P1 (up all l)$	
		AlonA E2 (down arm)	
	yaaycayciicayciiada i uca i aucaaaaa i aau i aaa i c		
		Дларк FI (up arm)	
BBC 423	gtcgacggatccccggaat1111CGA11GA1GCG1CCA1AG		
	GICTUGTCatCgtcatccttataatcCATAGGGGTATATCCTIGCC	$I \times FLAG-napK R1 (up arm)$	
BBC 408	gaagcagctccagcctacaAACIAGIIICIIGGGCAGCACAA	$\Delta napR + 2$ (down arm)	
CKP 510	AIGgattataaggatgacgatgacaaaGACGCAICAAICGAAAAACG	$I \times FLAG-hapR F2$ (down arm)	
BBC 409	GIICAIAATGATTTCCTTGGTGCC	$\Delta hapR$ R2 (down arm)	
BBC 374	TGGCAAAAAGCGAGAGAAGAAG	∆ <i>luxO</i> F1 (up arm)	

(Continued on next page)

# TABLE 1 (Continued)

BRC 375     grcgarcggatcCaggatCATCAGCGACATATTTCTTC     Alb       CKP 319     TCATATCTGCCAAACTAATCACCACAGA     Mux       BRC 376     gaagcagttccagctacaTAAGCCATCAGCAATGACTCAAC     Alu       BRC 377     TCACACCCGAATTCCATCAC     Alu       CKP 318     TGACTTAAGCCAGATATGACG     Alu       BRC 377     TCACACCCGAATTCCATCAC     Alu       CKP 353     grcgacggatcccaggatCTGTTCATCGCAGATATACCCAG     Acq       CKP 354     GTCTCTTGACCAGCGGTTGTTCATCGCAAAAAAACC     Acq       CKP 355     grcgacggatcccaggatGCCGggatCTGTGTCATCGCAAAAAAAACC     Acq       CKP 359     gaagcagtcccaggatGCCGggatGCCGCGG     Alu       CKP 350     grcgacggatCcCGggatGCCCGGCACTCATAGCCAAAAAAAAAAAAAAAAAAAAAA	Description	
CKP 519       TCATATCTGGCAAACGTAACGCAGGATTAAGTCAGGG       Intelligend of the second of the se	Δ <i>luxO</i> R1 (up arm)	
BRC 376     gaagcagtecagtecagtacaTAAGCGATAGAGGAATGAATCAAC     Abu       CKP 518     TGACTTAATCCTGTCGAGATAGATGGACG     Abu       BRC 377     TCACACCCGAATTTACTGC     Abu       CRP 554     GTCTCTTAATCGC     Acq       CRP 555     gatogargetecaggeatCTTGTCATGG     Acq       CRP 556     gaagcagtecaggeatCTTGTCATAGCCATATATCCTAG     Acq       CRP 557     AGTGGAACCACTTCTTGTC     Acq       CRP 558     gatogargetecaggeatCTTGTCATAGGCATATAATAACAAAAAC     Acq       CRP 559     gatogargetecaggeatCTTGTCATAATGCATAATAACAAAAAC     Acq       CRP 551     gaagcagtecaggeatCACCACTAAGGCAACTAGGCA     Au       CRP 552     TTCTTAAGCTGATACATTGC     Au       ABD 334     AGTGCTCCGCGAACTTGCTGTGAAACG     Au       ABD 255     gaagcagteccaggatCACTCGCCACATAAGCCAAGAGCCTAAAG     Au       ABD 255     gaagcaggateccaggatCACTCACCCACATAAGCCAAGAGCCTAAAG     Au       ABD 725     GGAAGCAACTCAACTCACCCACATAGCCAAGAGCCTAAAG     Au       ABD 725     GGAAGCAACACTCAACCTCACAC     Det       CRP 463     CACATATCGGCCAATTGGCG     Au       CRP 463     CACATATCGGCCAATTGGCG     Ac       CRP 463     CACATATCCAGCTCAACCCTCAC     Ac       CRP 463     CACTATACGCGAAACTTGGCGAATTTGGC     Ac       CRP 463     CACATGCCAGTAACTCAGCCTCACCCCAC     Ac <t< td=""><td>luxOD47E R1 (up arm)</td></t<>	luxOD47E R1 (up arm)	
CKP 518     TGACTTAATCGTGCTGAGATTACGATTGCCAGATATGACG     Juke       GRB 377     TCACACCCGAATTCCATCATGC     Muke       CKP 553     GTGTCTTAGCCGAGGTACTG     ACAC       CKP 555     gtagaggatcccoggattGTGTGTTCATAGCCAATTATACTCAG     ACAC       CKP 557     AGTGGAACACTTCTTGTC     ACAC       CKP 558     gtagaggatcccoggattGGCGC     ALI       CKP 559     gtagaggatcccoggattGGCGC     ALI       CKP 550     gtagaggatcccoggattGGCGC     ALI       CKP 551     gtagaggatcccoggattGGCGC     ALI       CKP 552     TTCTTAGCTGGAACTCCCTGTTCCTCTC     ALI       CKP 551     gtagaggatcccoggattGCGCGCAACTTGGCG     ALI       CKP 552     TTCTAGCGGAACTTTGCTTGCTGG     ALI       CKP 553     gtagaggatcccoggattGCCGCGGAGTTAGG     ALI       CKP 550     gtagatggatcccoggattGCCGGGAGTTAGG     ALI       CKP 551     gtagatggatcccoggattGCCGGGAGTTAGG     ALI       ABD 255     gtagatggatcccoggattGCCCGGAGTTAGG     ALI       ABD 255     gtagatggatcccoggattGCCCGGAGTTAGG     ALI       ABD 255     GtagatggattCGCGGgattGCCAATTAGGC     ALI       CKP 463     TTGCCAAATTAGGCCAATTAGG     ALI       CKP 464     TTGCCAGTATTGCACCAGG     ALI       CKP 465     CCATGTGGTGGAAAGCCTTAGC     ALI       CKP 464     TTGCCAGTATTGGCAGCAATT	$\Delta luxO$ F2 (down arm)	
BBC 377     TCACACCCGAATTTCCATCATCC     Aug       CKP 554     GTCCTTLAGCCGACGTACTG     Aug       CKP 555     gragagatrcacgotasTTTGTTAATTCATAGCATATATCCTAG     Aug       CKP 556     gragagatrcacgotasTTTGTTAATTGCATAATAACCAAAAC     Aug       CKP 557     AGTTGGAACCACTTCTTGTC     Aug       CKP 550     gragagatrcacgotasTTGGTAATTGCATAATAACCAAAAAC     Aug       CKP 551     gragaggatrcacgotasTGGCAATTTCCTTGTCTCC     Aug       CKP 551     gragaggatrcacgotasTGGCAACCACTGGGTGTGAAACG     Aug       CKP 552     TTCTTAGCGTGGATCAATTGC     Aug       ABD 334     AGTGGTGGGACATTGGCG     Aug       ABD 235     gragaggatrcacgotacCACAATAGCCCAGAAAAGG     Aug       ABD 235     gragaggatrcacgotacCACAATAGCCAGAAAAGGCCTAAAG     Aug       ABD 235     gragaggatrcacgotacCACACATAGGGC     Aug       CKP 461     TTGGTAGCAACTTGGGG     Aug       CKP 463     CCAATATACGGGCTTGAGAACTTGGGG     Aug       CKP 463     CAAACTATGCAGCAACTTGGGG     Aug       CKP 463     CAAGTATCCAGGCTAAGGG     Aug       CKP 464     TTGGTAGCAACTTGGGG     Aug       CKP 465     CCATATGAAGGCTGAGGAATTAGG     Aug       CKP 465     CCATGTGGGATAGAACGCT     Aug       CKP 465     CCATGTGGGATAGAACGCT     Aug       CKP 465     CCATATGAAGGCTGGAA	luxOD47E F2 (down arm)	
CPD 554     GTCTTTAGCCCAAGGTACTG     LCG       CPD 555     gtcgacgattaccggattaCTGTTATATGCAAATATATCCTAG     LCG       CPD 556     gtagtcagtcasgcattaCTTGTTAATGCAAAATAACAAAAAC     LCG       CPD 557     AGTTGGAACCACTTGTCC     LCG       CPD 559     gtagtcagtcasgcattaCTTGTTAATGCATAAATAACAAAAAC     LCG       CPD 559     gtagtcagtcasgcattaCTTGTTAAATGCATAAATAACAAAAAAC     LCG       CPD 550     gtagtcagtcasgcattaCCGGCATTGTCTCTC     LGG       CPD 551     gtagtcagtcasgcattaCCGGCATCTGTTGTAAAGCG     LLG       ABD 235     gtagtcagtcaggtaTCGCGGATGAGGCAGAGGATAAAG     LLG       ABD 235     gtagtcaggtagtcaggtaCCCGGAGATAAGCCAAGAGGCCAACGCCAAGAGGCCTAAGG     LLG       ABD 235     gtagtcagtcaggtaCCCGGATGAGCCAAGAGCCAACCCACCAAGAGCCCAACGCCAAGAGCCTAACGCAAGAGGCCTAAGC     LLG       ABD 235     gtagtcagtcaggtaCCCGGaTGACAGCCAAGAGCCAACCCACCAG     LLG       ABD 235     gtagtcagtcaggtaCCCGGaTGACAGCCAAGAGCCAACCCCAGC     LLG       BBC 082     gtcagtcaggtaCCCGGaTGACACCCACAAGCCCACCAGC     LLG       CKP 443     AAAGTCCACCTGCAGCGCCACCGC     LLG       CKP 445     CCATGTGTGATAGACACCCACGG     LLG       CKP 443     CAGTCGCACTGCTCGCACCGC     LLG       BBC 1911     CCTATACCACCAGAACCTGACCCAGAG     LLG       CKP 558     AATTCGTACCACGAAACCTGACCCAGAG     LLG       CKP 559     TTGCCAAAACGTGT	$\Lambda \mu x O B2$ (down arm)	
CKP 955     orgacgatzczeggaatCTGTCTCATCGCAATATATCCTAG     bcq       CKP 956     gaagcagtcczeggaatCTGTCTCATCGCAATATAACCAAAAAC     bcq       CKP 957     AGTTGGAACCACTCTTGTC     bcq       CKP 550     gragcagtcczggaatGGGATTCCTTGTC     bcq       CKP 551     gaagcagtcczggaatGGCATTCCTTGTC     bcq       CKP 550     gragcaggtcczggaatGGCATTCCTTGTCACC     blu       CKP 551     gaagcagtcczggaatGCAATTGCTTGTAAACG     blu       ABD 253     gaagcagtcczggaatGCACGATCTTGCTCG     blu       ABD 254     gtcgacggatccccggaatGCACGATCATTGCC     blu       ABD 255     gaagcagtcczggataCCAGCATAATGCCAAGGCAGGAGTAAAG     blu       ABD 256     CCCAAATACGGCAATCTGGCCA     bet       BBC 082     gtcgacggatccccggataCATGGACTAACTACACTTACCCCAG     bdu       CKP 463     CAATATCGGACATTGGCACTACGGGG     bdu       CKP 463     CAATATCGGACATTGGCACTG     bdu       BBC 010     TAAATGGGACATCGGACAACC     bdu       CKP 463     CAATGGGACATTGGTCACCGG     bdu       CKP 463     CAATGGCACTTGGTCACCG     bdu       CKP 463     CAATGCGAAACTGGCACAGC     bdu       BBC 010     TAACAGGGCATTGGTCACCG     bdu       CKP 453     CAGTGCACTTGGTCACCG     bdu       BBC 1910     CAATGCGAAACTGCACGG     bdu       BBC 744     cagttgcggtgatg	$\Delta casA = F1$ (up arm)	
Chi 322     gragagaticagicaciaTTCGTTAATGCATAATGACAAAAAC     Lac       CKP 356     gragagaticagiccagicataTTCGTTAATGCATAATGACAAAAAC     Lac       CKP 359     TICGAACCACTTCTTCC     Lac       CKP 350     gragagaticagiccacicaGCACCGC     Mui       CKP 351     gragagaticagiccacicaGCACACTTCCTTGTAAACG     Mui       CKP 352     TTCTTAGCGTGATCATTGC     Mui       CKP 353     gragaggaticagiccacicaGCACACTAATGCCCAGGAGTAAAG     Mai       ABD 334     ACTGCTCCGGACCTTTGCTCTG     Mai       ABD 255     gragaggaticagiccacicCACACATAGCCCAGACCCAAGAGCCTAACG     Mai       ABD 255     gragaggaticagiccacicCACAATAGCCCAACCCACACCACACCCAGGAGCTTAACG     Mai       ABD 255     GAAGCACCTCCACCCCCACAC     Det       BBC 082     gragaggaticagiccacicCAGACACCCAACACCCAAGAGCCCACCCAGA     Mai       CKP 463     AAAGTTCCAGCTCACGCGG     Mai       CKP 463     CAGTGCACTTGGGACTGCAGCGG     Mai       BBC 082     GCAGTGCACTTGGCACCTG     Mai       BBC 1911     CGTAATCCAACGCCAGACCCCACCCCGG     Mai       CKP 533     TGGACCACGAAACGTTAACGCCAGACTCAG     Mai       BBC 7759     TTGCAACTGAAGGAATTAG     Mai       BBC 744     cagcttcgggatagagaga     X       ABD 245     CCATATAGCAGAAAAGCATTTGCTCAGC     Mai       CKP 520     TCATATCTAACTGGAAGAAGGAATTAGAAAAGCCAAGA	$\Delta cqsA B1 (up arm)$	
Chr. 330     glaguaguaguctua ITTOTACIDA IDA IDA IDA IDA IDA IDA IDA IDA IDA	AcasA E2 (down arm)	
CM 337     AGTIGOLCACTICITICSTC     Diag       CM 349     TACAGGARCCACTCITICSTC     Diag       CM 549     TACAGGARCCACTGC     Diag       CKP 550     gtcgacgattccccggaatTGGCAGC     Diag       CKP 551     gacgagctccccgtcaccACTTAGTCGTTGTAAACG     Diag       ABD 334     AGTGCTCGGACTCTTTCGTCTG     Diag       ABD 354     gtcgacgagtccccgtcaccCCTACATTGC     Diag       ABD 255     gacgagctccccgtcaccCCACATAGCCCAGGGGGTAAAG     Det       ABD 255     GGACACGTCCCACCTTCGC     Det       BBC 082     gtcgacgagtccccgtcaccCTACA     Det       BBC 082     gtcgacgagtccccgtcacCTACA     Det       BBC 083     AAGTTGGTGCGATAATGTGC     Det       BBC 084     TTGGCAGAACATCTTTGGTC     Det       CKP 463     CCATGTGCGATAACGCC     Det       CKP 463     CCATGTGCGATAGACAACC     Det       BBC 082     CATGTGCGATAGACAACC     Det       BBC 101     TAAATGGGGCTTGGCACATG     Dia       BBC 1911     CGTAATCAAACTGGGAGATTTAG     Dia       BBC 759     TGGACACGAGACCTTTAACG     Dia       BBC 744     Caggttcggtgacgagagaga     IX       ABD 330     CACTGAGACAGTCTTAGGCAAACGTCACAGC     Dia       BBC 744     CaggttcggtgacGGGAAATGTAGAAAACCTAAG     Pad       CKP 505     TAAGCAAAC	Acquire A P2 (down ann)	
CKP 349     IACAAC USC INGCACGC     Mut       CKP 550     gragagateccaggattacGCATTICCTTCCCC     Mut       CKP 551     gaagcagtcccaggattacGACTTAAGTCGGTTCTGTAAACG     Mut       ABD 334     AGTGCTCCGGACTCTTTGCTCTG     Mut       ABD 254     gragagatcccaggattacagcattacCCCTCAAGCCGAGGATAAAG     Mat       ABD 255     gaagcagttccaggattacagcattacCCCTCAAGCCGAGGAGGACTAAG     Mat       ABD 256     CCCAAATACGGCAACTTGGCG     Mat       ABD 255     gaagcagttccaggattacagcattacCCCTAAGCAACAAGGGCCTTAAG     Mat       ABD 256     CCCAAATACGGCAACTTGGCG     Mat       ABD 255     GAAGCAGCTCCAGCCTACA     Det       BBC 082     gtgatgagttccaggatCATAACTTCAACCTTACCCACG     Mat       ABD 256     CCAATGTGTGGGATAACACC     Act       CKP 463     CAAGTGCCCACTGGCTACGG     Mat       CKP 463     CAAGTGCCCACTGGCAACGG     Mat       BBC 1911     CGTATCAAACTCGCGAAACG     Mat       CKP 558     CAATGCCACTGACCTTAACG     Mat       BBC 744     CAGTGCCACTGAGCACTTAACG     Mat       BBC 93     ttgattataaggatgagtag     1×       ABD 399     AACTGATGCGCAAAGTGC     Mat       ABD 399     AACTGATGCGCAAAGTGC     Mat       ABD 399     AACTGATGCGCAAAAGGCACTTAAGG     Mat       CKP 520     TCATATACTGGCAAAAGTGACCAGACCTACCTAG	$\Delta cqsA R2$ (down arm)	
CKP 550     gtcgacgattccccgaatTGGCATTTCCTTCCCC     Alu       CKP 551     gaaggattcccggtaatTGGCATTTGAAACG     Alu       ABD 334     AGTGCTCGGACTCTTGCTG     Alu       ABD 344     AGTGCTCGGACTCTTGCTG     Alu       ABD 255     gaaggattccaggtaatCATAACTGG     Alu       ABD 256     CCCAAATACGGCAACTTGGCG     Alu       ABD 256     GCCAAATACGGCAACTTGGCG     Alu       ABD 256     GCCAAATACGGCACTTGGCG     Alu       ABD 256     GCCAAATACGGCACCTTGGCG     Alu       ABD 256     GCCAAATACGGCACTTGGCG     Alu       CKP 461     TTGGCGAACTTGGCGATATTGG     Ack       CKP 463     CAATGTCCACGCTCACGCGG     Ack       CKP 463     CAATGTGCCACTTGGTCACTGGCG     Alu       BBC 682     CCATGTGCACTTGGCACTGG     Alu       CKP 463     CAATGCCACACACCC     Ack       CKP 463     CCATGTGCACTGGGGAATTTAG     Alu       BBC 610     TAAATGGGCTTGCAGCTG     Alu       BBC 611     CGTATCAAACTGCGAAAAGGCACTCAG     Alu       BBC 753     TGGCACACAGAACTCTGACGG     Alu       BBC 744     CGTGCCAAAGGAGCTTAAGG     Alu       BBC 744     CGGTGCTGCAGAAGGTTAGGCAACTAACGAAAGGCACCTAA     Pab       CKP 505     TAAGCAAACTGTAAGAAAAGGCACACTAACTAACAAACCTAA     Pab       CKP 506     TGCATTAGGG	$\Delta luxS$ F1 (up arm)	
CKP 551     gaagcagtccagctacaCATAAGTCGGTTCTGTAAACG     Alu       ABD 254     GTCTTAGCGGATCTTTGCTCTG     Alu       ABD 254     gtcgacgatcccaggatCCGCGACATTAAGC     Alu       ABD 255     gaagcagttccagcctacaCCACAATAAGCCAGAGAGCCTTAAG     Alu       ABD 256     CCCAAATACGGCAACTTGACCACAATAAGCCAGAGAGCCTTAAG     Alu       ABD 256     CCCAAATACGGCAACTTGACCACAATAAGCCAGAGAGCCTTAAG     Alu       ABD 256     CCCAAATACGGCAACTTGACCACAATAAGCCAGAGAGCCTTAAG     Det       BBC 062     gtcgacgatcccaggatCCGGGACCAA     Det       BBC 062     gtcgacgatCCCGGGC     Alu       CKP 463     CAAGTGCCACTGGCACCAG     Act       CKP 463     CAAGTGCCACTGGCACCTG     Alu       BBC 1911     CGTACCACTGACCTGGAAACTG     Alu       CKP 538     CAATGCCACTGACCTG     Alu       BBC 1911     CGTAACAACTCTGACCAGAACCTGA     Au       CKP 533     TGCAACAAGCACTTAAACG     Au       BBC 784     CATGCACTGAGCACTCAAG     Alu       BBC 784     CATGATGGCAGAAAAGGCTTTGCAC     Au       ABD 399     AACTGCAATGAGTCTAAGCG     Au       ABD 399     TGCAAAAAGGCAGAAACTCTAG     Au       ABD 399     TGCAAAAAGGCAGAAACTCTAGC     Au       ABD 399     AACTGCAATGAGTGACTGAG     Au       ABC 693     TTGCAAGAAAGGTTATAGCGCTGAAAGG     Au <td>Δ<i>lux</i>S R1 (up arm)</td>	Δ <i>lux</i> S R1 (up arm)	
CKP 552TICTTAGCGTGATCATTGCΔlaABD 334AGTGCTCCGACTCTTGCTCTGΔlaABD 255gagagatccaggaatCATTAGCCTGAGAGAGAGCCTTAAGΔlaABD 256CCCAAATAGCGCACTTGGCGΔlaABD 725GAAGCAGCTCCAGCCTACADetBBC 082gtgacggatcccaggaatCATAACTAACCACTTACTCACCCAGΔcbCKP 444TIGGTGGCATTATTGGΔcbCKP 445CCATGCAGCACTTGGCGCATTATTGGΔcbCKP 445CCATGCGCACTTGGCGCATTATTGGΔcbCKP 445CCATGCGCACTTGGCGCATTATTGGΔbbBBC 101TAAAGTGGCCTGGGAGATTTAGΔbbBBC 410TAAAGTGGCCTGGGAGATTTAGΔbbBBC 410TGAATGGGCAACTTGGGAAATTAGΔbbBBC 410TGAATGGGCAACTGGGAAATTAGΔbbBBC 410TGAATGCGCAACATGGAACTGΔbbBBC 410TGAATGCGGAACTTGGAGAATTAGΔbbBBC 410TGAACGAGCATCAAACTGGGAAATTAGΔbbBBC 410TGAACGAGCAACGAACAGGΔbbBBC 325TGGACCACGAACAACGGGTTCGCΔbbBBC 325TGGACCACGAACAACGGGTTCGCΔbbBBC 326CCATAACGGGTTCTGCΔbbBBC 328GCCAATAACATGTTCTGCACCACGCAAGGPabbBBC 744CagettcgcgtcctggtaGCGAAATTAACTCAGGCAAAGPabbCKP 865TAAGCAAACTGTACACTAACTAACTAAGCAAGGTAGPabbCKP 865TAAGCAAACTGTGACCTACAGPabbCKP 505TAAGCAAACTGTACATTACTCAGCTAGGAAGAPabbCKP 865TAAGCAAACTGTACAATTACTCAGCTAGCATAHapbCKP 950AACCTCTCCAAGCGCGTTCACAGCPabbCKP 865TAAGCAAA	Δ <i>luxS</i> F2 (down arm)	
ABD 334     AGTGCTCCGGACTCTTTGCTCG     Main       ABD 254     gtogacgagatcATCCCTCAACCCGAAGCGAGTAAAG     Main       ABD 255     gaagcagtccagctacaCACACATAAGCCGAGGAGTAAAG     Main       ABD 725     GAAGCAACTTGCAACCTGAGCG     Main       BBC 082     gtogacggatcccaggaatCATCCTCACCCTACACCCAG     Det       BBC 082     gtogacggatcccggagatCATACCTTACCCCAG     Main       CKP 443     CAAGTATCTAGGCAACATTTAGC     Main       CKP 443     CAAGTGCCACTGGCACTGG     Main       CKP 443     CAAGTGCCACTGGCACTG     Main       BBC 101     TAAATGGGGCTTGGAACATCTG     Main       BBC 110     CGTAATCGAAACTGCTGACAGG     Main       BBC 2759     TTGCACAACGGCATTCGAACTG     Main       BBC 2759     TTGCACAAACGGCAATTGAGCAGG     Main       BBC 2759     TTGCACAAAGCGGTTTCTGC     Main       BBC 2759     TTGCACAAAGCGCATCTAGGCA     Main       BBC 2759     TTGCACAAAGCGGTTTCTGC     Main       BBC 2759     TTGCACAAAGCGCTTAACGCA     Main       BBC 2759     TTGCACAACGGCATCTAGGCA     Main       BBC 276     CCATATACGGCAAAAGCGTTCTGC     Main       ABD 286     GCCATATACGGCAAACGGTTCTGC     Main       BBC 2759     TTGCACAAGCGGTTCTGCACGG     Pain       CKP 280     CATATATCGGCAAAAGGCTACGCAGGAGTAAGAACACCTAG	Δ <i>lux</i> S R2 (down arm)	
ABD 254     gtcgacggatcCTCCGGAGGAGTAAGG     Mar       ABD 255     gaagcatcccagcatcATCAACCCTAAGCCGAGGAGAGCCTTAAG     Mar       ABD 256     CCCAAATACGGCAACTTGGCG     Mar       ABD 256     GCCAAATACGGCAACTTGGCG     Mar       ABD 256     GCCAAATACGGCAACTTGGCG     Mar       ABC 082     gtcgacggatcCCCggatCATAACTAACTACACCTTACCCCAG     Mar       CKP 444     TGGCAGAACTTGTGGCGATTATTGG     Mar       CKP 445     CCATGGTGGATACTTGGTCACCC     Mar       CKP 445     CCATGTGTGGATGATTAGCACCC     Mar       BBC 1911     CGTAACAACTGGCGCTGGGACATG     Mar       BBC 1911     CGTAACAACTGGCACCAGG     Mar       CKP 558     AATTGTAACTCTGAGCAATG     Mar       BBC 1911     CGTAACAAACGCACTTGAACG     Mar       BBC 759     TGTCAAAAAGGGTTCTGC     Mar       ABD 399     AACTGATGAACGCCACTGAG     Mar       ABD 399     AACTGATGGCAGAAAAAGCCACTCAG     Mar       ABD 399     AACTGATGACGCAGAAAAAGCCACTCAG     Mar       ABD 399     AACTGATGGCAGAAAAAGCCACTCAG     Mar       ABD 399     AACTGATGGCAGAAAAAGCCACTCAG     Mar       ABD 399     AACTGATGGCAGAAAAAGCCACTCAG     Mar       ABD 30     CCCAATGAAATCAGTCAGCTAGCGTAG     Mar       ABC 744     CagcttgcggatcctggtgaGCAAATATAACTAACAGGAAAGCACTAG     Par <td colspan="2">∆<i>lacZ</i> F1 (up arm)</td>	∆ <i>lacZ</i> F1 (up arm)	
ABD 255     gaagcagretcagcitacagCACATAAGCCAGAGAGCCTTAAG     Max       ABD 256     CCCANATAGCGCAACTTGCCG     Max       ABD 725     GAAGCAGCTCCAGCCTACA     Det       BBC 082     gtcgacggatcCTAACTTACCACCTTACTCACCCAG     Act       CKP 441     TTGTTGGGCAGACATTTGCG     Act       CKP 443     CAAGTATCCAGCGCA     Act       CKP 443     CAAGTGCCACTGGC     Act       CKP 443     CAAGTGCCACTGGCACTG     Adot       BBC 1910     TAAATGGGGCTTGGCAACTG     Adot       BBC 1911     CGTACTGCACTGGCGAAAGTG     Adot       BBC 1911     CGTACTGCACTGGCGAAAGTG     Adot       BBC 7553     TGGACCACGAACCTTAAACG     Act       BBC 759     TTGCACAAAGCGCATTGAC     Act       BBC 759     TGCACAAGGCGTTCTGC     ADot       BBC 759     TGCACAAGGCGTTCTGC     ACV       ABD 286     CATAAACAGTGCTCTGAGCAAAAAGCCACTCAG     Act       BBC 993     ttgatatagagtagacgagtaca     1 ×       BBC 993     ttgatatagagtagacgacgacgaca     1 ×       BBC 993     ttgatatagacgagtacaCTTAGGCAAATTAACTCAGGCAAAG     Pad       CKP 850     TAAGCTAACTGGCAAAATTAGCCACCTAAG     Pad       CKP 902     CCATTACTGGCAAAATTAGCCACCTAGCAG     Pad       CKP 903     ttgattagagatgacagataca     1 ×       BBC 990     <	Δ <i>lacZ</i> R1 (up arm)	
ABD 256     CCCAATACGCCAATGCCG     Max       ABD 725     GAAGCAGCTCCAGCTTCACA     Det       BBC 082     gtcgacggatcCaTAACTTACATACCTACACCCAG     Act       CAK 411     TTGTTIGGTGCGATTATTGG     Act       CKP 464     TTGGCAGAACATCTTTGATC     Act       CKP 465     CCCATGCTGGATACACCACC     Act       CKP 463     CACTGCCACTTGGTGACACTG     Add       BBC 101     TAAATGGGGCTTGGGAGATTTAG     Add       BBC 410     TAAATGGGGCTTGGGAGATTAGG     Add       BBC 1911     CGTAATCAAACTGGGAATGG     Add       CKP 558     AATCGTACCTGGACACTG     Add       BBC 993     TTGGACACACGACACCTTAACG     Add       BBC 993     ttgattataaggatgacgatgac     1×       ABD 399     AACTCATGGCACACTGACGG     Add       BBC 993     ttgattataaggatgacgatgac     1×       ABD 399     AACTCATGGCACAAAAGGCACTCAAG     Add       BBC 744     CagcttcgcgtcctcggtaCGCAAATAAAAACACCTA     Hag       CKP 505     TAAGCAAACTGTTTGGCAAACGAAACGCACTA     Padd       CKP 505     TAAGCAAACTGTACGCAAACGTAAACAACCTAA     Padd       CKP 506     TCATATCTGGCACAACGTAAAACCTAAACAAACCTAA     Padd       CKP 505     TAAGCAAACTGTAACCAAACCTAAAACCTAA     Padd       CKP 506     TAAGCAAACTGTAAACTAAACTAAAACCTAAACTAAACT	$\Delta lacZ$ F2 (down arm)	
ABD 725GAAGCAGCTCCAGCCTACADetBBC 082gtcgacggatcCCCqggatCATAATGCACCTTACACCCAGCAc/cBBC 082gtcgacggatcCCCqgatCATAATGGAc/cCKP 464TTGGCAGATACATCTTGGCAc/cCKP 463AAAGTATCCAGCTCACGGCGAc/cCKP 465CCATGTGTGGATAGACACCCAc/cCKP 485CCATGTGTGGATAGACAACCAc/cCKP 485CCATGTGTGGATAGACAACCAc/cCKP 583CATTCGTAACTCGGAAAGTGAl/oBBC 101TAAATGGGGCTTGGAAAATTAGAl/oBBC 1911CGTAATCAAACTGGAAAGTGAl/oBBC 2759TTGCACAAAGCGTTTCTGCAl/oBBC 2759TTGCACAAAGCGTTTCTGCAl/oBBC 2759TTGTCACAAAGCGTTTCTGCAl/oBBC 2759TTGTCACAAAGCGTTTCTGCAl/oBBC 2759TTGTCACAAACGGTTTCTGCAl/oBBC 2759TTGTCACAAACGGTTTCTGCAl/oBBC 2760CCATAAACATTCTTGGCAAAAGGCATCTAAGAl/oBBC 2780GCCAATAGAATGAGCTATTGGCTGAl/oBBC 2744cagcttcgcgtcctcggtaGCGAAATATAACTCAGGCAAAGPabCKP 865TAAGCAAACTGTAGTGACGAACGAAGCTAAHagCKP 505TAGGATTGATGAGTAGAAACACCTAAHagCKP 505TAGGATTGATGGTGAGCGTAGCAAACGTAGPabCKP 507ACACCTGCCGGTAGAAPabCKP 507ACACCTGCCGGTGCAAACGTGPabBBC 299GCATCTAGGTTTTGACGTAGCAGGGTGTHagABD 133TTCTACCAAGCGCGGTAGCAAGGPabJDN 92agcagcggcctggtgccgcgcgcaccaatggaaaaaClorJDN 93tcagtggtggtggtggtggtggtGCCGA	$\Delta lacZ R2$ (down arm)	
BBC 082     gtcgacggatccccgggatCATAACTTACACCTACCCAG     Action       BBC 082     gtcgacggatcccccggaatCATAACTTACACCCTACCCAG     Action       CAK 411     TTGTTIGGTGCGATTATTGG     Action       CKP 463     AAAGTATICCAGCTCACGGGG     Action       CKP 463     CAGTGCCACTTGGTACCCTG     Action       BBC 1913     CAGTGCCACTTGGTCACCTG     Action       BBC 1911     CGTATCTAAACTGCGAAAGTG     Muin       CKP 558     AATTCGTAACTGCAACAGG     Action       BBC 2759     TTGCAGAAAGCGTTTCTGC     Action       BBC 993     ttgattataaggatgacgatgac     1×       ABD 399     AACTGATGGCACACGAAAAGCCATCAG     Action       BBC 993     ttgattataaggatgacgatgac     1×       ABD 399     AACTGATGGCACACAGAAAAGCCATCAG     Action       BBC 980     GCCAATAGAAAGCTGTTTCTGC     Muin       BBC 980     CCAATAACATGTTTCTGCAGAAAAAGCCATCAG     Pain       BBC 980     GCACATAACATGACGCACAGA     Naction       CKP 850     TAAGCAACATGTTGGCAAATAGAACCAGG     Pain       CKP 965     TAAGCAAACTGTAGCGTAAGA     Pain       CKP 505     TAAGCAAACTGAACCATAACAAACCTA     Hap       CKP 506     AATTGCAATGACACATAACAAACCTA     Pain       CKP 505     TAAGCAAATGAACTGTAACGAAAACCTAA     Pain       CKP 505     TAAGCAAATGAACTGTAACGAAAACCTAA<	Detect F for all deletions	
CAX 411 TIGTTIGGTGCGATTATTGG CAGATAGTGG AAAAAAAAAAAAAA	Achn detect B	
CKP 444 TTGGCAGAACATCITTGATC ACG CKP 443 AAAGTATCCAGCTCACGGG ACC CKP 443 CCATGGTGGAACATCITTGATC ACG CKP 443 CCATGGTCACCTG ACC CKP 443 CCATGGCGAACATCACG CKP 433 CCGTGCACCTGAACACC ACC Aba BBC 101 TAAATGGGGCTTGGAGAAGTG Aba BBC 1911 CGTAATCAAACTGCGAAAGTG Aba BBC 1911 CGTAATCAAACTGCGAACTG Aba BBC 1911 CGTAATCAAACGCGCAACTTAAACG Aba BBC 2759 TTGGACCACGACCTTAAACG Aba BBC 2759 TTGGACCACGACCTTAAACG Aba BBC 93 ttgattataagagtacgatgac 1× ABD 399 AACTGATGGCAAGAATAACG Aba BBC 93 ttgattataaggatgacgatgac 1× ABD 390 CCAATGGAACTGTTGCAGCAG Aba BBC 93 ttgattataggatgacgatgac 1× ABD 846 CCATAAACATGTTTCTGATCAGCAG CKP 520 TCATATCTGGCAAACGAGT CKP 520 TCATATCTGGCAAACGAGAATAAACCCACTCAG CKP 520 TCATATCTGGCAAACGAGAA BBC 391 CGCAATAGAATGATTATGGCTG bac CKP 520 TCATATCTGGCAAACGAGAAAACCCACTAG P <sub>chb</sub> CKP 555 TAAGCAAACTGTAGCAGAAAGCCTA CKP 505 TAAGCAAACTGTAGCGAAAGGCAAACGCTAG P <sub>chb</sub> CKP 505 TAAGCAAACTGTAGCGAAAGCTAATTAAACTCAGGCAAAGG P <sub>chb</sub> CKP 505 TAGGTTTGTGAGGAGGAAATTATACCTAGCAAACCCTA Hap CKP 505 TAGGCTTGAGTAGTAGATAATAACCTAACAAACCCTA Hap CKP 505 TAGGCATTGAGTAGTAAGTAAATCACTAACAACCTA Hap CKP 506 AATTGCAATGGTAGTAGATTATTATCTGGCT CKP 507 ACCCCAAGCAAAGTAGATCATTAACCATAACACTA ACACCTAGCCAAGGAAATTTATCTGCAGGTGT Hap CKP 505 TAGGCTTGAGGAGGTAAGACACTTA ABD 132 CTGTCTCAAGCCAGGAGAGTAAGACACTTA ABD 132 CTGTCTCAAGCCAGGAGAGTAAG CKP 867 TCGTGTCTGAGGCGTTACAA pod ABD 132 CTGTCTCAAGCCGGTTACAA ABD 132 CTGTCTCAAGCCGGTTACAA ABD 132 CTGTCTCAAGCCGGTACGAGAGT CKP 867 TCGTATGAGCGCGCGCCCACG P <sub>chb</sub> JDN 92 agcagcggcctggtgccgcgcgcgcagcCatatggacgcatcaatcgaaaaa Clor P JDN 92 CTGGAGGaccacccacca Am DDN 23 atGgctgccgcggcgccaca Am	AcinA detect R	
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BBC 1911     CGTAATCAAACTGGGAAAGTG     Δ/Li       CKP 558     AATTGGTAACTGTGAGCATG     Δ/Li       BC 2759     TGGACCACGAACGTTCAACG     Δ/Li       BBC 993     ttgattataaggatgacgatgac     1×       ABD 399     AACTGATAGAAAAGCCACTCAG     Δ/Li       BBC 993     ttgattataaggatgacgatgac     1×       ABD 399     AACTGATAGAATAGATGCAGAAAAAGCCACTCAG     Δ/Li       BBC 993     ttgattataaggatgacgatgac     1×       ABD 390     GCCAATAGAATGAGCTATTGGCTG     LuxC       CKP 520     TCATATCTGGCAAAAGTCTATGGCAGA     P,ab       BBC 744     CagcttcgcgtcctcggtaCGCAAATATAACTCAGGGCAAAG     P,ab       CKP 855     TAAGCAAACTGTAGCGTAGAGAGAAAAAACACCACTAG     P,ab       CKP 865     TAAGCAAACTGTAGCGTAGAAAAAACACCTAA     P,ab       CKP 866     TGCATTTTTTCTGGTAGC     P,ab       CKP 505     TAAGCAAACTGTAGCGTAGAAAAACACTAACAAACCTA     Hag       CKP 506     AATTGCAATGAAAGTAAACTAAACAAACCTA     Hag       CKP 506     AATTGCAAGAAAATTTATCAATTGCAATT     Hag       CKP 507     ACACCTAGCCAAGAAATTTAACCATGTGCA     P,ab       BBC 990     AACACTCTCCAAGACCTACCTC     P,ab       BBC 990     AACACTCTAGGTTAGAAGTAAAG     PoD       ABD 132     CTGTCTAAGCCGGTTACAA     PoD       CKP 865     TAAGCAAACTGTAGCGTAGAAAG	∆ <i>hapR</i> detect R	
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CKP 072cccgggatcctgtgtgaaatgCTTTGGCAGGAGTAAGAAAACACCTAGP chpCKP 865TAAGCAAACTGTAGCGTAGAAGP chpCKP 866TGCATTTTTTCTGGTAGCP chpCKP 504GCCAAATAAGTAAGTAAACAACTAACAAAACCTAHapCKP 505TAGGTTTGATGTTTAGCTTACTTACTTATTTGGCHapCKP 506AATTGCAATTGATAAATTTCTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAATTTACTAATTGCAATTHapUsed for qPCR and qRT-PCRBBC 989GCATCTAGGTTTGACGTTTTAACGP chbBBC 989GCATCTAGGCTTCCAAGACCTACCTCP chbP chbABD 132CTGTCTCAAGCCGGTTACAArpodABD 133TTTCTACCAGTGCAGAGAAGCrpodCKP 865TAAGCAAACTGTAGCGTAGAAGP chpiCKP 867TCGTATGAACGCTCACCGP chpiJDN 92agcagcggcctggtgccgcgcggcagcCatatggacgcatcaatcgaaaaaClorJDN 22CTCGAGcacaccaccaaAm b bJDN 23atGgtgcgcgcggggaccaAm	P F	
CKP 865TAAGCAAACTGTAGCGTAGAAGPCKP 866TGCATTTTTTCTTGGTAGCPCKP 504GCCAAATAAGTAAGTAAACACTAACAAACCTAHapCKP 505TAGGTTTGTTAGTGTTTACTTACTTACTTACTTAGGCHapCKP 506AATTGCAATTGATAAATTTCTTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAAATTTATCAATTGCAATTGHapUsed for qPCR and qRT-PCRGCATCTAGGTTTTGACGTTTTTAACGPBBC 989GCATCTAGGTTTTGACGTTTTTAACGPABD 132CTGTCTCAAGCCGGTTACAArpolABD 133TTTCTACCAGTGCAGAGAAGrpolCKP 865TAAGCAAACTGTAGCGTAGAAGPCKP 867TCGTATGAACGCTCACCACPJDN 92agcagcggcctggtgccgcgcggcagcCatatggacgcatcaatcgaaaaaClorJDN 22CTCGAGcaccaccaccacaAmJDN 23atGgctgccgcggggaccaAm	P R	
CKP 805TAGCAAACTOTAGCGTAGAGPCKP 806TGCATTITTITCTIGGTAGCPCKP 504GCCAAATAAGTAAGTAAACACTAACAAACCTAHapCKP 505TAGGTTIGTTAGTGTTTACTTACTTACTTAGTGGCHapCKP 506AATTGCAATTGATAAATTTCTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAATTTATCAATTGCAATTHapUsed for qPCR and qRT-PCRBBC 989GCATCTAGGTTTTGACGTTTTAACGPBBC 989GCATCTAGGTTTTGACGTTTTAACGPBBC 980AACACTCTCCAAGACCTACCTCPABD 132CTGTCTCAAGCCGGTTACAArpolABD 133TTTCTACCAGTGCAGGAGAGCPCKP 865TAAGCAAACTGTAGCGTAGAAGPCKP 867TCGTATGAACGCTCACCACGPUsed for purification vectorJDN 92agcagcggcctggtgcgcgcgcgcgccatcaatcgaaaaaClorJDN 22CTCGAGcaccaccaccaaAmbJDN 23atGgctgccgcgcggcgccacaAm	D E	
CKP 300TICATIATITICITIGGTAACFreipiCKP 504GCCAAATAAGTAAGTAAACACTAACAAACCTAHapCKP 505TAGGTTIGTTAGTGTTTACTTACTTATTTGGCHapCKP 506AATTGCAATTGATAAATTTCTTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAATTTATCAATTGCAATTHapUsed for qPCR and qRT-PCRgCCATCTAGGTTTTGACGTTTTAACGP_chbBBC 989GCATCTAGGTTTGACGTTTTAACGP_chbABD 132CTGTCTCAAGCCGGTTACAArpolABD 133TTTCTACCAGTGCAGAGATGCrpolCKP 865TAAGCAAACTGTGAGCGTAGAAGP_chbCKP 867TCGTATGAACGCTCACCACGP_chbUsed for purification vectorJDN 92agcagcggcctggtgcgcgcgcgcagcCatatggacgcatcaatcgaaaaaClorJDN 93tcagtggtggtggtggtggtggtggtggtggtgcTCGAGctagttcttatagatacacagcatattgaggClorJDN 22CTCGAGcaccaccaccaaAmJDN 23atGgctgccgcgcggcagccaaAm		
CKP 504GCCAAATAAGTAAGTAAGTAAGTAACACTAAHapCKP 505TAGGTTTGTTAGTGTTTACTTACTTATTTGGCHapCKP 506AATTGCAATTGATAAATTTCTTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAATTTATCAATTGCAATTHapUsed for qPCR and qRT-PCRgCATCTAGGTTTTGACGTTTTTAACGP_{chb}BBC 989GCATCTAGGTTTTGACGTTTTTAACGP_{chb}BBC 990AACACTCTCCCAAGACCTACCTCP_{chb}ABD 132CTGTCTCAAGCCGGTTACAArpolCKP 865TAAGCAAACTGTAGGCGTAGAAGP_{chp}CKP 865TAAGCAAACTGTAGCGTAGAAGP_{chp}Used for purification vectorJDN 92agcagcggcctggtgccgcgcgcgcgccatatggacgcatcaatcgaaaaaClorJDN 93tcagtggtggtggtggtggtggtggtggtgCTCGAGctagttcttatagatacacagcatattgaggClorJDN 22CTCGAGcaccaccaccacAmJDN 23atGgctgccgcgcggcaccaAm		
CKP 505IAGGITIGITAGIGITIACITACITATIGGCHapCKP 506AATTGCAATTGATAAATTTCTTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAATTTATCAATTGCAATTHapUsed for qPCR and qRT-PCRBBC 989GCATCTAGGTTTTGACGTTTTAACGP_{chb}BBC 990AACACTCTCCAAGACCTACCTCP_{chb}ABD 132CTGTCTCAAGCCGGTTACAArpolABD 133TTTCTACCAGTGCAGAGATGCrpolCKP 865TAAGCAAACTGTAGCGTAGAAGP_{chp}CKP 867TCGTATGAACGCTCACCACGP_{chp}JDN 92agcagcggcctggtgccgcgcgcgcagcCatatggacgcatcaatcgaaaaaClorJDN 93tcagtggtggtggtggtggtggtggtggtggtgctCGAGCatatggacgcatattgaggClorJDN 22CTCGAGcaccaccaccaaAmJDN 23atGgctgccgcggcggcagccaAm	Нарк вз т Р	
CKP 506AATTGCAATTGATAAATTTCTTGGCTAGGTGTHapCKP 507ACACCTAGCCAAGAAATTTATCAATTGCAATTHapUsed for qPCR and qRT-PCRBBC 989GCATCTAGGTTTTGACGTTTTAACGPBBC 990AACACTCTCCAAGACCTACCTCPABD 132CTGTCTCAAGCCGGTTACAArpolABD 133TTTCTACCAGTGCAGAGATGCrpolCKP 865TAAGCAAACTGTAGCGTAGAAGPCKP 867TCGTATGAACGCTCACCACGPUsed for purification vectorJDN 92agcagcggcctggtgccgcgcgcgcagcCatatggacgcatcaatcgaaaaaClorJDN 93tcagtggtggtggtggtggtggtggtggtggtgctCGAGctagttcttatagatacacagcatattgaggClorJDN 22CTCGAAGcaccaccaccaaAmJDN 23atGgctgccgcgcgcgcagcCataAm	HapR BS 1 R	
CKP 507ACACCTAGCCAAGAAATTTATCAATTGCAATTHapUsed for qPCR and qRT-PCRGCATCTAGGTTTTGACGTTTTTAACGP_chbBBC 989GCATCTAGGTTTTGACGTTTTTAACGP_chbBBC 990AACACTCTCCAAGACCTACCTCP_chbABD 132CTGTCTCAAGCCGGTTACAArpolABD 133TTTCTACCAGTGCAGAGATGCrpolCKP 865TAAGCAAACTGTAGCGTAGAAGP_chpiCKP 867TCGTATGAACGCTCACCACGP_chpiUsed for purification vectorJDN 92agcagcggcctggtgccgcgcgcagcCatatggacgcatcaatcgaaaaaClorJDN 93tcagtggtggtggtggtggtggtggtggtggtggtgctCCGAGctagttcttatagatacacagcatattgaggClorJDN 22CTCGAAGcaccaccaaccaAmjJDN 23atGgctgccgcggcggcagcCataAmj	HapR BS 2 F	
Used for qPCR and qRT-PCR BBC 989 GCATCTAGGTTTTGACGTTTTAACG P <sub>chb</sub> BBC 990 AACACTCTCCAAGACCTACCTC P <sub>chb</sub> 132 CTGTCTCAAGCCGGTTACAA rpol ABD 133 TTTCTACCAGTGCAGAGATGC rpol CKP 865 TAAGCAAACTGTAGCGTAGAAG P <sub>clp</sub> CKP 867 TCGTATGAACGCTCACCACG P <sub>clp</sub> Used for purification vector JDN 92 agcagcggcctggtggccgcgcgcagcCatatggacgcatcaatcgaaaaa Clor p JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtggtggtg	HapR BS 2 R	
BBC 989 GCATCTAGGTTTTGACGTTTTAACG P <sub>chb</sub> BBC 990 AACACTCTCCAAGACCTACCTC P <sub>chb</sub> ABD 132 CTGTCTCAAGCCGGTTACAA rpol ABD 133 TTTCTACCAGTGCAGAGATGC rpol CKP 865 TAAGCAAACTGTAGCGTAGAAG P <sub>chp</sub> CKP 867 TCGTATGAACGCTCACCACG P <sub>chp</sub> Used for purification vector JDN 92 agcagcggcctggtgccgcgcgcagcCatatggacgcatcaatcgaaaaa Clor p JDN 93 tcagtggtggtggtggtggtggtggtggtggtgctCGGAGctagttcttatagatacacagcatattgagg Clor JDN 22 CTCGAGcaccaccacca Amj JDN 23 atGgctgcgcgcggcagcCata		
BBC 990     ACACTCTCCCANGACCTACCTC     P       BBC 990     ACACTCTCCCAAGACCTACCTC     P       ABD 132     CTGTCTCAAGCCGGTTACAA     rpol       ABD 133     TTTCTACCAGTGCAGAGATGC     rpol       CKP 865     TAAGCAAACTGTAGCGTAGAAG     P       CKP 867     TCGTATGAACGCTCACCACG     P       Used for purification vector     JDN 92     agcagcggcctggtgccgcgcgcagcCatatggacgcatcaatcgaaaaa     Clor       JDN 93     tcagtggtggtggtggtggtggtggtggtggtgcCTCGAGctagttcttatagatacacagcatattgagg     Clor       JDN 22     CTCGAGcaccaccaaccaa     Amj       JDN 23     atGgctgccgcggcggcagcca     Amj	P amplify F	
ABD 132 CTGTCTAAGACCGGTTACAA rpol ABD 132 CTGTCTCAAGACCGGTTACAA rpol ABD 133 TTTCTACCAGTGCAGAGATGC rpol CKP 865 TAAGCAAACTGTAGCGTAGAAG P <sub>clpi</sub> CKP 867 TCGTATGAACGCTCACCACG P <sub>clpi</sub> Used for purification vector JDN 92 agcagcggcctggtgccgcggcagcCatatggacgcatcaatcgaaaaa Clor p JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtggtgCTCGAGctagttcttatagatacacagcatattgagg Clor p JDN 22 CTCGAGcaccaccacca Amj	P amplify D	
ABD 132 CIGICICAAGCCGGIAGCAA rpoi ABD 133 TTTCTACCAGTGCAGAGATGC rpoi CKP 865 TAAGCAAACTGTAGCGTAGAAG P <sub>clpi</sub> CKP 867 TCGTATGAACGCTCACCACG P <sub>clpi</sub> Used for purification vector JDN 92 agcagcggcctggtgccgcggcagcCatatggacgcatcaatcgaaaaa Clor p JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtgctCGAGctagttcttatagatacacagcatattgagg Clor p JDN 22 CTCGAGcaccaccacca Amj b	P <sub>chb</sub> amplify R	
ABD 133       TTICTACCAGTGCAGAGATGC       rpol         CKP 865       TAAGCAAACTGTAGCGTAGAAG       P_clpi         CKP 867       TCGTATGAACGCTCACCACG       P_clpi         Used for purification vector       gcagcggcctggtgccgcgcggcagcCatatggacgcatcaatcgaaaaa       Clor         JDN 92       agcagtggtggtggtggtggtggtggtggtggtgccccatatggacgcatcaatcgaaaaa       Clor         JDN 93       tcagtggtggtggtggtggtggtggtggtgccccacaccaacca	rpoB amplify F	
CKP 865     TAAGCAAACTGTAGCGTAGAAG     P_dpl       CKP 867     TCGTATGAACGCTCACCACG     P_dpl       Used for purification vector     JDN 92     agcagcggcctggtgccgcgcgcgcagcCatatggacgcatcaatcgaaaaa     Clor       JDN 93     tcagtggtggtggtggtggtggtggtggtggtggtgccccacaccaacca	rpoB amplify R	
CKP 867     TCGTATGAACGCTCACCACG     P <sub>clpi</sub> Used for purification vector     agcagcggcctggtgccgcggcagcCatatggacgcatcaatcgaaaaa     Clor       JDN 92     agcagtggtggtggtggtggtggtggtggtggtggtggtggtg	P <sub>clpP</sub> amplify F	
Used for purification vector JDN 92 agcagcggctggtgccgcggcggcagcCatatggacgcatcaatcgaaaaa Clor p JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtggtggtg	P <sub>clpP</sub> amplify R	
JDN 92 agcagcggctggtggcggcggcagcCatatggacgcatcaatcgaaaaa Cloi p JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtggtggtg		
JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtggtggtg	Cloning 6×His-hanR into	
JDN 93 tcagtggtggtggtggtggtggtggtggtggtggtggtggtg		
p JDN 22 CTCGAGcaccaccacca b JDN 23 atGgctgccgcggcacca Ami	Cloning 6×His-hapR into	
JDN 23 atGgctgccgcggcacca Ami	pET28b vector via IDA, R	
JDN 23 atGgctgccgcggcacca Ami	backbone for IDA F	
JDN 25 aldyclycycycycycacca Allia	Amplify pET29b voctor	
b	backbone for IDA. R	
~	······································	
Used for complementation constructs		
BBC 832 GCTTTTGCTACAACGACCG ΔVC	ΔVCA0692 F1 for up arm	
BBC 828 CACCATACCCACGCCGAAACAACAGTGATGTAGCGAATCGGAC ΔVC	ΔVCA0692 R1 for up arm	
BBC 243 TIGTTICGGCGTGGGTATGGTG Tm <sup>r</sup>	Tm <sup>r</sup> or Zeo <sup>r</sup> F for middle arm	
BBC 647 tttttctatttctgaatcgattcatacgaCTCATTAGGCACCCCAGGC Tm <sup>r</sup>	Tm <sup>r</sup> F or Zeo <sup>r</sup> for middle arm	
CKP 948 tratataatraatraaaaaaACCATICTCGTIGTGTGGGG P	P han R F for middle arm	
BRC 729 totagerengenergenergenergenergenergenergene	$P_{intro and R}$ $P_{intro a$	

#### TABLE 1 (Continued)

Primer	Sequence <sup>a</sup>	Description
CKP 949	tcgtatgaatcgattcagaaatagaaaaaCCCTCATGCATTTTATAACTG	P <sub>clpSA</sub> -clpSA F for middle arm
CKP 950	tgtaggctggagctgcttcCTAGTGGACCACCTCTTCGC	P <sub>clpSA</sub> -clpSA R for middle arm
BBC 830	gaagcagctccagcctacaGTTGAGTTGGATGCAGCACC	ΔVCA0692 F2 for down arm
BBC 834	CACAATTTCTCGCTTAAAATGTCC	ΔVCA0692 R2 for down arm
CKP 509	gcaggtggagcaggtggaGACGCATCAATCGAAAAACG	∆VCA0692::P <sub>hapR</sub> -hapR detect F
BBC 1101	CAGACGTACTATTAACAGGACTGAC	ΔVCA0692::P <sub>hapR</sub> -hapR detect R
CKP 235	gtcgacggatccccggaatCAAATATATCCTCCTCACTATTTTGATTAG	ΔVCA0692::P <sub>clpSA</sub> -clpSA detect F
CKP 447	gtcgacggatccccggaatTTTACTCATGACACTTCAATATTTG	$\Delta VCA0692::P_{clpSA}$ -clpSA detect R

<sup>a</sup>Lowercase letters in primers indicate the sequence of overlap sequences needed for SOE PCR. <sup>b</sup>IDA, isothermal DNA assembly.

were placed under a 0.2% Gelzan pad and imaged on an inverted Nikon Ti2 microscope with a Plan Apo  $60 \times$  objective, yellow fluorescent protein (YFP) and mCherry filter cubes, a Hamamatsu ORCA-Flash 4.0 camera, and Nikon NIS-Elements imaging software.

The strains used to examine  $P_{chb}$  expression across a population contained (i) a  $P_{chb}$ -mCherry reporter and (ii) a reporter that drove constitutive expression of GFP,  $P_{const2}$ -GFP. By using these reporters, the expression of the  $P_{chb}$ -mCherry could be normalized to GFP expression in each cell. Images were analyzed on Fiji using the MicrobeJ plugin (60) to determine mean intensity of cells in the YFP and mCherry channels. GFP was assessed using a YFP filter set to avoid background fluorescence from chitin beads, which was stronger in the GFP channel. Background fluorescence was subtracted from each channel, and the mCherry/GFP fluorescence was determined for each individual cell.

HapR protein purification. A plasmid expressing a hexahistidine-tagged hapR wild-type (WT) allele was generated using Gibson assembly. The hapR insert was amplified from V. cholerae E7946 and inserted into a pET28b vector (Novagen) using the primers listed in Table 1. The plasmid was then electroporated into E. coli BL21(DE3) for protein overexpression. This strain was grown overnight in LB medium with kanamycin, back-diluted 1:100 into 1 liter of LB medium with kanamycin, and grown to an OD<sub>600</sub> of 0.4 to 0.6 at 30°C. Expression of HapR was induced by IPTG to a final concentration of 1 mM, and cultures were grown for 4 h shaking at 30°C. The cells were pelleted and frozen at -80°C. The pellet was resuspended in 25 ml buffer A (25 mM Tris, pH 8, 500 mM NaCl), and an Avestin EmulsiFlex-C3 emulsifier was used to lyse cells. The soluble lysate was applied to a HisTrap HP nickel nitrilotriacetic acid (Ni-NTA) column using an Äkta Pure fast protein liquid chromatography (FPLC) system in buffer A and eluted from the column with a gradient of buffer B (25 mM Tris, pH 8, 500 mM NaCl, 1 M imidazole). The purified protein was concentrated to approximately 5 ml using Sartorius Vivaspin turbo 10,000 molecular weight cutoff (MWCO) centrifugal concentrators. The sample was manually injected into the Äkta Pure and separated via size exclusion chromatography on a HiLoad 16/600 Superdex 75-pg column equilibrated with gel filtration buffer (25 mM Tris, pH 7.5, 200 mM NaCl). Eluted fractions were analyzed by SDS-PAGE (15% gel), pooled, and concentrated using the same centrifugal concentrators previously mentioned. The samples were then immediately frozen in liquid nitrogen with a final concentration of 10% glycerol and stored at -80°C.

**Electrophoretic mobility shift assay.** Binding reactions contained 10 mM Tris HCl, pH 7.5, 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol (DTT), 50  $\mu$ g/ml bovine serum albumin (BSA), 0.1 mg/ml salmon sperm DNA, 5% glycerol, 1 nM Cy5-labeled DNA probe, and HapR at the indicated concentrations (diluted in 10 mM Tris, pH 7.5, 10 mM KCl, 1 mM DTT, and 5% glycerol). Reaction mixtures were incubated at room temperature for 20 min in the dark and then electrophoretically separated on polyacrylamide gels in 0.5× Tris-borate-EDTA (TBE) buffer. Gels were imaged for Cy5 fluorescence on a Typhoon 9210 instrument. Short DNA probes (30 bp) were made by end-labeling one primer of a complementary pair (primers listed in Table 1) using 20  $\mu$ M Cy5-dCTP and terminal deoxynucleotidyl transferase (TdT; Promega). Complementary primers (one labeled with Cy5 and the other unlabeled) were annealed by slow cooling at equimolar concentrations in annealing buffer (10 mM Tris, pH 7.5, and 50 mM NaCl). P<sub>chb</sub> and P<sub>cipP</sub> probes were made by Phusion PCR, where Cy5-dCTP was included in the reaction at a level that would result in incorporation of 1 to 2 Cy5-labeled nucleotides in the final probe as previously described.

ChIP-qPCR assays. Assays were carried out exactly as previously described (10). Briefly, overnight cultures were diluted to an OD<sub>600</sub> of 0.08 and then grown for 6 h at 30°C. Cultures were cross-linked using 1% paraformaldehyde and then quenched with a 1.2 molar excess of Tris. Cells were washed with phosphate-buffered saline (PBS) and stored at -80°C overnight. The next day, cells were resuspended in lysis buffer (1 $\times$  FastBreak cell lysis reagent [Promega], 50  $\mu$ g/ml lysozyme, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 $\times$  protease inhibitor cocktail; 100 $\times$  inhibitor cocktail contained the following: 0.07 mg/ml phosphoramidon [Santa Cruz], 0.006 mg/ml bestatin [MP Biomedicals/ Fisher Scientific], 1.67 mg/ml AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; DOT Scientific], 0.07 mg/ml pepstatin A [GoldBio], and 0.07 mg/ml E64 [Gold Bio]) and then lysed by sonication, resulting in a DNA shear size of ~500 bp. Lysates were incubated with anti-FLAG M2 magnetic beads (Sigma), washed to remove unbound proteins, and then bound protein-DNA complexes were eluted off with SDS. Samples were digested with proteinase K, then cross-links were reversed. DNA samples were cleaned up and used as a template for quantitative PCR (qPCR) using iTaq Universal SYBR green supermix (Bio-Rad) and primers specific for the genes indicated (primers are listed in Table 1) on a StepOne qPCR system. Standard curves of genomic DNA were included in each experiment and were used to determine the abundance of each amplicon in the input (derived from the lysate prior to ChIP) and output (derived

# TABLE 2 Strains used in this study

Strain			
designation	Reference(s) in this report	Genotype	Reference
SAD 030	V. cholerae E7946 WT; parent for all	V. cholerae E7946 O1 El Tor	50
SAD 2825	Activator screen strain	V. cholerae E7946 ΔVC1807::P <sub>chb</sub> -mCherry, Kan <sup>r</sup> ; P <sub>chb</sub> -lacZ, Spec <sup>r</sup> ; ΔVCA0692::chiS, Tm <sup>r</sup> ; Δ3' cbp, pDL1086 Cm <sup>r</sup> Carb <sup>r</sup>	This study
SAD 2826	$\Delta clpA$ strain counterscreen	V. cholerae E7946 ΔVC1807::P <sub>chb</sub> -mCherry, Kan <sup>r</sup> ; P <sub>chb</sub> -lacZ, Spec <sup>r</sup> ; ΔclpA; Δcbp::Tm <sup>r</sup> ; pDL1086, Cm <sup>r</sup> Carb <sup>r</sup>	This study
SAD 1309	Fig. 1 and 4 parent; Fig. S5 HapR WT	V. cholerae E7946 ΔlacZ::P <sub>cbb</sub> -GFP, Kan <sup>r</sup> ; Δcbp::Spec <sup>r</sup>	11
SAD 2827	Fig. 1 and 4 $\Delta clpA$ strain	V. cholerae E7946 $\Delta lacZ::P_{chb}$ -GFP, Kan'; $\Delta clpA::Carb'; \Delta cbp::Spec'$	This study
SAD 2828	Fig. 1 $\Delta clpP$ strain	V. cholerae E7946 ΔlacZ::P <sub>cbb</sub> -GFP, Kan <sup>r</sup> ; ΔclpP::Carb <sup>r</sup> ; Δcbp::Tm <sup>r</sup>	This study
SAD 2829	Fig. 1 $\Delta clpAP$ strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔclpA::Carb <sup>r</sup> ; ΔclpP::Tm <sup>r</sup> ; Δcbp::Spec <sup>r</sup>	This study
SAD 2830	Fig. 1 $\Delta clpS$ strain	V. cholerae E7946 AlacZ::P_++-GFP, Kan': AclpS::Carb': Acbp::Tm'	This study
SAD 2831	Fig. 1 $\Delta clpX$ strain	V. cholerae E7946 AlacZ::P-++-GFP, Kan'; AclpX::Carb'; Acbp::Tm'	This study
SAD 2832	Fig. 1 $\Delta lonA$ strain	V. cholerae E7946 ΔlacZ::P <sub>skb</sub> -GFP, Kan <sup>r</sup> ; ΔlonA::Tm <sup>r</sup> ; Δcbp::Carb <sup>r</sup>	This study
SAD 2833	Fig. 1 $\Delta hapR \Delta clpA$ strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔhapR::Spec <sup>r</sup> ; ΔclpA:: Carb <sup>r</sup> : Δchp:Tm <sup>r</sup>	This study
SAD 2834	Fig. 1 <i>∆hapR</i> strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔhapR::Spec <sup>r</sup> ; Δcbp::Carb <sup>r</sup>	This study
SAD 2835	Fig. 2 parent	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -mCherry, Kan <sup>r</sup> ; ΔVCA0692::P <sub>const2</sub> - GFP, Spec <sup>r</sup>	This study
SAD 2836	Fig. 2 ∆ <i>hapR</i> strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -mCherry, Kan <sup>r</sup> ; ΔVCA0692::P <sub>const2</sub> - GFP, Spec <sup>r</sup> ; ΔhapR::Cm <sup>r</sup>	This study
SAD 2837	Fig. 2 Δ <i>cbp</i> strain and Fig. S3 E7946 parent	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -mCherry, Kan <sup>r</sup> ; ΔVCA0692::P <sub>const2</sub> - GFP, Spec <sup>r</sup> ; Δcbp::Tm <sup>r</sup>	This study
SAD 2838	Fig. 2 Δ <i>hapR</i> Δ <i>cbp</i> strain and Fig. S3 E7946 Δ <i>hapR</i> strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -mCherry, Kan <sup>r</sup> ; ΔVCA0692::P <sub>const2</sub> - GFP, Specr; ΔhapR::Cm <sup>r</sup> ; Δcbp::Tm <sup>r</sup>	This study
SAD 2839	Fig. 3 ChIP strain and Fig. S5 $1 \times$ FLAG-HapR	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; 1× FLAG-hapR; Δcbp::Tm <sup>r</sup>	This study
SAD 2840	Fig. 4 $\Delta luxO$ strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔluxO::Spec <sup>r</sup> ; Δcbp::Carb <sup>r</sup>	This study
SAD 2841	Fig. 4 luxOD47E strain	V. cholerae E7946 ΔlacZ::P <sub>cbb</sub> -GFP, Kan <sup>r</sup> ; luxO <sup>D47E</sup> ; Δcbp::Spec <sup>r</sup>	This study
SAD 2842	Fig. 4 $\Delta luxS$ strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔluxS::Cm <sup>r</sup> ; Δcbp::Spec <sup>r</sup>	This study
SAD 2843	Fig. 4 $\Delta cqsA$ strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔcqsA::Tm <sup>r</sup> ; Δcbp::Spec <sup>r</sup>	This study
SAD 2844	Fig. 4 $\Delta cqsA \Delta hapR$ strain	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP, Kan <sup>r</sup> ; ΔcqsA::Tm <sup>r</sup> ; ΔhapR::Cm <sup>r</sup> ; Δcbp::Spec <sup>r</sup>	This study
SAD 306	V. cholerae A1552 WT; parent for all other A1552 strains	V. cholerae A1552 O1 El Tor	51
SAD 2908	E. coli strain used to mate pMMB tfoX-qstR into complementation strains used in Fig. S1	E. coli S17 harboring pMMB67EH tfoX qstR, Cm <sup>r</sup>	This study
SAD 2909	Fig. S1 $\Delta hapR$ strain $P_{hapR}$ -hapR	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP Kan <sup>r</sup> ; ΔhapR::Spec <sup>r</sup> ; Δcbp::Carb <sup>r</sup> ; ΔVCA0692::P <sub>bage</sub> -hapR, Tm <sup>r</sup>	This study
SAD 2910	Fig. S1 ΔhapR ΔclpA strain P <sub>hape</sub> -hapR	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP Kan <sup>τ</sup> ; ΔhapR::Spec <sup>τ</sup> ; ΔclpA:: Carb <sup>τ</sup> ; Δcbp::Tm <sup>τ</sup> ; ΔVCA0692::P <sub>hap8</sub> -hapR, Zeo <sup>τ</sup>	This study
SAD 2911	Fig. S1 $\Delta clpA$ strain $P_{clpSA}$ -clpSA	V. cholerae E7946 ΔlacZ::P <sub>chb</sub> -GFP Kan'; ΔclpA::Carb'; Δcbp::Spec'; ΔVCA0692::P <sub>closa</sub> -clpSA, Tm <sup>r</sup>	This study
SAD 2912	Fig. S2 $\Delta hapR$ strain	V. cholerae E7946 ΔhapR::Cm <sup>r</sup>	This study
SAD 2845	Fig. S3 A1552 parent	V. cholerae A1552 ΔVCA0692::P <sub>const2</sub> -GFP, Spec <sup>r</sup> ; ΔlacZ::P <sub>chb</sub> - mCherry, Kan <sup>r</sup> ; Δcbp::Tm <sup>r</sup>	This study
SAD 2846	Fig. S3 A1552 <i>∆hapR</i> strain	V. cholerae A1552 ΔVCA0692::P <sub>const2</sub> -GFP, Spec <sup>r</sup> ; ΔlacZ::P <sub>chb</sub> - mCherry, Kan <sup>r</sup> ; ΔhapR::Cm <sup>r</sup> ; Δcbp::Tm <sup>r</sup>	This study
SAD 2847	Fig. S4 in WT <i>lacZ</i> <sup>+</sup> strain	V. cholerae E7946 ΔVC1807::Cm <sup>r</sup> ; ΔVC0995	This study
SAD 2848	Fig. S4 WT $\Delta lacZ$ strain	V. cholerae E7946 ΔlacZ::Spec <sup>r</sup> ; ΔVC1807::Cm <sup>r</sup> ; ΔVC0995	This study
SAD 2849	Fig. S4 $\Delta hapR \Delta lacZ$ strain	V. cholerae E7946 ΔlacZ::Spec <sup>r</sup> ; ΔVC1807::Cm <sup>r</sup> ; ΔVC0995; ΔhapR::Tm <sup>r</sup>	This study
JDN 71	Fig. 3B and C purified HapR	E. coli BL21(DE3) harboring pET28b-6×His-hapR, Kan <sup>r</sup>	This study

from the samples after ChIP). Primers to amplify *rpoB* served as a baseline control in this assay because HapR does not bind this locus. Data are reported as "fold enrichment," which is defined as the ratio of the test promoter ( $P_{chb}$  or  $P_{cloP}$ )/*rpoB* found in the output divided by the same ratio found in the input.

**Chitobiose competition.** Overnight cultures grown in LB were washed with M9 medium and mixed 1:1. For each growth reaction mixture, 10<sup>2</sup> cells of this mixture was added to M9 medium supplemented with 0.2% chitobiose and 10  $\mu$ M synthetic CAI-1 and grown shaking at 30°C for 24 h. After 24 h,  $\sim$ 10<sup>2</sup> cells from this mixture was used to inoculate fresh growth reactions to achieve additional generations of growth on chitobiose. This was repeated a third time after another 24 h. CAI-1 was supplemented

throughout these experiments to ensure consistently high levels of HapR expression throughout transfer steps. After each 24-h growth period, the CFU per milliliter was determined for each strain in the mixture by dilution plating on LB agar supplemented with X-Gal. Competing strains were discerned by blue/ white screening as one was a *lacZ*<sup>+</sup> strain and the other was a *ΔlacZ* strain. A *lacZ*<sup>+</sup> strain was competed against a *ΔlacZ* strain (*ΔnapR* strain-parent competition). *V. cholerae* can grow on chitobiose through the activity of the following two sugar transporters: the GlcNAc phosphotransferase system (PTS) transporter (VC095) or the chitobiose ABC transporter (VC0616-0620). Because we wanted to study the regulation of the transporter encoded within the *chb* locus, all strains for this assay had a deletion in VC0995 that renders growth on chitobiose dependent on the *chb* locus as previously described (11). Competitive indices were calculated as the CFU ratio of the *ΔlacZ* strain/*lacZ*<sup>+</sup> strain after growth for the indicated number of generations divided by the CFU ratio of the *ΔlacZ* strain/*lacZ*<sup>+</sup> strain in the initial inoculum.

**Statistics.** Statistical comparisons were determined using Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's posttest using GraphPad Prism software.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only. **SUPPLEMENTAL FILE 1**, PDF file, 0.9 MB.

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