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The response regulator PhoP negatively regulates *Yersinia pseudotuberculosis* and *Yersinia pestis* biofilms

Yi-Cheng Sun[1], **Alexandra Koumoutsi**, and **Creg Darby**[*]
Department of Cell and Tissue Biology, Program in Microbial Pathogenesis, University of California, San Francisco CA 94143

Abstract

A few *Yersinia pseudotuberculosis* strains form biofilms on the head of the nematode *Caenorhabditis elegans*, but numerous others do not. We show that a widely used *Y. pseudotuberculosis* strain, YPIII, is biofilm positive because of a mutation in *phoP*, which encodes the response regulator of a two-component system. For two wild-type *Y. pseudotuberculosis* that do not make biofilms on *C. elegans*, deletion of *phoP* was sufficient to produce robust biofilms. In *Yersinia pestis*, a *phoP* mutant made more extensive biofilms *in vitro* than did the wild type. Expression of HmsT, a diguanylate cyclase that positively regulates biofilms, is diminished in *Y. pseudotuberculosis* strains with functional PhoP.

Introduction

Yersinia pseudotuberculosis and *Yersinia pestis* are closely related bacteria (Achtman, *et al.*, 1999, Chain, *et al.*, 2004). *Y. pseudotuberculosis*, a prototroph, is a relatively mild gastrointestinal pathogen (Smego, *et al.*, 1999). *Y. pestis*, the agent of bubonic plague, is a frequently lethal bacterium that evolved recently (Achtman, *et al.*, 1999); it is an auxotroph that that infects mammals (usually rodents) and their fleas and is believed to be an obligate parasite (Perry & Fetherston, 1997). An important part of the *Y. pestis* life cycle is formation of a biofilm to colonize the digestive tracts of fleas (Jarrett, *et al.*, 2004). *Y. pseudotuberculosis* is apparently incapable of forming biofilms in fleas (Erickson, *et al.*, 2006), and a role for biofilms in its natural life cycle has not been reported.

In a laboratory model, both species form biofilms on the head of the nematode *Caenorhabditis elegans* (Darby, *et al.*, 2002). Multiple *Y. pestis* strains make these *in vivo* biofilms (Darby, *et al.*, 2002, Joshua, *et al.*, 2003), but under the same conditions relatively few *Y. pseudotuberculosis* strains do so. A study that examined 40 *Y. pseudotuberculosis* strain backgrounds found that only four made “severe” biofilms, while four others had an intermediate phenotype, two made barely detectable biofilms and 30 produced none (Joshua, *et al.*, 2003).

Y. pseudotuberculosis strains that fail to make biofilms in fleas or on *C. elegans* are nevertheless capable of forming biofilms under some conditions. In one study, strains that failed to make biofilms in fleas formed biofilms *in vitro* on borosilicate glass (Erickson, *et al.*, 2006). In another report, deletion of *rcsA*, encoding a component of a phosphorelay signaling system, resulted in robust biofilm formation on *C. elegans* (Sun, *et al.*, 2008).

*Corresponding author: Department of Cell and Tissue Biology, Box 0640, University of California, San Francisco, CA 94143-0640. Telephone: 415-476-3104. Fax: 415-476-1499, email: creg.darby@ucsf.edu.
[1]Present address: Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT 59840

YPIII, a widely used laboratory strain, is among the few *Y. pseudotuberculosis* that make strong biofilms on *C. elegans* (Darby, *et al.*, 2002, Joshua, *et al.*, 2003). YPIII has an inactivating mutation in *phoP*, which encodes the response regulator of a two-component signal transduction system (Grabenstein, *et al.*, 2004). In the present study we show that PhoP negatively regulates *Y. pestis* and *Y. pseudotuberculosis* biofilms.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used are shown in Table 1. Bacteria were grown in LB except as noted below, and plasmids were maintained with 100 μg/ml ampicillin. CDY664 and CDY665 were made by electroporating plasmid pCBD26 into the indicated parent strains. CDY618 was made using the λ Red recombination method (Datsenko & Wanner, 2000,Sun, *et al.*, 2008) to delete *phoP* from *Y. pestis* KIM6+ and replace it with a kanamycin resistance gene; the PCR primers used were 5′-GATAATATCCTGTTATCCGGTTAACGTTTTATCAAGGATTGGTGTATTCCGGGGATCCGTCGACC and 5′-CTAGTTGACGTCAAAACGATATCCCTGACTACGAATAGTCGTAATGTGTAGGCTGGAGCTGCTTCG. All *Y. pestis* strains in this study are avirulent in mammals due to absence of the pCD1 virulence plasmid. To make pCBD26, the *hmsT* gene and its promoter were PCR amplified from *Y. pestis* KIM6+ using the primers 5′-CGCGGATCCACGTGGTACAACATGCTGAC and 5′-CTTCGGCCGTCCCGAGTGATAAGGTGTGG, then cloned into pCR2.1-TOPO (Invitrogen). The fidelity of the open reading frame was verified by DNA sequencing.

C. elegans biofilms

For *Y. pseudotuberculosis* YPIII and IP2666c and their derivatives, nematode growth was assayed as described (Darby, *et al.*, 2005). Briefly, adult *C. elegans* were allowed to lay eggs on lawns of bacteria for 2–4 h; after the adults were removed, the plates were incubated for 2 d at 20°C, and the worm development to fourth larval (L4) stage was scored. To assay the biofilm capability of IP32777 and its derivatives, and for photographs of all *Y. pseudotuberculosis* strains, adult *C. elegans* were placed on bacterial lawns for approx. 16 h.

In vitro biofilms

Y. pestis biofilms grown on polystyrene culture dishes were assayed as described (Sun, *et al.*, 2008). Briefly, bacteria were grown in the dishes in 40% brain-heart infusion broth with shaking for 16 h at 26°C. The wells were washed gently with water, adherent biofilms were stained with crystal violet, and the wells were washed again. Crystal violet was resolubilized in ethanol-acetone and quantified by spectrophotometry. For each strain, four replicates were grown in parallel; wild-type and isogenic mutants were grown in a single 24-well dish. Mean and standard deviations were plotted, and significance was determined using the two-tailed Student's t-test.

Western blotting

Bacteria were grown in LB at 26°C to an OD_{600} of 4 (YPIII strains) or 2 (IP2666c strains), with 1mM IPTG added for strains with a *phoP* plasmid or empty vector. Approx. 20 μg of total protein per strain was electrophoresed on 12% Bis-Tris polyacrylamide gels (Invitrogen) and blotted onto nitrocellulose. Primary rabbit polyclonal antibody against HmsT (Perry, *et al.*, 2004) was diluted 1:10,000 in either phosphate-buffered saline (PBS) plus 5% skim milk or TBST buffer (20mM Tris HCl pH 7.5, 150mM NaCl, 0.05% Tween-20) plus 5% skim milk and incubated overnight at 4°C. After three buffer washes, goat anti-rabbit secondary antibody

conjugated to horseradish peroxidase (Invitrogen) was diluted 1:2,000 in buffer plus skim milk and the membrane incubated for 1 h, then washed three times in buffer. Bound antibody was detected with Immobilon Western HRP Substrate (Millipore).

Results

Because *Yersinia* sp. biofilms cover the mouth of *C. elegans* and inhibit the nematode's feeding, the ability of newly hatched *C. elegans* to grow on a bacterial lawn is a measure of the bacteria's biofilm formation (Darby, *et al.*, 2002, Darby, *et al.*, 2005, Sun, *et al.*, 2008). Beginning with eggs deposited on a lawn, almost all animals will develop to the fourth larval (L4) stage if feeding on biofilm-negative bacteria, while almost none will become L4 if the bacteria make robust biofilms.

We used this *C. elegans* growth assay to determine whether the *phoP* mutation in *Y. pseudotuberculosis* YPIII is responsible for the biofilm phenotype. Consistent with previous reports (Darby, *et al.*, 2002, Darby, *et al.*, 2005), YPIII made large biofilms on the worms and strongly inhibited growth (Fig. 1). When YPIII was transformed with a plasmid expressing wild-type *phoP*, it failed to make biofilms detectable by microscopy, and worms grew normally (Fig. 1). The phenotype of the *phoP*+ strain was essentially the same as YPIII with a mutation in the positive regulator *hmsT* (Fig. 1). To further establish the role of *phoP* in biofilm regulation, we examined another *Y. pseudotuberculosis* strain background. IP2666c failed to make biofilms and failed to inhibit growth, but an isogenic strain with a *phoP* deletion made visible biofilms and inhibited growth almost as well as YPIII (Fig. 1). Plasmid complementation restored biofilm repression, confirming that biofilm production was due to the *phoP* mutation (Fig. 1).

We also tested a third *Y. pseudotuberculosis* strain background, IP32777. The growth assay could not be used, because the strain has an uncharacterized activity that kills *C. elegans* eggs before they hatch. Instead, we placed adult *C. elegans* on IP32777 lawns for approx. 16 h and scored biofilm accumulation by microscopy. IP32777 failed to make a detectable biofilm. When *phoP* was deleted, substantial biofilms were made; complementation with plasmid-expressed *phoP* restored the biofilm-negative phenotype (data not shown). Judged by this visual assay, biofilm production by the IP32777 *phoP* mutant was somewhat weaker than that of its counterpart in the IP2666c background.

HmsT is a diguanylate cyclase required for both *Y. pestis* and *Y. pseudotuberculosis* biofilms (Darby, *et al.*, 2002, Kirillina, *et al.*, 2004); few other positive regulators have been identified. To examine whether PhoP negatively regulates biofilms by repressing *hmsT*, we transformed biofilm-negative IP2666c and IP32777 with a high-copy *hmsT* plasmid (pHmsT). Biofilms were made on *C. elegans*, similar to those observed when *phoP* is deleted (Fig. 1 and data not shown).

To confirm that PhoP represses HmsT, we used Western blotting. YPIII, a *phoP* mutant, produced substantial HmsT when transformed with an empty vector, but when wild-type *phoP* was on the plasmid, the protein level was reduced to a nearly undetectable level (Fig. 2A). With IP2666c, which has wild-type *phoP*, HmsT was not observed at all, but deletion of *phoP* produced detectable protein (Fig. 2B). We conclude that PhoP inhibits biofilms at least in part by negatively regulating HmsT. It has not been established whether the regulation is direct or through intermediates.

To determine whether PhoP negatively regulates *Y. pestis* biofilms, we deleted *phoP* from the biofilm-positive strain KIM6+. Because KIM6+ makes biofilms that completely inhibit *C. elegans* growth (Sun, *et al.*, 2008), an increase in biofilms in the nematode assay would not be detectable. Instead, we examined *Y. pestis* biofilms *in vitro* by growing them on polystyrene

dishes and staining with crystal violet. The values for an *hmsS* mutant were barely above background (Fig. 3), confirming that the *in vito* biofilm is *hmsHFRS*-dependent. The *phoP* mutant biofilms were consistently about twice as extensive as the wild-type (Fig. 3), confirming that PhoP negatively regulates *Y. pestis* biofilms. During initial trials it was observed that biofilms made by the *phoP* mutant adhered more loosely to the dish than did wild-type biofilms, and gentle washing was required to avoid dislodging them.

Discussion

The role of biofilms in the *Y. pestis* life cycle is known. *Y. pestis* forms biofilms in the digestive tracts of fleas that block the insect's feeding, stimulating it to bite repeatedly in search of food and thereby transmit the bacteria to new hosts (Jarrett, *et al.*, 2004, Darby, 2008). Biofilms play no apparent role in *Y. pestis* mammalian pathogenesis. A mutation in *hmsR*, a gene required for biofilms, did not reduce virulence in a mouse infection model (Lillard, *et al.*, 1999), and biofilm formation *in vitro* is repressed at 37°C (Kirillina, *et al.*, 2004).

Despite the close relationship between the two *Yersinia* species, multiple *Y. pseudotuberculosis* strains failed to form biofilms in fleas, including those that were able to make biofilms *in vitro* (Erickson, *et al.*, 2006). No published reports examine biofilms in *Y. pseudotuberculosis* mammalian infections. However, an *hmsT* mutant that was completely defective for biofilm formation in the *C. elegans* assay (Darby, *et al*., 2002) was fully virulent in a mouse oral infection model (J.W. Hsu, C. Darby and S. Falkow, unpublished data), suggesting that biofilms are not required in a mammalian host.

Y. pestis and *Y. pseudotuberculosis* have virtually identical exopolysaccharide biosynthetic operons, *hmsHFRS* (Darby, 2008). This operon is required for *Y. pestis* biofilms in fleas (Hinnebusch, *et al.*, 1996, Jarrett, *et al.*, 2004) and for either species to make biofilms on *C. elegans* (Darby, *et al.*, 2002). Multiple *C. elegans* mutants isolated on the basis of resistance to *Y. pseudotuberculosis* biofilm attachment were all cross-resistant to *Y. pestis* (Darby, *et al.*, 2007). Together, these findings suggest that differences between the two bacteria in the *C. elegans* biofilm model are not at the level of exopolysaccharide composition and structure. Consistent with this interpretation, we recently reported a regulatory difference affecting biofilms. During *Y. pestis* evolution, a mutation occurred that inactivated *rcsA*, encoding a phosphorelay accessory protein; the mutation relieved biofilm repression and was apparently crucial for the bacterium's ability to form biofilms in fleas (Sun, *et al.*, 2008).

In the current work we examined another regulatory signaling pathway, the PhoP-PhoQ two-component system. Our investigation was prompted by the finding that the widely used *Y. pseudotuberculosis* laboratory strain YPIII has an inactivating mutation in *phoP* (Grabenstein, *et al.*, 2004). Because YPIII makes robust biofilms in the *C. elegans* assay while many other *Y. pseudotuberculosis* do not (Darby, *et al.*, 2002, Joshua, *et al.*, 2003, Sun, *et al.*, 2008), we asked whether the mutation in *phoP* is responsible. The answer was affirmative. Restoration of functional *phoP* in YPIII resulted in almost complete absence of biofilms in the *C. elegans* model (Fig. 1). In two other *Y. pseudotuberculosis* wild-type backgrounds that do not make biofilms on *C. elegans*, deletion of *phoP* was sufficient to produce extensive biofilms that blocked nematode feeding (Fig. 1 and data not shown).

We conclude that PhoP negatively regulates biofilms in *Y. pseudotuberculosis*. This appears to occur at least in part by downregulation, directly or indirectly, of the diguanylate cyclase HmsT (Fig. 2). In two different backgrounds, HmsT was absent or barely detectable with wild-type *phoP* present but highly expressed when *phoP* was mutated or deleted.

In *Y. pestis*, a *phoP* deletion resulted in a doubling of biofilm production, as assayed by crystal violet staining of biofilms adhering to polystyrene (Fig. 3). This indicates that in *Y. pestis* as

well as *Y. pseudotuberculosis*, PhoP negatively regulates biofilms. We also observed that the *Y. pestis phoP* biofilms are less adherent than normal. Interestingly, Erickson *et al.* observed that *Y. pseudotuberculosis* biofilms in glass flow cells tend to slough off the surface more readily than those made by *Y. pestis*. Differences in adhesion are not easily explained by mere changes in the amount of exopolysaccharide; rather, they suggest variation in composition or structure of the extracellular matrix. Our findings suggest that in *Y. pestis*, PhoP regulates both the volume and the adhesive properties of the biofilm.

PhoP, a response regulator, functions with the sensor kinase PhoQ in a two-component system. PhoP-PhoQ has been studied most extensively in *Salmonella enterica* serovar Typhimurium, where it responds to Mg^{2+} and Ca^{2+} concentrations and is essential for virulence (Groisman, 2001). Of particular relevance to the present study, a *S. enterica phoP* null mutant had enhanced biofilm production on gallstones and on glass, while a *phoP* constitutive mutant was biofilm defective, indicating that PhoP negatively regulates *S. enterica* biofilms (Prouty & Gunn, 2003).

In *Y. pseudotuberculosis* as in *S. enterica*, PhoP mediates a response to Mg^{2+} (Grabenstein, *et al.*, 2004). A *Y. pseudotuberculosis phoP* mutant was attenuated in a mouse infection model, with a 100-fold higher LD_{50} (Grabenstein, *et al.*, 2004), and *phoP* is a virulence factor in a *Y. pseudotuberculosis* lung infection model (Fisher, *et al.*, 2007). In both *Y. pseudotuberculosis* and *Y. pestis*, PhoP increases survival inside macrophages (Oyston,*et al.*, 2000, Grabenstein, *et al.*, 2004). Although PhoP has not been examined as extensively in *Yersinia* sp. as it has in *S. enterica*, the available evidence indicates that it functions as a positive virulence regulator in mammalian infections.

Y. pestis is an obligate parasite with two disparate hosts, and as it shuttles between fleas at lower temperatures and mammals at higher ones, it regulates its functions accordingly (Prentice & Rahalison, 2007). In fleas, *Y. pestis* makes biofilms required to persist in the insect, while repressing the Yop proteins that disrupt and evade mammalian immunity. When the bacteria are transmitted to a mammalian host, the profile is reversed: biofilms are repressed while Yops are expressed at high levels. Our finding that *Y. pestis* PhoP negatively regulates biofilms is consistent with this bimodal life cycle. During mammalian infections, PhoP positively regulates multiple genes that promote survival and virulence (Grabenstein, *et al.*, 2006), and our results suggest that at the same time PhoP negatively regulates biofilm functions that are unneeded in mammalian hosts.

The place of biofilms in the *Y. pseudotuberculosis* life cycle is not known. However, the two *Yersinia* have a close evolutionary relationship, and they use many of the same genetic elements (*hmsHFRS*, *hmsT*, *rcs*, *phoP*) in making biofilms. In addition, the genomes of both species encode multiple insecticidal proteins (Parkhill, *et al.*, 2001, Gendlina, *et al.*, 2007, Pinheiro & Ellar, 2007, Waterfield, *et al.*, 2007). Thus, even though *Y. pseudotuberculosis* appears unable to make biofilms in fleas, it likely encounters insects in its life cycle. The specialized ability of *Y. pestis* to make biofilms in fleas may have evolved from a more general capability in the ancestral *Y. pseudotuberculosis* to make biofilms in an invertebrate host.

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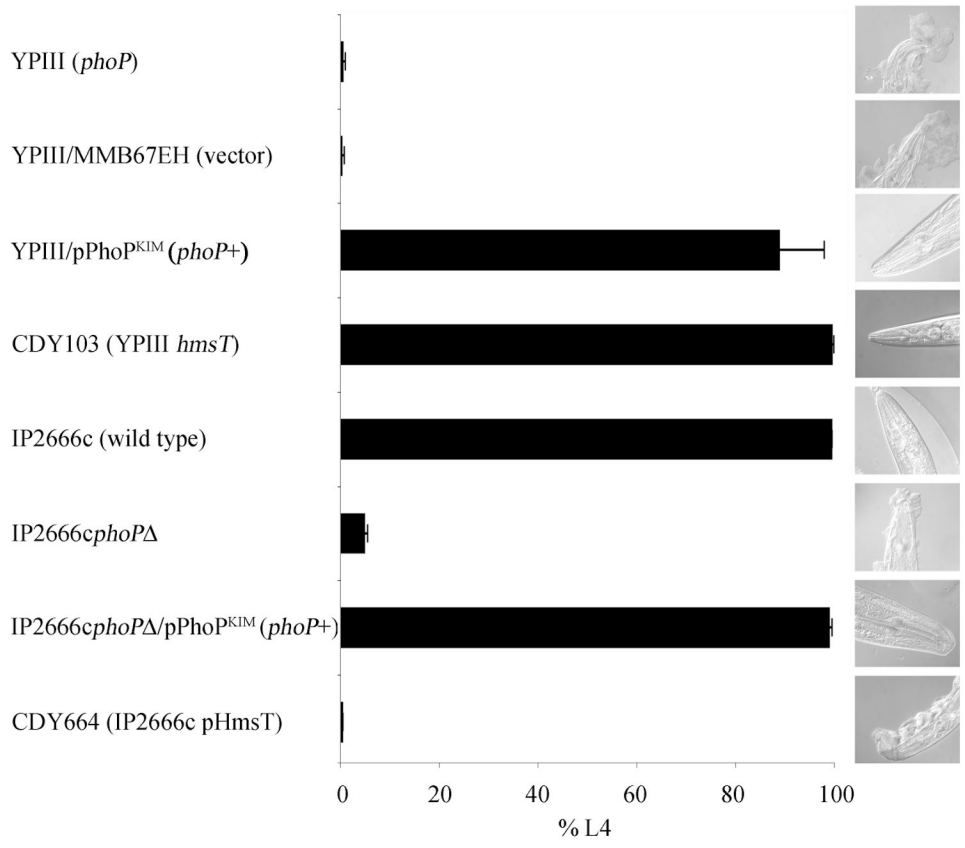

Fig. 1.

PhoP represses *Y. pseudotuberculosis* biofilms on *C. elegans*. Growth of *C. elegans* to L4 stage in 2 d on lawns of the indicated strains is plotted; data are mean ± S.D. of assays performed on three separate occasions, with at least 1,200 worms per sample. Low growth indicates biofilm is made that inhibits feeding, while high growth indicates reduction or absence of biofilm. Photographs show typical adult worms after 16 h on bacterial lawns.

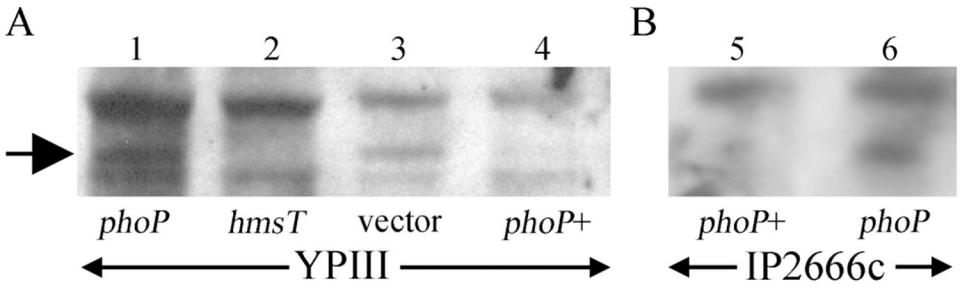

Fig. 2.

phoP mutations increase HmsT expression in *Y. pseudotuberculosis*. Total protein from strains in the YPIII (A) and IP2666c (B) backgrounds was immunoblotted with a rabbit polyclonal antibody against HmsT. The antibody is known to cross-react with multiple other proteins (Perry, *et al.*, 2004); the HmsT band (arrow) is identified by its absence in the *hmsT* mutant. 1, YPIII; 2, CDY103; 3, YPIII/MMB67EH; 4, YPIII/pPhoP^{KIM}; 5, IP2666c; 6, IP266c*phoP*Δ.

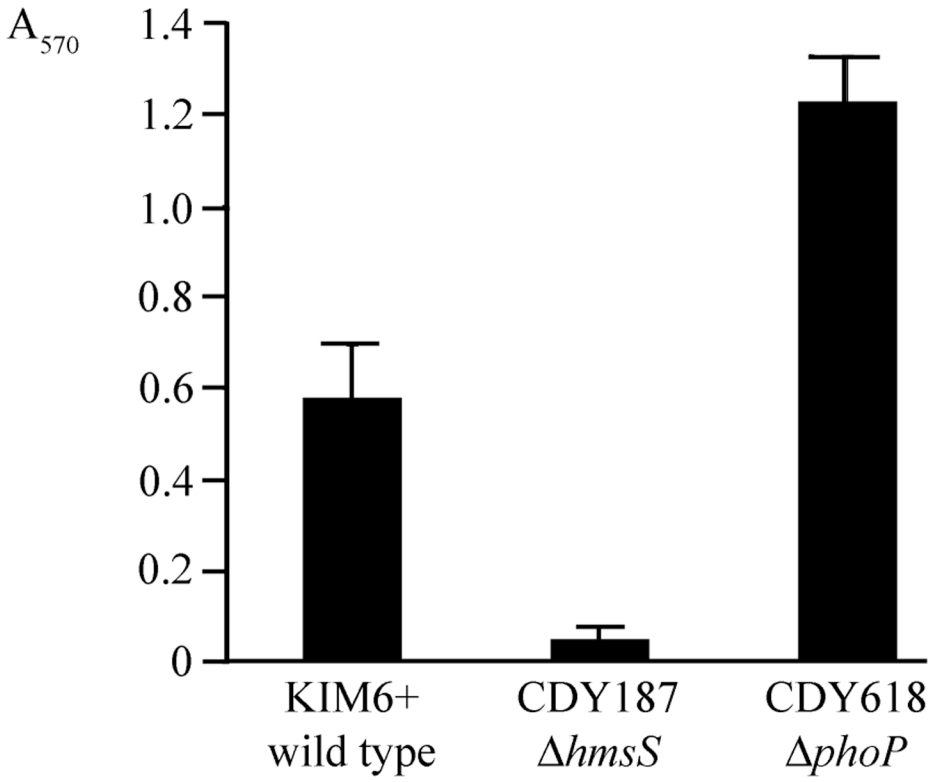

Fig. 3.

phoP negatively regulates *Y. pestis* biofilms *in vitro*. Broth cultures were grown in a multi-well polystyrene culture dish for 16 h. After washing, the adherent material was stained with crystal violet, which was resolubilized and quantified by absorption at 570 nm. The *hmsS* mutant is a negative control; *hmsS* is known to be required for *Y. pestis* biofilms *in vitro* and *in vivo* (Forman, *et al.*, 2006, Sun, *et al.*, 2008). The *hmsS* ($p = 0.002$) and *phoP* ($p = 0.0016$) mutants both differed significantly from wild type. The experiment was repeated with similar results.

Table 1

Bacterial strains and plasmids

Strain or plasmid	Genotype and/or description	Reference or source
Y. pseudotuberculosis		
YPIII	Serogroup O3, *phoP*^{T160P}	(Gemski, *et al.*, 1980)
YPIII/MMB67EH	*phoP*^{T160P}/pMMB67EH	(Grabenstein, *et al.*, 2004)
YPIII/pPhoP^{KIM}	*phoP*^{T160P}/pPhoP^{KIM}	(Grabenstein, *et al.*, 2004)
CDY103	YPIII*phoP*^{T160P}/*hmsT*::Tn*phoA*	(Darby, *et al.*, 2002)
IP2666c	Serogroup O3, wild type	(Simonet & Falkow, 1992)
IP2666c*phoP*Δ	*phoP*Δ127–429	(Grabenstein, *et al.*, 2004)
IP2666c*phoP*Δ/pPhoP^{KIM}	*phoP*Δ127–429/pPhoP^{KIM}	(Grabenstein, *et al.*, 2004)
CDY664	IP2666c/pCBD26	This study
IP32777	Serogroup O1, wild type	(Collyn, *et al.*, 2002)
IP32777*phoP::kan*	*phoP*::*kan*	(Grabenstein, *et al.*, 2004)
IP32777*phoP::kan*/pPhoP^{KIM}	*phoP*::*kan*/pPhoP^{KIM}	(Grabenstein, *et al.*, 2004)
CDY665	IP32777/pCBD26	This study
Y. pestis		
KIM6+	wild type (ΔpCD1)	(Deng, *et al.*, 2002)
CDY618	KIM6+ Δ*phoP::kan*	This study
CDY187	Δ*hmsS::cat*	(Sun, *et al.*, 2008)
Plasmids		
pMMB67EH	expression vector	(Furste, *et al.*, 1986)
pPhoP^{KIM}	*phoP* in pMMB67EH	(Grabenstein, *et al.*, 2004)
pCR2.1-TOPO	cloning vector	Invitrogen
pCBD26 (pHmsT)	*hmsT* in pCR2.1-TOPO	This study