Mutation-induced remodeling of BfmRS two-component system in 

*Pseudomonas aeruginosa*

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**One Sentence Summary:** Spontaneous mutations induce remodeling of TCSs

**Abstract:** Genetic mutations are a primary driving force behind the adaptive evolution of bacterial pathogens. Here, we show that in *Pseudomonas aeruginosa*, an important human pathogen, the naturally evolved L181P/E376Q missense mutations in the two-component sensor BfmS gene increases the phosphorylation level and thus the regulatory activity of its cognate response regulator BfmR, which in turn directs this pathogen toward a chronic infection state. The elevated phosphorylation of BfmR appears at least in part due to the reduced phosphatase activity of BfmS, which also allows the cross-phosphorylation of BfmR by GtrS, a non-cognate sensor kinase. We documented that not only the L181P/E376Q but also other spontaneous missense mutations in *bfmS*, such as A42E/G347D, T242R, and R393H, cause a similar...
remodeling of the BfmRS two-component system in *P. aeruginosa*. This study thus exemplifies the plasticity of two-component systems mediated by spontaneous mutations, also suggesting that mutation-induced activation of BfmRS may contribute to the host adaptation of *P. aeruginosa* during chronic infections.

**Key words:** *Pseudomonas aeruginosa*, spontaneous mutation, two-component system, gene expression, adaptation

**Introduction**

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen and frequently causes life-threatening infections in humans (1, 2). This pathogen is a leading cause of chronic pulmonary infections and high mortality in cystic fibrosis (CF) patients (2, 3). Once establishing a chronic infection, *P. aeruginosa* is nearly impossible to eradicate with current antibiotic regimens (3, 4). In order to design better strategies for clinical intervention, we need to improve our understanding of the evolution and adaptation of *P. aeruginosa* during infections (5-7).

During chronic infections, *P. aeruginosa* undergoes numerous genetic changes, leading to the development of mutants with altered phenotypes such as conversion to the mucoid colony, reduced production of acute infection-associated virulence factors, a transition to a biofilm-associated lifestyle, and enhanced antibiotic resistance (5, 6). Genome sequencing analyses have revealed recurrent patterns of mutations in CF isolates of *P. aeruginosa*. In particular, mutations are commonly found in the genes encoding global regulators such as MucA and LasR (5-13), indicating that, in *P. aeruginosa*, transcriptional reprogramming may be a key element of adaptation to the CF airway.

The survival of *P. aeruginosa* in the CF airways depends, at least in part, on its ability to sense and respond to changes in its environment. In fact, in the CF isolates of *P. aeruginosa*, genetic alterations are often found in genes, such as *pmrB*, *bfmS*, *retS*, *amgR*, *amgS*, *parS*, and *cbrB*, encoding proteins of two-component systems (TCS) (9, 11-16) that serve as a common bacterial mechanism for sensing and responding to the extracellular environment. The canonical TCS system consists of a sensor histidine kinase (SK) and a cognate response regulator (RR) (17, 18). Upon stimulus detection, the SK phosphorylates its conserved histidine residue and subsequently transfers the phosphoryl group to the conserved aspartate residue in RR. Then the phosphorylated RR coordinates changes in bacterial behavior, often through its activity as a transcriptional regulator (17, 18). Many SK proteins have both kinase and phosphatase activities (17, 18). Depending on the status of those activities, SK can behave as a net kinase (kinase activity > phosphatase activity) or a net phosphatase (phosphatase activity > kinase activity) (17, 18). Cross-regulation
between different TCSs is rare (17); however, in a few cases, it does occur, and many more examples of cross-regulation have been seen in genetically perturbed organisms (17, 19-21).

In various clonal types of P. aeruginosa, the SK BfmS gene is a frequent target of adaptive mutations (9, 12, 13, 16). It is also worthy to note that in MPAO1, a laboratory strain of P. aeruginosa, the wild-type BfmS negatively controls its cognate RR BfmR, which is a positively auto-regulated transcription factor and plays important roles in biofilm maturation (22, 23), rhl quorum sensing (24), and acute bacterial infections (24) in P. aeruginosa. Inspired by these observations, we decide to examine the effect of spontaneous missense mutations in bfmS on the physiology of P. aeruginosa and the underlying mechanisms.

Results

L181P/E376Q missense mutations in bfmS enhance the regulatory functions of BfmR

In our previous study, although the wild-type (WT) bfmS allele inhibited the regulatory activity of BfmR, the bfmS mutant alleles, bfmS<sup>L181P</sup> and bfmS<sup>L181P/E376Q</sup>, activated it (24). In the study, the bfmS alleles were provided by a multi-copy plasmid. To exclude the plasmid-copy number effect and to further assess the consequences of the bfmS missense mutations, we generated a bfmS<sup>L181P/E376Q</sup> mutant allele at the native bfmS site in the chromosome of the laboratory strain MPAO1. As compared with its isogenic parent strain MPAO1, the resulting mutant, bfmS<sup>DK2</sup>, exhibited much higher expression levels of bfmR-<i>lux</i> (Fig. 1A, fig. S1), an indicator for the transcriptional regulatory activity BfmR (24), demonstrating that the mutant allele can activate BfmR regardless of the copy number. The bfmR-<i>lux</i> reporter exhibited an activation surge in either WT MPAO1 or bfmS<sup>DK2</sup> mutant that is following the growth curve. This is in line with the observation that BfmRS is auto-regulated (24) and indicates that the induction of the BfmRS system is growth-dependent.

Introduction of a plasmid-borne WT bfmS into the bfmS<sup>DK2</sup> mutant reduced the bfmR-<i>lux</i> expression to a WT level (Fig. 1A and fig. S1), confirming the inhibitory role of the WT BfmS on BfmR. A similar effect was observed with the chromosomal <i>pa4103-lux</i> transcriptional reporter fusion (fig. S2A), another indicator of BfmR transcriptional regulatory activity (24). In a Western blot analysis, the bfmS<sup>DK2</sup> mutant produced a much higher amount of BfmS proteins than the WT MPAO1 strain (Fig. 1B), which can be explained by the fact that bfmR and bfmS forms an operon (Fig. S2B). We also obtained consistent results in Western blot analysis in which the inner membrane fractions were analysed by immunoblotting (Fig. 1B), and this is in line with the prediction that BfmS is an inner membrane protein (1). Collectively, these
results suggest that the missense mutation (L181P/E376Q) in bfmS enhances the transcriptional regulatory activity of BfmR in *P. aeruginosa* MPAO1.

Since the function of BfmR is activated by phosphorylation (24), we next tested if the phosphorylation status of BfmR is affected by the *bfmS* missense mutation. With a plasmid (pAK1900-bfmR-flag, table S1), Flag-tagged BfmR was expressed in WT and the *bfmS* L181/E376 missense mutant (*bfmS*DK2) of MPAO1 strain; then, the total bacterial proteins were resolved on SDS-PAGE gels containing Phos-tag™ acrylamide (25) and subjected to Western blotting with an anti-FLAG antibody. In the WT strain, only the non-phosphorylated BfmR-flag was observed, whereas, in the *bfmS*DK2 mutant, a large amount of phosphorylated BfmR-flag (P~BfmR-flag) was also detected (Fig. 1C), suggesting that the phosphorylation level of BfmR is elevated by the L181P/E376Q missense mutations in *bfmS*. The phosphorylation level of BfmR-flag in the *bfmS*DK2 mutant was reduced to the WT level by introduction of a WT *bfmS* with an integration-proficient vector (mini-*bfmS*, table S1) (Fig. 1C), confirming an inhibitory role of the WT BfmS in the phosphorylation of BfmR.

As compared with the WT MPAO1 strain, the *bfmS* L181/E376 missense mutant (*bfmS*DK2) exhibited lesser production of quorum-sensing (QS) signal molecules N-butanoyl-l-homoserine lactone (C4-HSL) (Fig. 1D), reduced virulence in either lettuce leaf (Fig. 1E) or *Drosophila melanogaster* model (Fig. 1F), and increased biofilm formation (Fig. 1G and H). Each of these phenotypes could be restored to the WT level by *in trans* expression of a plasmid-borne WT *bfmS* (Fig. 1D-G). These observations indicate that the naturally evolved missense mutations (L181P/E376Q) in *bfmS* may confer multiple important phenotypes relevant to the adaptation of *P. aeruginosa* to CF patients (5, 6). Moreover, deletion of *bfmR* totally suppressed all the observed phenotypes caused by the *bfmS* L181P/E376Q missense mutations (fig. S2 C-G), indicating that the functional activation of BfmR has an important role in directing *P. aeruginosa* toward enhanced adaptive performance.

**BfmR is highly activated in the CF-adapted isolates of *P. aeruginosa* DK2 lineage**

As aforementioned, BfmR is activated upon *bfmS* L181P/E376Q missense mutations (Fig. 1). In DK2 isolates from the long-term-infected CF patients (CF-adapted isolates, sampled between 1979 and 2008), *pa4107* (*efhp*), an BfmR-dependent gene (24), is the most abundant mRNA transcript, and also the most increased gene (with a >256-fold increase), as compared with the non-CF-adapted isolate PAO1 (26). In light of these observations, we compared the transcriptome of 12 CF-adapted isolates of DK2 with that of three DK2 isolates from an early infection (non-adapted isolates, sampled in 1973) (9, 12). The comparison identified that the CF-adapted DK2 isolates share common gene expression profiles, where the expression of 78 genes were increased, whereas the expression of 49 genes were decreased (Sheet 1 of Data file 1). To our surprise, genes activated by BfmR including *bfmR* itself, *pa4102* (*bfmS*),
pa4103, pa4104, pa4105, pa4106, and pa4107 were found among the most increased genes in the CF-adapted DK2 isolates (Fig. 2A, Sheet 1 of Data file 1), indicating that BfmR is highly activated in the CF-adapted isolates of *P. aeruginosa* DK2 lineage. Similar results were seen in the microarray expression dataset GSE62970, which contains the gene expression profiles of two CF-adapted isolates (DK2-91 and DK2-07) and a non-adapted isolate (DK2-WT) of DK2 lineage (Sheet 2 of Data file 1).

To confirm that the increased transcriptional regulatory activity of BfmR in the CF-adapted clinical isolates is due to the L181P/E376Q mutation in BfmS, we replaced the *bfmS*<sup>L181P/E376Q</sup> allele of the *P. aeruginosa* DK2 strain (27) with the WT *bfmS* by allelic exchange. When compared to its isogenic parent DK2 strain, the resulting mutant *bfmS*<sup>PAO1</sup> exhibited phenotypes indicative of reduced BfmR activity, such as enhanced production of QS signal C4-HSL and virulence factor pyocyanin (Fig. 2B and C), and decreased biofilm formation (Fig. 2D). The Phos-tag mobility shift assay showed that the *bfmS*<sup>PAO1</sup> mutant produces a much smaller amount of the P–BfmR-flag, as compared with the WT DK2 strain (Fig. 2E). As expected, in both *bfmS*<sup>PAO1</sup> and ∆*bfmRS* mutants, the activity of *pa4103-lux* was much lower than that of the WT DK2 strain (Fig. 2F). Thus, in the CF-adapted clinical isolates of the DK2 lineage, an activation of BfmR and the resulting adaptive changes (such as virulence-associated traits and gene expression) are due, at least in part, to the L181P/E376Q missense mutations in *bfmS*.

*gtrS* is essential for the transcriptional regulatory activity of BfmR in the absence of *bfmS*

It appears that increased regulatory activity of BfmR, which resulted from L181P/E376Q missense mutations of *bfmS*, may be critical for the pathogenesis of *P. aeruginosa* DK2 in CF patients (Fig. 2). To further identify whether or not BfmS is the only regulator controlling the regulatory activity of BfmR, we performed a suppressor screening using a transposon mutant library of ∆*bfmS*. In the screening, the activity of BfmR was measured by the *bfmR* promoter-*lacZ* reporter fusion (table S1). On M8-glutamate minimal agar medium supplemented with glucose and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), the reporter fusion brings about intense blue colonies (indicative of high activity of BfmR) in the ∆*bfmS* mutant; however, in the WT MPAO1 strain, it causes very weak blue colonies (indicative of low BfmR activity). From a library of ~30,000 ∆*bfmS* transposon mutants, we identified 12 weak blue colonies. Of the 12 mutants, 10 had a transposon insertion in either the promoter or coding region of *bfmR*. The other two had a transposon insertion in *pa3190* (*gltB*) and *pa3191* (*gtrS*), respectively. The *gltB* gene encodes a periplasmic binding protein GltB involved in glucose transport (28), whereas the *gtrS* gene encodes the sensor kinase GtrS that forms a TCS with the glucose uptake response regulator GltR (29-31) (fig. S3A).
To verify the role of GltB in the transcriptional regulatory activity of BfmR, we deleted gltB in the bfmS deletion mutant (ΔbfmS) background. Like the original transposon mutant, the resulting bfmS-gltB double deletion mutant (ΔbfmSΔgltB) showed a WT level of low bfmR promoter activity (fig. S3B). Ectopic expression of gltB in the ΔbfmSΔgltB mutant strain significantly increased the activity of bfmR promoter to a level similar to that of the bfmS single deletion mutant (ΔbfmS) (fig. S3B), demonstrating that GltB is a potent activator of BfmR in the ΔbfmS mutant. Likewise, we verified that, in ΔbfmS, gtrS is also crucial for the transcriptional regulatory activity of BfmR, as evidenced by the facts that deletion of both gltR and gtrS decreases the bfmR-lux activity of the ΔbfmS mutant to a level of the WT MPAO1 strain (Fig. 3A), while the introduction of a plasmid containing gtrS (p-gtrS) into the ΔbfmSΔgltR-gtrS triple mutant increases the bfmR-lux activity to the high level seen in the ΔbfmS mutant (Fig. 3A). These results also suggest that the increased transcriptional regulatory activity of BfmR by GtrS does not require GltR. On the other hand, ectopic expression of gtrS failed to induce the level of pa4103-lux activity when bfmR was further deleted (ΔbfmSΔgltR-gtrS) (Fig. 3B), supporting the idea that the activation of pa4103-lux expression by GtrS requires BfmR. Taken together, these results suggest that GltB and GtrS are critical in the regulatory activity of BfmR in the bfmS deletion mutant (ΔbfmS). Also, we found that GtrS can increase the regulatory activity of BfmR independently of GltB, but not vice versa (fig. S3C). These observations prompted us to investigate the molecular pathway(s) that links GtrS to the functional activation of BfmR.

Glucose signals the transcriptional regulatory activity of BfmR through GtrS in the ΔbfmS mutant

In GtrS, the conserved H280 residue is predicted to be autophosphorylated (http://www.pseudomonas.com/). In order to examine the functional importance of this His residue, we examined the ability of the GtrSH280A protein to activate the expression of pa4103-lux. The H280A amino acid substituent had no obvious effect on the expression/stability of the GtrS protein (fig. S3D). However, unlike the WT GtrS, the GtrSH280A failed to promote the expression of pa4103-lux in a bfmS-gtrS double deletion mutant strain (ΔbfmSΔgtrS) (fig. S3E), indicating that the autokinase activity is required for GtrS in order to activate BfmR.

Since the uptake of glucose in P. aeruginosa is likely regulated by GtrS (32), we next sought to examine the effects of glucose on the expression of the pa4103-lux reporter in WT MPAO1 strain, bfmS deletion mutant (ΔbfmS), and the ΔbfmS mutant complemented with p-bfmS, in order to further examine the role of GtrS in the transcriptional regulatory activity of BfmR. Although the following 16 carbohydrates failed to induce the promoter activity of pa4103 in all strain backgrounds: glycerol, D-mannitol, D-fructose, D-(+)-trehalose, succinate, pyruvate, citrate, sucrose, α-ketoglutarate, maltose, D-gluconate, DL-malate, D-Glucose-6-Phosphate, 5-Keto-D-
gluconic acid, and D-(+)-xylose, the addition of glucose promoted $pa4103$-lux expression in the $bfmS$ deletion mutant strain ($\Delta bfmS$) but not in either the WT MPAO1 strain or the complemented strain of $\Delta bfmS$ (fig. S4A). In $\Delta bfmS$, glucose can induce the $pa4103$-lux activity in a concentration-dependent manner, even at low micromolar levels (fig. S4B); however, in the $\Delta bfmS\Delta gtrS$ double mutant, it gave no effect (fig. S4C), indicating that, in the $\Delta bfmS$ mutant, the glucose-mediated activation of BfmR requires GtrS.

Additionally, we observed that glucose induces the promoter activity of $gltB$ ($gltB$-lux) in a GtrS- and concentration-dependent manner (fig. S4 D and E), consistent with the notion that the high-affinity glucose transport system of $P$. aeruginosa is induced by glucose and the GtrS-GltR TCS (32). Deletion of $gltB$ in $P$. aeruginosa MPAO1 was associated with lower expression of $gltB$-lux, indicative of reduced GtrS-GltR TCS activity (fig. S4F). The introduction of p-$gltR$-$gtrS$, but not p-$gtrS$, into the $\Delta gltB$ strain was able to enhance the activity of $gltB$-lux (fig. S4F). These results suggest that GltB may activate GtrS-GltR TCS by a hitherto unknown mechanism.

GtrS forms a TCS with BfmR in $P$. aeruginosa mutant deficient in $bfmS$

Since GtrS is a SK, most likely, it activates BfmR through phosphorylation. To examine this possibility, BfmR-flag was expressed, and the phosphorylation of the BfmR protein was analyzed by Phos-tag assay in WT MPAO1, $\Delta bfmRS$ double mutant, and the $\Delta bfmRS\Delta gltR$-$gtrS$ tetra mutant. As expected, only non-phosphorylated BfmR was detected in the WT MPAO1 strain (lane 6 in Fig. 3C), whereas both phosphorylated and non-phosphorylated forms of BfmR were detected in the $\Delta bfmRS$ double mutant (lane 1). In the $\Delta bfmRS\Delta gltR$-$gtrS$ tetra mutant, however, only a slight amount of phosphorylated BfmR was detected (lane 2). The level of P-BfmR was restored by the introduction of either $gltR$-$gtrS$ (lane 3) or $gtrS$ (lane 5), but not $gltR$ alone (lane 4). Thus, GtrS has a substantially positive effect on the phosphorylation of BfmR in $P$. aeruginosa deficient in $bfmS$.

When BfmR-flag was expressed in the $\Delta bfmRS$ double mutant, a high amount of phosphorylated BfmR was observed only in the presence of glucose (fig. S5A, lane 2 and 6). A similar result was obtained with the $\Delta bfmRS\Delta pta$-acka mutant (fig. S5A, lanes 3 and 7), indicating that the acetyl phosphate does not play a major role in the phosphorylation of BfmR under the experimental condition employed. The in vivo phosphorylation of BfmR was eliminated by substituting the predicted phosphorylation site Asp55 with alanine (fig. S5A, lane 4), confirming that BfmR is phosphorylated at Asp55 (24).

To examine whether GtrS directly interacts with BfmR, we performed co-immunoprecipitation (co-IP) experiments. Both HA-tagged GtrS (GtrS-HA) and Flag-
tagged BfmR (BfmR-flag) were expressed in the bfmS deletion mutant (ΔbfmS). After GtrS-HA was immunoprecipitated with an anti-HA antibody, the co-precipitation of BfmR-flag was examined with anti-Flag antibody. BfmR-flag was detected only when GtrS was co-expressed (lane 6 in Fig. 3D). Conversely, the immunoprecipitation of BfmR-flag with anti-Flag antibody was also able to bring down HA-tagged GtrS (GtrS-HA) (fig. S5B, lane 5), indicating that GtrS directly interacts with BfmR in vivo.

Next, to investigate whether GtrS can directly phosphorylate BfmR, we purified the N-terminally 6His-tagged cytosolic fragment GtrS (hereafter referred to as His6-GtrSc) and examined whether it can phosphorylate the His6-BfmR. In the presence of ATP, His6-GtrSc was autophosphorylated (Fig. 3E). When the His6-BfmR was further added, the phosphoryl group was transferred from His6-GtrSc to BfmR (Fig. 3E). His6-BfmR alone was unable to autophosphorylate (fig. S5C, see also in lane 1 in Fig. 5B). Heating the sample converted all P~His6-BfmR to His6-BfmR (fig. S5D, lane 3), consistent with the heat-liability of aspartyl-phosphate bonds (17, 18). His6-BfmR$_{D55A}$ was not phosphorylated by GtrSc (fig. S5D), however, indicating that the phosphorylation of BfmR by GtrS depends on the phosphorylation residue (Asp55). Using Pro-Q Diamond stain as an indicator of phosphorylation, we also observed that His6-BfmR can be phosphorylated by His6-GtrSc (Fig. 3F). Taken together, these results support that, in ΔbfmS, GtrS forms a TCS with BfmR.

**GtrS and BfmR still forms a TCS in the presence of BfmSL181P/E376Q**

We next sought to examine whether GtrS contributes to the activation of BfmR in the bfmS$_{DK2}$ mutant background. We deleted gtrS in the bfmS$_{DK2}$ and measured the pa4103-lux expression. In the presence of glucose, the expression of pa4103-lux was high in bfmS$_{DK2}$; however, it was obviously, although not completely, reduced (~75% reduction) by the deletion of gtrS (+ Glucose in Fig. 4A). The reduced expression of pa4103-lux was restored upon introduction of gtrS with a plasmid (Fig. 4A). In contrast to the glucose-replete condition, however, in the absence of glucose, the expression of pa4103-lux was lower, and it was not obviously affected by the gtrS deletion (- Glucose in Fig. 4A). Similar results were obtained when the expression level of BfmS was used as an indicator of the BfmR regulatory activity (Fig. 4B). These results suggest that GtrS is an important contributor to the highly active status of BfmR in the bfmS$_{DK2}$ mutant, and, for the contribution, glucose is essential. Such a notion was further supported by the fact that the gtrS deletion suppressed the phenotypes conferred by the bfmS L181P/E376Q missense mutations, including reduced production of C4-HSL, reduced virulence against either lettuce leaf or D. melanogaster, and increased biofilm formation (Fig. 4C-F).

When co-expressed with BfmR-flag in the bfmS$_{DK2}$ mutant, GtrS-HA co-immunoprecipitated with BfmR-flag (Fig. 4G), indicating the direct interaction
between GtrS and BfmR in the presence of BfmS^{L181P/E376Q}. A similar result was obtained when the use of a bacterial two-hybrid system to study the interactions of GtrS and BfmR in *Escherichia coli* (fig. S6). These results are consistent the fact that in *P. aeruginosa* MPAO1, GtrS is the closest homolog of BfmS with 38.5% sequence identity. What is more, 85.7% (6 out of 7) of the partner specificity-determining residues, which inferred from studies on EnvZ (33) and HK853 (34), are completely conserved in BfmS and GtrS (fig. S7). Thus, the interacting interfaces of BfmS and GtrS may share common features that enable similar protein-protein interactions with the BfmR. Additionally, we observed that exogenously applied glucose, even at low micromolar concentrations, could induce the expression of *pa4103-lux* in *bfmS^{DK2}* mutant, but fail to do this in the WT MPAO1 (Fig. 4H). Thus, GtrS and BfmR still form a functional TCS in the presence of BfmS^{L181P/E376Q}.

To further explore whether the GtrS-mediated activation of BfmR also occurs in the clinical isolate DK2 strain, we repeated the *pa4103-lux* activity assay for the WT DK2 strain and its isogenic gtrS deletion mutant (DK2-ΔgtrS). Because the DK2 strain grows poorly in minimal medium, in this assay, bacterial cells were grown in PB medium. As with the MPAO1 strain background, in the DK2 background, the *pa4103-lux* activity was lower in the absence of glucose (PB versus PB + Glucose in Fig. 4I). The deletion of gtrS significantly decreased the *pa4103-lux* activity (~70%) when glucose was present in the growth medium (PB + Glucose in Fig. 4I). Similar results were also observed in the Western blot analysis of BfmS (Fig. 4J and K). These data verify that the functional GtrS-BfmR interaction in the presence of BfmS^{L181P/E376Q} is not limited to the genetic background of MPAO1.

**L181P/E376Q substitutions promote the autophosphorylation level of BfmS in vivo**

When grown without glucose, *bfmS^{DK2}* mutant exhibited a much higher *pa4103-lux* activity than the Δ*bfmS* mutant (fig. S8A). The *bfmS^{DK2}ΔgtrS* mutant also displayed much higher *pa4103-lux* activity than Δ*bfmSΔgtrS* strain regardless of the presence of glucose (fig. S8A). These results indicate that, although the BfmS protein inhibits the transcriptional regulatory activity of BfmR, the BfmS^{L181P/E376Q} mutant protein can activate it in a GtrS-independent manner. Indeed, the co-IP experiment showed that BfmS^{L181P/E376Q} directly interacts with BfmR in vivo (Fig. 5A). The purified N-terminal His6/GST-double tagged cytosolic fragment of BfmS^{L181P/E376Q} (hereafter referred to as BfmSc^{DK2}) protein was able to autophosphorylate (Fig. 5B, lane 3; fig. S8B) and, subsequently, phosphorylated His6-BfmR (Fig. 5B, lane 5; fig. S8B). As expected, BfmR^{D55A} was not phosphorylated by BfmSc^{DK2} (Fig. 5B, lane 6). In accordance with the previous report (24), the BfmS inhibited the regulatory function of BfmR in various growth media such as minimal medium (Fig. 3 A and B), LB medium, and PB medium (fig. S8D). However, like BfmSc^{DK2}, the cytosolic fragment of BfmS (BfmSc) was able to autophosphorylate and, subsequently, phosphorylated
BfmR \textit{in vitro} (fig. S8C), indicating that BfmS have the potential ability to activate BfmR under certain conditions.

BfmS is predicted to autophosphorylate the His238 residue (\url{http://www.pseudomonas.com/}). Following the prediction, the purified cytosolic fragment BfmS^{DK2}_{H238A} protein (that is, the BfmSc^{DK2} protein with H238A substitution) was unable to autophosphorylate (Fig. 5B, lane 4). Moreover, in the MPAO1 strain background, the H238A substitution completely abolished the ability of BfmS^{L181P/E376Q} to induce the expression of \textit{pa4103-lux} (Fig. 5C), to reduce the bacterial virulence in either \textit{D. melanogaster} (Fig. 5D) or lettuce leaf (Fig. 5E), and to enhance biofilm formation (Fig. 5F), indicating that the autophosphorylation at His238 is essential for mediating the molecular and cellular effects of BfmS^{L181P/E376Q}. Although non-detectable levels of P~BfmS was observed in WT MPAO1, a very high level of P~BfmS^{DK2} was seen in the \textit{bfmS}^{DK2} mutant (Fig. 5G). Similar results were obtained in the DK2 strain background as well (Fig. 5H). In addition, we observed that BfmSc^{DK2} and SUMO-BfmSc^{DK2} proteins (N-terminal His6-SUMO tagged cytosolic segment of BfmS^{DK2}) purified from \textit{E. coli}, respectively, display much higher level of autophosphorylation compared with BfmSc and SUMO-BfmSc (N-terminal His6-SUMO tagged cytosolic segment of BfmS) (fig. S9 A and B), and as expected, higher level of phosphorylation of BfmR by SUMO-BfmSc^{DK2} was also seen (fig. S9C). These results suggest that the L181P/E376Q amino acid substitutions caused elevated phosphorylation of BfmS at His238 and thus provide a more significant number of phosphoryl groups for the transfer to BfmR, thereby contributing to the increased regulatory activity of BfmR.

**L181P/E376Q substitutions compromises the phosphatase activity of BfmS**

It is known that the phosphatase activity of a HK is a key mechanism of preventing cross-talk between two-component systems (17). We therefore examined the effect of L181P/E376Q amino acid substitutions on the phosphatase activity of BfmS by using \textit{in vitro} assays with the truncated form of recombinant BfmS (SUMO-BfmSc), given that BfmS is a membrane protein (Fig. 1B) and is thus difficult to overexpress and purify. When transphosphorylation reactions were performed at a 1:6 (SUMO-BfmSc/His6-BfmR) molar ratio and then ATP was removed from the assays, P~His6-BfmR showed a half-life more than 30 min (Fig. 5I). When SUMO-BfmSc was added after ATP removal to reach a 4:1 (SUMO-BfmSc/His6-BfmR) molar ratio, the half-life of P~His6-BfmR decreased to 2 min, with a full reduction of phosphorylation of within 30 min (Fig. 5J). These results suggest that BfmS has significant phosphatase activity for P~BfmR, and are consistent with the previous observation that BfmS is a negative regulator of BfmR in vivo (24). However, when SUMO-BfmSc^{DK2} was added, the half-life of P~His6-BfmR was more than 5 min, a 2.5-fold higher than that in the presence of SUMO-BfmSc (Fig. 5J). These results indicate that L181P/E376Q amino acid substitutions may reduce the phosphatase activity of BfmS. Moreover, the
BfmR phosphorylation wanes over times (Fig. 5J), indicating that BfmSDK2 not only acts as a kinase (fig. S8) but also acts as a phosphatase against BfmR. Using surface plasmon resonance assay, we observed that SUMO-BfmSc and SUMO-BfmScDK2 display similar binding activity to the His6-BfmR (fig. S9 D and E), which suggests that L181P/E376Q amino acid substitutions may have no significant effect on the ability of BfmS to sequester BfmR from GtrS. Additionally, using Pro-Q Diamond stain, we found that the kinetics of the phosphorylation of His6-BfmR by SUMO-BfmSc and SUMO-BfmScDK2 are similar, indicating that L181P/E376Q amino acid substitutions have no significant effect on the ability of SUMO-BfmSc to transfers its phosphoryl group to His6-BfmR (figS9 F and G).

GtrS mediates changes in gene expression profile of \textit{P. aeruginosa} evoked by \textit{bfmS} mutations

To further investigate the roles of \textit{gtrS} in \textit{ΔbfmS} and \textit{bfmSDK2} mutants, we performed RNA-seq analysis on WT, \textit{ΔbfmS} mutant, \textit{ΔbfmSΔgtrS} mutant, \textit{bfmSDK2} mutant, \textit{bfmSDK2ΔgtrS} mutant, and the \textit{ΔbfmRS} mutant strain of MPAO1. Deletion of \textit{bfmS} in MPAO1 decreased the transcript levels of 490 genes (designated as \textit{bfmS}-activated genes, Sheet 3 in Data file 2), while it increased the expression of 201 genes (designated as \textit{bfmS}-repressed genes, Sheet 4 in Data file 2) (Fig. 6A). Of note, deletion of \textit{gtrS} in the \textit{bfmS} deletion mutant (\textit{ΔbfmS}) background increased the transcript levels of 60.2% (295 out of 490) of the \textit{bfmS}-activated genes, while it decreased the transcript levels of 75% (151 out of 201) of the \textit{bfmS}-repressed genes (Fig. 6A and Sheet 3-4 in Data file 2). These results indicate that GtrS can has an opposite role to BfmS in regulating the expression of a number of \textit{P. aeruginosa} genes, which also suggest that a functional \textit{gtrS} is key in determining the alterations in gene expression levels caused by \textit{bfmS} deletion, and this is well in line with our biochemical data (Fig. 3).

By comparing the transcriptome of \textit{bfmSDK2} mutant to that of WT PAO1, we identified 887 L181P/E376Q-induced genes and 1350 L181P/E376Q-reduced genes (Fig. 6B, Sheet 5-6 in Data file 2). These genes represent approximately 39.3% of the total number of annotated genes in the \textit{P. aeruginosa} PAO1 genome. We found that the expression of 27.2% (241 out of 887) of the L181P/E376Q-induced genes was decreased in the \textit{bfmSDK2} mutant due to the deletion of \textit{gtrS}, whereas the transcript levels of 17.5% (236 out of 1350) of the L181P/E376Q-reduced genes were increased (Fig. 6B, Sheet 5-6 in Data file 2). These results suggest that GtrS contributes to the L181P/E376Q-induced alterations of gene expression in \textit{P. aeruginosa} MPAO1, albeit to a lesser extent than it does in the \textit{bfmS} deletion mutant strain (\textit{ΔbfmS}).

We identified 417 BfmR-activated genes and 409 BfmR-repressed genes, by comparing the transcriptome of \textit{ΔbfmS} to that of \textit{ΔbfmRS} (Sheet 7 in Data file 2). As expected, the transcript levels of most, if not all, of the BfmR-activated and -repressed genes in the \textit{ΔbfmS} mutant were either decreased or induced by the deletion of \textit{gtrS},
respectively (Fig. 6C, lane 3; Sheet 7 in Data file 2), supporting the notion that GtrS has an essential role for the transcriptional regulatory activity of BfmR in the $\Delta$bfmS mutant. We also found that deletion of gtrS in the $bfmS^{\text{DK2}}$ mutant has a similar effect on the expression of some BfmR-activated and -repressed genes (Fig. 6C, genes in clusters I and II; Sheet 7 in Data file 2). For instance, the transcript levels of genes directly activated by BfmR, including pa4103, pa4104, pa4105, pa4106, and pa4107, were significantly decreased by more than 65% in the $bfmS^{\text{DK2}}$ mutant upon the deletion of gtrS, and a ~84% decrease was observed for the mRNA level of pa4107 (Fig. 6C, Sheet 7 in Data file 2).

Additionally, in both $\Delta$bfmS and $bfmS^{\text{DK2}}$ mutants, like in the CF-adapted isolates, the most abundant mRNA transcript was the BfmR-dependent gene pa4107, which was also the most up-regulated gene (with a >2000-fold increase) in these two mutants as compared with the WT MPAO1 strain (Sheet 1-2 in Data file 2). Thus, it is very likely that BfmR is highly activated in these two mutant strain. In all, these transcriptome data support the conclusion that BfmR can be activated by GtrS in either $\Delta$bfmS or $bfmS^{\text{DK2}}$ mutant.

**Activation of BfmR by missense mutations in bfmS is not limited to L181P/E376Q**

Not only $bfmS^{\text{L181P/E376Q}}$ but also other $bfmS$ missense variants exist in the P. aeruginosa CF isolates (table S3). To examine whether those missense mutations in bfmS affect the activity of BfmR, we generated 22 missense variants of bfmS and introduced them with the pAK1900 plasmid into the $\Delta$bfmS strain. Of these 22, the following 10 bfmS missense variants have a more than 2-fold increase in the expression of $pa4103$-lux: L181Q, A42E/G347D, F31L, F31L/D295N, T242R, T120K/L164F/G179D/Y280H, A4T/R393H, L168-L, Q92E/L184P, and T120K/L164F/A281T, as compared to the wild type bfmS (Fig. 6D). Along with the 10 bfmS missense variants, the L181P, L181P/E376Q, and R393H also significantly increased the $pa4103$-lux activity in the $\Delta$bfmS strain (Fig. 6D), this is consistent with the result of our previous study where the BfmR activity was measured by $bfmR$-lux (24). All of these 13 bfmS variants only have a slight effect (<1.2-fold) on the expression of $pa4103$-lux in the $\Delta$bfmRS mutant (fig. S10A), indicating that those BfmS variant proteins require BfmR in order to activate the expression of $pa4103$-lux. Those variants bear amino acid substituent at different domains of the BfmS (Fig. 6E), implying that the regulatory activity of BfmS can be modulated by diverse mechanisms.

Among the 25 bfmS alleles tested, $bfmS^{A42E/G347D}$ (found in AU15431 and AU7032 isolates) (35) was most potent in increasing the $pa4103$-lux activity (>300-fold increase) (Fig. 6D). However, a single A42E missense mutation alone has a much weaker effect (< 3-fold increase) than the A42E/G347D missense mutation, while the
G347D missense mutation alone has no obvious effect (fig. S10B). These observations suggest that the combination of these two amino acid substituents (A42E and G347D) synergistically enhances the regulatory functions of BfmS. Similar results were observed for the L181P/E376Q missense mutations (Fig. 6D, Fig. S10B) and the A4T/R393H missense mutations (Fig. 6D), indicating that the interaction of point mutations in bfmS can manifest in positive epistasis. Together, these results indicate that bfmS mutation-induced activation of BfmR is not limited to either DK2 lineage or the certain types of amino acid changes.

**A42E/G347D, T242R, and R393H substituents promote the activation of BfmR by GtrS**

In the AU15431 isolate, a high amount of P–BfmR-flag was detected, whereas no P–BfmR-flag was detected in the AU15431::bfmS<sup>PAO1</sup> (in which the WT bfmS replaced the bfmS<sup>A42E/G347D</sup>) (fig. S10C). The AU15431::bfmS<sup>PAO1</sup> mutant strain also exhibited a much lower pa4103-lux activity, as compared with its isogenic parent AU15431 (fig. S10D). Moreover, the deletion of gtrS in AU15431 reduced the expression of both pa4103-lux (fig. S10D) and the BfmS protein (fig. S10E). These results suggest that, as with the L181P/E376Q missense mutations in DK2 strain, the A42E/G347D missense mutations in bfmS induces an GtrS-mediated activation of BfmR in the AU15431 isolate.

Like L181P/E376Q, the A42E/G347D amino acid substituents also caused an elevated autophosphorylation levels of BfmS in <i>P. aeruginosa</i> (fig. S10 F and G). Similar results were observed for the T242R missense mutation of bfmS (fig. S10F), which is the second potent mutation in increasing the regulatory activity of BfmR (Fig. 6D). Although glucose did not increase the expression of pa4103-lux in the presence of WT bfmS or bfmS<sup>DK2 H238A</sup> allele, it did so with either a bfmS<sup>A42E/G347D</sup> (fig. S10H), a bfmS<sup>A42E/G347D/H238A</sup> (fig. S10H), a bfmS<sup>T242R</sup> (fig. S10I), or a bfmS<sup>T242R/H238A</sup> allele (fig. S10I). These results suggest that A42E/G347D and T242R amino acid substituents in BfmS, respectively, enables bacteria to modulate the activity of BfmR in response to glucose. When gtrS was further deleted in ΔbfmS carrying either bfmS<sup>A42E/G347D/H238A</sup> (fig. S10H) or bfmS<sup>T242R/H238A</sup> (fig. S10I), glucose fails to induce the expression of pa4103-lux. Thus, as with the L181P/E376Q missense mutations, other missense mutations in bfmS can enable the activation of BfmR by GtrS. This notion is further supported by the observation that gtrS is required for the ability of the bfmS<sup>R393H</sup> mutant of MPAO1 (in which the bfmS<sup>R393H</sup> replaced the wild type bfmS allele, table S1) to increase the expression of pa4103-lux in response to glucose (fig. S10J).

Using Pro-Q Diamond stain, we showed that the T242R substitution nearly completely abolish the phosphatase activity of BfmSc against the phosphorylated BfmR (fig. S11A and B). This is in line with the fact that Thr242 is at a known
location required for phosphatase activity of the HisKA subfamily of bacterial SKs (37). In addition, like the L181P/E376Q amino acid substitutions, R393H substitution caused a decrease in the phosphatase activity of BfmSc as well (fig. S11A and B). These results suggest bfmS mutation-induced cross-phosphorylation of BfmR by GtrS is not limited to the L181P/E376Q (Fig. 6F).

Discussions

In this study, we showed that bfmS spontaneous mutations induce remodeling of TCSs and allow P. aeruginosa to integrate information from multiple sources to activate BfmR, which in turn directs this bacterium toward a chronic infection state (Fig. 6F). Like many TCSs, the WT BfmRS TCS is auto-regulated (24). We observed that the mutated BfmRS TCS (BfmR-BfmSDK2) is auto-regulated as well and responds to certain factor(s) during bacterial growth, as evidenced by the fact that the bfmR-lux reporter exhibited an activation surge in the bfmSDK2 mutant grown in different media (Fig. 1A, fig. S1). The change in the opposing biochemical activities (kinase and phosphatase) of BfmSDK2 (Fig. 5J, fig. S8B) may result in such an effect. Additionally, autoregulation may affect the BfmR-BfmSDK2 system by controlling the relative concentrations of BfmR and BfmSDK2, and thus provide a threshold for gene regulation, as it did for some other TCS systems (36).

It is worthy to note that during adaptation to the CF lung environment, L181P and E376Q missense mutations in bfmS were fixed in the P. aeruginosa DK2 lineage, which is a dominating clone in Denmark (9, 12, 37). However, bfmS mutant alleles were also found in more than three hundred P. aeruginosa CF isolates from different continents and countries, in addition to at least 50 DK2 CF-adapted isolates (table S3). Of note, bfmS mutant alleles encoding BfmS variants that bear amino acid substituent at position 181 (L181Q), which causes an increase in the regulatory activity of BfmR (Fig. 6D), was also observed in all 26 isolates belong to the M3L7 sub-lineage emerged in Australia (16). These would suggest that, the prevalence of bfmS mutations is not limited to the DK2 CF-adapted isolates and may be higher than we have found.

Mutations in regulatory genes are important in the adaptation process (5, 37). When mutations affect global regulatory genes, considerable phenotypic divergence can be rapidly achieved, thereby setting the scene for adaptive radiations (5-7). The evolution of a new trait has been proposed to occur in three-steps: potentiation, actualization, and refinement (38). We showed that naturally evolved mutations in bfmS are sufficient to direct P. aeruginosa toward a chronic state (decreased QS-signal production, attenuation of bacterial acute virulence, and enhanced biofilm formation), by activating a single protein (BfmR) (Fig. 1, Fig. 2, fig. S2, and Fig. 6F). The functional activation of BfmR can also explain some adaptive gene expression changes in the DK2 lineage (Fig. 2A, Fig. 6C). Therefore, it is possible that
L181P/E376Q (or L181P) represents an actualizing mutation, producing various origins and diversity in phenotypes and gene expression of the DK2 isolates. Given these, mutation in bfmS, and/or its combination with mutations in other global regulatory genes such as pmrB, rpoN, mucA, and lasR (9, 12, 37), may have a critical role in directing P. aeruginosa DK2 toward increased adaptive performance, and thus resulting in lineages that are highly successful in the CF lungs and have the capacity to be transmitted among individuals with CF.

We showed that L181P/E376Q, A42E/G347D, T242R, or R393H missense mutations in bfmS can enable P. aeruginosa to exploit the cross-regulation between GtrS and BfmR in order to alter its response to glucose (Fig. 6F), although TCSs typically insulated from one another (17). Moreover, L181P/E376Q, T242R, and R393H substituents respectively decreased the phosphatase activity of SUMO-BfmSc (Fig. S11). Therefore, it is not a far-fetched idea that the phosphatase activity of BfmS may be a key mechanism of preventing the phosphorylation of BfmR by GtrS. This notion is further supported by the fact that when bfmS is absent, both the phosphorylation level and the regulatory activity of BfmR are largely dependent on the gtrS (Fig. 3C, Fig. 6 A and C).

In airway surface liquid (ASL), glucose concentrations (~ 0.4 mM) are about 12 times lower than that in the blood (39), which might be a homeostatic mechanism inhibiting bacterial proliferation by depriving the bacteria of an essential nutrient (39, 40). Indeed, from studies in animals and patients with either chronic obstructive pulmonary disease (COPD) or cystic fibrosis, an association has been reported between airway glucose concentrations and increased susceptibility to bacterial infection (39-42). Given these, the functional interaction between GtrS and BfmR, which contributes to the transcriptional regulatory activity of BfmR, may be physiologically relevant. Since BfmR is highly activated in a number of CF-isolates grown both in vitro and in vivo (GDS2869, GDS2870, GDS4249, GSE62970, and GSE31227) and has a crucial function in biofilm formation (22-24) (fig. S2 F and G), targeting BfmR function might be of particular interest.

In conclusion, in this study, we have provided evidence that, during chronic infections, the P. aeruginosa can remodel its signaling cascades to integrate different signals to regulate a major lifestyle switch (between virulence and biofilm formation) (Fig. 6C). Our data also suggest that, in addition to cis- and trans-regulatory changes (43-46), mutation-induced remodeling of signaling cascades may also contribute to changes in gene expression during the evolution (Fig. Data files 1-2, Fig. 6C). More in-depth knowledge about the adaptive changes in signaling pathways will provide clues to the selective forces driving pathogen evolution in the host environment and help to improve the treatment of the infection.

Materials and Methods
Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in table S1. Unless noted otherwise, *P. aeruginosa* MPAO1 and its derivatives were grown in Luria-Bertani (LB) medium, Pyocyanin production broth (PB medium) (20 g peptone, 1.4 g MgCl₂, 10 g K₂SO₄, 20 ml glycerol per liter; pH 7.0), or M8-glutamate minimal medium (6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 0.24 g MgSO₄, 0.5 g glutamate per liter; pH 7.4) supplemented with or without glucose (2 mM), as indicated. DK2 and AU15431 and their derivatives were grown in PB medium. *E. coli* were grown in LB medium. All cultures were incubated at 37°C with shaking (250 rpm) unless noted otherwise. For plasmid maintenance in *P. aeruginosa*, the medium was supplemented with 100 µg/ml carbenicillin, 50 µg/ml tetracycline, or 100 µg/ml kanamycin when required. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 µg/ml carbenicillin, 50 µg/ml kanamycin, 10 µg/ml tetracycline, or 10 µg/ml gentamicin, as appropriate. For marker selection in *P. aeruginosa* DK2 and AU15431 isolates, 500 µg/ml gentamicin and 150 µg/ml tetracycline were used when required.

Transposon mutagenesis

The ΔbfmS::bfmR-lacZ mutant strain was subjected to transposon mutagenesis using the mariner transposon vector pBT20 (47). To construct the strains ΔbfmS::bfmR-lacZ, the bfmR promoter region (-995 to +20 of the start codon) was amplified by PCR using the primers pro-bfmR-F (with *Xho*I site) and pro-bfmR-R (with *Bam*HI site) (table S2) and cloned into the *Xho*I and *Bam*HI sites of the integration-proficient mini-CTX-lacZ (48) in order to generate mini-CTX-bfmR-lacZ (mini-bfmR-lacZ). The resulting plasmid was conjugated into ΔbfmS and the construct was integrated into the attB site though a diparental mating using *E. coli* S17 λ-pir as the donor, following excision of the mini-CTX backbone from the chromosome using a flippase (FLP) recombinase encoded on the pFLP2 plasmid (49).

For transposon mutagenesis, the transposon in pBT20 was conjugally transferred by biparental mating into the ΔbfmS::bfmR-lacZ mutant, following a protocol previously described (50). Briefly, the donor strain (*E. coli* SM10-λ, pir) containing the pBT20 and the recipient ΔbfmS::bfmR-lacZ strain were scraped from overnight plates and suspended in 1 ml of M8-glutamate minimal medium. Concentrations of the bacteria in the suspensions were adjusted to OD₆₀₀ of 40 for the donor and OD₆₀₀ of 20 for the recipient. Next, each donor and recipient were mixed together and spotted on a LB agar plate and incubated at 37°C for 7 h. Mating mixtures were scraped and resuspended in 1 ml of M8-glutamate minimal medium. Transposon-mutagenized bacteria were selected by plating on PIA plates containing gentamicin at 150 µg/ml. A sterile tip was used to pick up individual colonies and dip them into the M8-glutamate minimal agar plates (supplying 1.5% agar and 2 mM glucose) with 20 µg/ml X-gal. Approximately 30,000 colonies were screened for the appearance of
blue color. The localization of the Mariner transposon with respect to the *P. aeruginosa* genome was determined using an established protocol (47).

**Plasmid construction for the constitutive expression of *P. aeruginosa* genes**

All the primers used for plasmid construction are listed in table S2. To construct the plasmid for constitutive expression of *gtrS*, a ~1.5 kb PCR product covering 72 bp of the *gtrS* upstream region, the *gtrS* gene, and 9 bp downstream of *gtrS* was amplified from MPAO1 genomic DNA using primers *gtrS-comp-F* (with *HindIII* site) and *gtrS-comp-R* (with *BamHI* site). For generating pAK1900-*gltR* (p-*gltR*), a ~0.77 kb PCR product covering 38 bp of the *gltR* upstream region, the *gltR* gene, and 7 bp downstream of *gltR* was amplified MPAO1 genomic DNA using primers *gltR-comp-F* (with *HindIII* site) and *gltR-comp-R* (with *KpnI* site). For generating pAK1900-*gltB* (p-*gltB*), a ~1.3 kb PCR product covering 40 bp of the *gltB* upstream region, the *gltB* gene, and 15 bp downstream of *gltB* was amplified MPAO1 genomic DNA using primers *pa3190-comp-F* (with *HindIII* site) and *pa3190-comp-R* (with *KpnI* site). All the products were digested with the indicated enzymes and cloned into pAK1900 (41), and the direction of transcription of the cloned genes is in the same orientation as *plac* on pAK1900.

In order to generate pRK415-*bfmS*, a ~1.4 kb PCR product covering 20 bp of the *bfmS* upstream region, the *bfmS* gene, and 15 bp downstream of *bfmS* was amplified from MPAO1 genomic DNA using primer pair *bfmS-comp-F*/bfmS-comp-R (*HindIII*/*BamHI* sites). For generating pRK415-*gtrS*, the primer pair *gtrS-comp-F*/gtrS-comp-R and MPAO1 genomic DNA were used. All the PCR products were cloned into pRK415 (52), and the direction of transcription of the cloned genes was in the same orientation as the *plac* promoter on pRK415.

For generating mini-*bfmS*, mini-*gltR-gtrS*, mini-*gltR*, and mini-*gtrS*, primers pAK1900-mini-*F* (with *XhoI* site) and pAK1900-mini-*R* (with *XbaI* site) were used. Briefly, a ~1.7 kb PCR product covering the *lac* promoter of pAK1900 plasmid and the *bfmS* was amplified from p-*bfmS* plasmid (table S1) DNA, and the PCR products were cloned into integrated mini-CTX-lacZ vector. For generating mini-*gltR-gtrS*, a ~2.5 kb PCR product covering the *lac* promoter of pAK1900 plasmid and the *gltR-gtrS* was amplified from p-*gltR-gtrS* plasmid (table S1) DNA. For generating mini-*gltR*, a ~1 kb PCR product covering the *lac* promoter of the pAK1900 and the *gltR* was amplified from p-*gltR* plasmid (table S1). For generating mini-*gtrS*, a ~1.8 kp PCR product covering the *lac* promoter of the pAK1900 and the *gtrS* was amplified from p-*gtrS* plasmid DNA (table S1). The PCR products were cloned into integrated mini-CTX-lacZ vector (48).
All constructs were sequenced to ensure that no unwanted mutations resulted.

Plasmid construction for the expression of FLAG- or HA-tagged genes

For generating pAK1900-\textit{bfmR-flag} (\textit{p-bfmR-flag}), a \textasciitilde 0.8 kb PCR product covering the region from 15 bp upstream and the \textit{bfmR} gene (not including the stop codon) was generated from MPAO1 genomic DNA with primers \textit{bfmR-comp-F} (with \textit{HindIII} site) and \textit{bfmR-flag-R} (with \textit{BamHI} site) (table S2). The \textit{HindIII} and \textit{BamHI} digested PCR product was cloned into pAK1900 to generate pAK1900-\textit{bfmR-flag} (\textit{p-bfmR-flag}). The pAK1900-\textit{bfmR^{D55A}-flag} (\textit{p-bfmR^{D55A}-flag}, the aspartate 55 of BfmR was replaced by alanine) was obtained by using the primer pair BfmR(D55A)-F/BfmR(D55A)-R and a QuikChange II site-directed mutagenesis kit (Stratagene, Catalog#: 200518). For generating p RK415-\textit{bfmR-flag}, primers \textit{bfmR-comp-F} and \textit{bfmR-flag-R} were used again, and the PCR product was cloned into pRK415 plasmid. For generating pAK1900-\textit{gtrS-HA}, primers gtrS-comp-F (with \textit{HindIII} site) and gtrS-HA-R (with \textit{BamHI} site) (table S2) were used to perform PCR of the GtrS gene that was meant to fuse with a C-terminal HA-tag, and a \textasciitilde 1.55 kb PCR product covering the region from 72 bp upstream and the GtrS gene (not including the stop codon) was generated from MPAO1 genomic DNA and cloned into the pAK1900. For generating pAK1900-\textit{gtrS^{H280A}-HA}, the primer pair GtrS(H280A)-F/GtrS(H280A)-R and a QuikChange II site-directed mutagenesis kit (Stratagene, Catalog#:200518) were used. All constructs were sequenced to ensure that no unwanted mutations resulted.

Construction of gene deletion mutant strains of \textit{P. aeruginosa} MPAO1

For gene replacement, a SacB-based strategy was employed as previously described (49). To construct the \textit{gltB} null mutant (\textit{\Delta gltB}), polymerase chain reactions (PCRs) were performed in order to amplify sequences upstream (~1.2 kb) and downstream (~1.3 kb) of the intended deletion. The upstream fragment was amplified from MPAO1 genomic DNA using primers D-pa3190-up-F (with \textit{EcoRI} site) and D-pa3190-up-R (with \textit{KpnI} site), while the downstream fragment was amplified with primers D-pa3190-down-F (with \textit{KpnI} site) and D-pa3190-down-R (with \textit{HindIII} site). These two PCR products were digested and then cloned into the \textit{EcoRI/HindIII} digested gene replacement vector pEX18Ap, yielding pEX18Ap::\textit{pa3190UD}. A 1.8 kb gentamicin resistance cassette was cut from pPS858 with \textit{KpnI} and then cloned into pEX18Ap::\textit{pa3190UD}. The resultant plasmid, pEX18Ap::\textit{pa3190UGD}, was electroporated into MPAO1 with selection for gentamicin resistance. Colonies were screened for gentamicin sensitivity and loss of sucrose (5%) sensitivity, which typically indicates a double-cross-over event and thus marks the occurrence of gene replacement.
A similar strategy was used to construct the ΔgtrS, ΔgltR-gtrS, and ΔbfmR as described above. Briefly, for the construction of ΔgtrS, the upstream fragment (~1 kb) of the intended deletion was amplified with primers D-gtrS-up-F (with EcoRI site) and D-gtrS-up-R (with BamHI site), and the downstream fragment (~1 kb) was amplified with primers D-gtrS-down-F (with BamHI site) and D-gtrS-down-R (with HindIII site). For the construction of ΔgltR-gtrS, the upstream fragment (~1.3 kb) of the intended deletion was amplified with primer pair D-gltR-up-F/D-gltR-up-R (EcoRI/BamHI sites) and the downstream fragment (~1 kb) was amplified with primer pair D-gltR-down-F/D-gltR-down-R (BamHI/HindIII). For the construction of ΔbfmR, the upstream fragment (~1 kb) of the intended deletion was amplified with primer pair D-bfmR-up-F/D-bfmR-up-R (EcoRI/BamHI sites) while the downstream fragment (~1.2 kb) was amplified with primer pair D-bfmR-down-F/D-bfmR-down-R (BamHI/HindIII). A 1.8 kb gentamicin resistance cassette was cut from pPS858 with BamHI and then cloned into the pEX18Ap::gtrSUD, pEX18Ap::gltR-gtrSUD, and pEX18Ap::bfmRUD, yielding pEX18Ap::gtrSUGD, pEX18Ap::gltR-gtrSUGD, and pEX18Ap::bfmRUGD, respectively, as described above.

For the construction of ΔbfmSΔgltB, the gentamicin resistance cassette of ΔgltB was excised by using the plasmid pFLP2 that encoded Flp recombinase, and then the pEX18Ap::bfmSUGD plasmid (table S1) was electroporated into the ΔgltB (without gentamicin resistance cassette) in order to generate ΔbfmSΔgltB. The same strategy was used to construct the ΔbfmSΔgtrS and ΔbfmSΔgltR-gtrS mutants. For generating ΔbfmRSΔpta-acka mutant, pEX18Ap::acka-ptaUTD (24) was electroporated into the ΔbfmRS. For the construction of ΔbfmRSΔgltR-gtrS, the gentamicin resistance cassette of ΔgltR-gtrS was excised by using the plasmid pFLP2 that encoded Flp recombinase, and the pEX18Ap::bfmRSUGD (table S1) was electroporated into the ΔgltR-gtrS (without gentamicin resistance cassette) in order to generate ΔbfmRSΔgltR-gtrS.

The primers used for PCRs are listed in table S2, and all the mutant strains were confirmed by PCR.

Construction of gene deletion mutants of P. aeruginosa clinical isolates

For construction of ΔgtrS mutant of DK2 strain and AU15431 isolate, pEX18Ap::gtrSUGD plasmid (table S1) was electroporated into DK2 and AU15431, respectively, and the transformants were screened on an LB plate containing 500 μg/ml gentamicin and 5 % sucrose. For the construction of ΔbfmRS in DK2, a similar strategy as described above and pEX18Ap::bfmRSUGD plasmid (table S1) were used. For the construction of ΔbfmS mutant strain of either DK2 strain or AU15431 isolate, pEX18Ap::bfmSUGD plasmid (table S1) was used. All the mutant strains were confirmed by PCR and DNA sequencing.
Construction of allelic exchange mutants

To construct the \textit{bfmS}^{DK2} (MPAO1-\textit{bfmS}^{DK2}) allelic exchange mutant, a ~3.3 kb PCR product covering ~1 kb upstream of \textit{bfmS}, the \textit{bfmS} gene, and ~0.95 kb downstream of \textit{bfmS} was amplified from \textit{P. aeruginosa} DK2 genomic DNA using primers \textit{bfmS}-allelic-DK2-F (with EcoRI site) and \textit{bfmS}-allelic-DK2-R (with HindIII site). The fragments were subsequently digested and cloned into EcoRI/HindIII digested gene replacement vector pEX18Tc (table S1). The resultant plasmid, pEX18Tc-For-\textit{bfmS}^{DK2} were electroporated into the Δ\textit{bfmS} mutant (with gentamicin resistance cassette) (table S1) with selection for both tetracycline and gentamicin resistance, which typically indicates a single-cross-over event. Colonies were further screening for tetracycline and gentamicin sensitivity, and loss of sucrose (5%) sensitivity, which typically indicates a double-cross-over event thus marks the occurrence of gene replacement. The \textit{bfmS}^{DK2} mutant was further confirmed by PCR and DNA sequencing. For generating MPAO1-\textit{bfmS}^{DK2}^{R393H}, plasmid pEX18Tc-For-\textit{bfmS}^{DK2}^{H238A} was constructed by using the primer pair BfmS(H238A)-F/BfmS(H238A)-R, a QuikChange II site-directed mutagenesis kit (Stratagene, Catalog#: 200518), and MPAO1-\textit{bfmS}^{DK2}^{H238A} plasmid DNA.

To construct the \textit{bfmS}^{R393H} (MPAO1-\textit{bfmS}^{R393H}) allelic exchange mutant, PCR product was amplified from \textit{P. aeruginosa} MPAO1 genomic DNA using primers \textit{bfmS}-allelic-DK2-F (with EcoRI site) and \textit{bfmS}-allelic-DK2-R (with HindIII site) and then cloned into pEX18Tc, yielding pEX18Tc-For-\textit{bfmS}^{PAO1} plasmid. Subsequently, the resultant plasmid, pEX18Tc-For-\textit{bfmS}^{R393H} (table S1), was constructed by using the primer pair R393H-F/R393H-R and a QuikChange II site-directed mutagenesis kit (Stratagene, Catalog#: 200518). The pEX18Tc-For-\textit{bfmS}^{R393H} was further electroporated into the Δ\textit{bfmS} mutant (with gentamicin resistance cassette) (table S1) in order to generate the \textit{bfmS}^{R393H} allelic exchange mutant as described above.

To construct \textit{bfmS}^{DK2}Δ\textit{bfmR}, pEX18Ap::\textit{bfmRU}GD plasmid (table S1) was electroporated into \textit{bfmS}^{DK2} allelic exchange mutant with selection for gentamicin resistance. To construct \textit{bfmS}^{DK2}Δ\textit{gtrS} and \textit{bfmS}^{R393H}Δ\textit{gtrS} mutants, pEX18Ap::\textit{gtrSU}GD plasmid (table S1) was electroporated into \textit{bfmS}^{DK2} and allelic exchange mutants, respectively, with selection for gentamicin resistance. Colonies were screened for gentamicin sensitivity and loss of sucrose (5%) sensitivity, which typically indicates a double-cross-over event and thus marks the occurrence of gene replacement. PCR and DNA sequencing further confirmed the replacement of the gene allele.

A similar strategy was used to construct the DK2-\textit{bfmS}^{PAO1} and AU15431-\textit{bfmS}^{PAO1} mutants. Briefly, pEX18Tc-For-\textit{bfmS}^{PAO1} plasmid was electroporated into DK2-Δ\textit{bfmS} and AU15431-Δ\textit{bfmS} (table S1), respectively. Colonies were screened...
for tetracycline and gentamicin sensitivity and loss of sucrose (5%) sensitivity.

**Overexpression of recombinant proteins in *E. coli* and their purifications**

The following ten recombinant proteins were expressed in *E. coli*: His$_6$-GtrSc, the N-terminal His$_6$-tagged cytosolic segment of GtrS (residues 269-465); BfmSc, the N-terminal His$_6$- and GST-tagged cytosolic segment of BfmS (residues 175-435); BfmSc$^{DK2}$, a BfmSc mutant in which the leucine 181 (L181) and glutamic acid 376 (E376) of BfmS were respectively replaced by proline (P) and Q (glutamine); BfmSc$^{DK2}$ H$^{238\text{A}}$, a BfmSc$^{DK2}$ mutant in which the histidine 238 (H238) of BfmS was replaced by alanine (A); SUMO-BfmSc, the N-terminal His$_6$-SUMO tagged cytosolic segment of BfmS (residues 175-435); SUMO-BfmSc$^{DK2}$, a SUMO-BfmSc variant in which the leucine 181 (L181) and glutamic acid 376 (E376) of BfmS were respectively replaced by proline (P) and Q (glutamine); SUMO-BfmSc$^{T242\text{R}}$, a SUMO-BfmSc variant in which the tyrosine 242 (T242) of BfmS was replaced by arginine (R); SUMO-BfmSc$^{R393\text{H}}$, a SUMO-BfmSc variant in which the arginine 393 (R393) of BfmS was replaced by histidine (H); His$_6$-BfmR, N-terminal His$_6$-tagged BfmR; His$_6$-BfmR$^{D55\text{A}}$, a His$_6$-BfmR variant in which the Aspartic acid 55 (D55) of BfmR was replaced by alanine (A).

For the construction of expression plasmids of the His$_6$-GtrSc proteins, primers gtrS-KD-F (with *BamHI* site) and gtrS-KD-R (*EcoRI*) were used. The DNA fragment amplified from *P. aeruginosa* MPA01 genomic DNA encoding the kinase domain of *gtrS* (residues 269-465) was cloned into pET28a (table S1), yielding pET28a-GtrSc plasmid. For the expression of BfmSc proteins, primers bfmS-KD-F (with *BamHI* site) and bfmS-KD-R (*XhoI*) were used and the corresponding DNA fragment encoding the kinase domain of WT BfmS (residues 175-435) was cloned into the prokaryotic expression vector pGEX-6p-1-6His (table S1), yielding pGEX-6p-1-BfmSc plasmid (the gene cassette was fused in-frame to 3'-terminal of glutathione S-transferase gene of the pGEX-6p-1-6His). For generating pGEX-6p-1-BfmSc$^{DK2}$, the primer pairs BfmS(L181P)-F/BfmS(L181P)-R and BfmS(E376Q)-F/BfmS(E376Q)-R, and a QuikChange II site-directed mutagenesis kit (Stratagene, Catalog#: 200518) were used. For generating pGEX-6p-1-BfmSc$^{DK2}$ H$^{238\text{A}}$-Cter, the primer pair BfmS(H238A)-F/BfmS(H238A)-R and a QuikChange II site-directed mutagenesis kit were used. For the expression of SUMO-BfmSc proteins, primers bfmS-KD-2F (with *KpnI* site) and bfmS-KD-2R (*HindIII*) were used and the corresponding DNA fragment encoding the kinase domain of WT BfmS (residues 175-435) was cloned into the prokaryotic expression vector pET28a(+-sumo), yielding pET28a(+-sumo)-BfmSc plasmid (table S1). For generating pET28a(+-sumo)-BfmSc$^{DK2}$, the primer pairs BfmS(L181P)-F/BfmS(L181P)-R and BfmS(E376Q)-F/BfmS(E376Q)-R, and a QuikChange II site-directed mutagenesis kit were used. For generating pET28a(+-)}
sumo-BfmSc\textsuperscript{T242R} and pET28a(+)-sumo-BfmSc\textsuperscript{R393H}, the primer pairs T242R-F/T242R-R and R393H-F/R393H-R were used, respectively. All constructs were sequenced to ensure that no unwanted mutations resulted.

The protein was expressed in \textit{E. coli} strain BL21 star (DE3) and purifications were performed as previously described (24, 50, 53, 54). Briefly, bacteria were grown at 37°C overnight in 10 ml of LB medium with shaking (250 rpm). The cultures were transferred into 1 L of LB medium incubated at 37°C with shaking (200 rpm) until the OD\textsubscript{600} reached 0.6, and then IPTG (isopropyl-1-thio-β-d-galactopyranoside) was added to a final concentration of 1.0 mM. After 4 h incubation at 30°C with shaking (200 rpm), the cells were harvested by centrifugation and stored at -80°C. The cells were lysed at 4°C by sonication in lysis buffer [50 mM Tris (pH 7.4), 200 mM NaCl, 1 mM PSMF and 2 mM DTT]. Clarified cell lysate was loaded onto a HisTrap HP column (Code#: 17-5247-01, GE Healthcare), washed with Ni-NTA washing buffer and eluted with Ni-NTA elution buffer. The fractions containing BfmSc proteins were loaded onto the HiTrap Desalting 5 x 5 ml (Sephadex G-25 S) (Code#: 17-1408-01, GE Healthcare) with a running condition of 50 mM Tris (pH 7.4), 200 mM NaCl and 2 mM DTT to remove the imidazole. The purified protein was > 90% pure as estimated by a 12% (wt/vol) SDS/PAGE gel.

\textit{In vitro} phosphorylation assays

\textit{In vitro} autophosphorylation assays were carried out in phosphorylation reaction mixtures (100 μl) contained purified proteins (~20 μg) in phosphorylation reaction buffer [50 mM Tris-HCl, pH 7.5; 50 mM KCl, 1 mM DTT, 5 mM MgCl\textsubscript{2}]. The reaction was initiated by addition of 10 μCi of [γ\textsuperscript{-32P}]ATP (PerkinElmer, BLU002A500UC) and then incubated at room temperature for 5 min (or for the indicated time). 10 μl aliquots were removed following the addition of 2 μl 5 × SDS loading buffer, and the sample was resolved onto a 12% SDS-PAGE, visualized by autoradiography and Coomassie blue staining.

To examine the transphosphorylation of BfmR, the His\textsubscript{6}-GtrSc, BfmSc, and BfmSc\textsubscript{DK2} proteins (20-40 μg) were incubated in phosphorylation reaction buffer [50 mM Tris-HCl, pH 7.5; 50mM KCl, 1 mM DTT, 5 mM MgCl\textsubscript{2}] containing 10 μCi [γ\textsuperscript{-32P}]ATP for 5 min (or for the time indicated) as described above. Either purified His\textsubscript{6}-BfmR or His\textsubscript{6}-BfmR\textsuperscript{D55A}, as indicated, was added to the reaction mixture in a final volume of 100 μl. After incubation at room temperature for 5 min (or for the time indicated), 10 μl aliquots were removed, in which the transphosphorylation reaction was stopped by adding 2 μl of 5 × SDS loading buffer, and analyzed by SDS-PAGE, visualized by autoradiography and Coomassie blue staining.

\textit{In vitro} phosphorylation were also monitored by Pro-Q stain according to the protocol of the manufacturer (Invitrogen). For \textit{in vitro} autophosphorylation assays,
either BfmSc (~3 μg), BfmScDK2 (~1.5 μg), or His6-BfmR (~2 μg) was incubated in phosphorylation reaction mixtures (20 μl) supplemented with 2 mM ATP; For in vitro phosphorylation of BfmR by BfmS, SUMO-BfmSc (~2 μg) and SUMO-BfmScDK2 (~2 μg) were respectively incubated with His6-BfmR (~2 μg) in phosphorylation reaction mixtures (20 μl) supplemented without or with 2 mM ATP, as indicated. After incubation at on ice for the indicated times, 10 μl aliquots were removed and the reaction was stopped by adding 2 μl of 5 × SDS loading buffer, and analyzed by SDS-PAGE. To examine the phosphorylation of BfmR by GtrS, the His6-GtrSc (~15 μg) was first incubated in phosphorylation reaction buffer containing 5 mM ATP for 10 min and then the purified His6-BfmR (~15 μg) was added to the reaction mixture in a final volume of 100 μl. After incubation at room temperature for the indicated times, 10 μl aliquots were removed, in which the phosphorylation reaction was stopped by adding 2 μl of 5 × SDS loading buffer, and analyzed by SDS-PAGE. After staining with Pro-Q Diamond, the gels were stained with Coomassie blue to determine total protein content. The proteins stained with Pro-Q Diamond were visualized using a Tanon-5200 multi (Tanon) at an excitation wavelength of 535 nm and a band pass emission filter of 605 nm. Protein phosphorylation level was quantified by determining the ratio of the intensity of phosphoprotein in Pro-Q Diamond image to its intensity of total protein in Coomassie blue image using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) when required.

In vitro dephosphorylation assays

To examine the dephosphorylation of P~BfmR, His6-BfmR was first transphosphorylated by SUMO-BfmSc (at a SUMO-BfmSc/His6-BfmR ratio of 1:6) for 10 min and then the excess of radiolabeled ATP was removed by desalting tubes Bio-Spin®6 (BioRad). To examine the dephosphorylation of P~BfmR in the absence of additional BfmS or BfmSDK2, P~His6-BfmR samples were fractionated by 12% SDS-PAGE, and radiolabeled proteins were visualized by autoradiography. To examine BfmS- or BfmSDK2-mediated dephosphorylation of P~BfmR, SUMO-BfmSc and SUMO-BfmScDK2 was respectively added into the reaction to reach a 4:1 (SUMO-BfmSc/His6-BfmR or SUMO-BfmScDK2/His6-BfmR) ratio, the reactions were initiated by adding 3 mM ADP at a final concentration and then incubated at room temperature for the indicated time. Reactions were stopped by the addition of 5 μl 5 × SDS loading buffer into a 20 μl reaction mixtures, and a 10 μl aliquot was resolved onto a 12% SDS-PAGE, visualized by autoradiography and Coomassie blue staining.

In vitro dephosphorylation were also monitored by Pro-Q stain. To examine the dephosphorylation of BfmR, His6-BfmR proteins (~ 40 μg) were first transphosphorylated by His6-GtrSc (at a His6-GtrSc/His6-BfmR ratio of 1:1) for 30 min phosphorylation reaction buffer supplemented with 5 mM ATP. The excess of ATP was removed by desalting tubes Bio-Spin®6 (BioRad). SUMO-BfmSc and its
variants (BfmSc^{DK2}, BfmSc^{T242R}, and BfmSc^{R393H}) was respectively added into the reaction to reach a 2:3 (SUMO-BfmSc/His6-BfmR) ratio. The reactions were initiated by adding 3 mM ADP at a final concentration and then incubated at room temperature for the indicated times, stopped by the addition of 2 μl 5 × SDS loading buffer. 10 μl aliquot was resolved onto a 12% SDS-PAGE. Phosphoproteins were stained with the Pro-Q Diamond.

Localized Surface Plasmon Resonance

An OpenSPR localized surface plasmon resonance (Nicoya Lifesciences) was used to analyzed the interaction of BfmS with BfmR. His6-BfmR protein was fixed on the COOH sensor chip by capture-coupling, then SUMO-BfmSc at concentrations of 12.5 nM, 40 nM, 80nM, 100nM and SUMO-BfmSc^{DK2} at concentrations of 25 nM, 50nM, 80nM, 100nM were injected sequentially into the chamber in PBS running buffer at 25°C. The binding time and disassociation time were both 240 s, the flowrate was 20 μl/min, the chip was regenerated with 0.02% SDS. A one to one diffusion corrected model was fitted to the wavelength shifts corresponding to the varied drug concentration. The data was retrieved and analyzed with TraceDrawer software (Ridgeview Instruments AB). Kinetic parameters were calculated using a global analysis, and the data was fitted to a one to one model.

Sample preparation and the Phos-tag gel electrophoresis

For the detection of in vivo BfmR phosphorylation, *P. aeruginosa* MPAO1 and its derivatives were grown at 37°C for 24 h on M8-glutamate minimal agar plate supplemented with 0.2 % glucose. To prepare cell lysates for the Phos-tag (APExBIO, Code#: F4003) gel assay, bacteria cells were scraped from the plate and immediately resuspended in 60 μl of lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM MgCl₂, 0.1% Triton X-100, 15 μg/ml DNase I, 0.5 mM PMSF, 1 mM DTT] with 0.1% (vol/vol) Lysonase. Sufficient lysis was achieved by repeated pipetting up and down for 10 s followed by addition of 20 μl of 4 × SDS loading buffer. 15 μl aliquots of the resulting cell lysates were immediately loaded onto a Phos-tag gel (10% acrylamide gels containing 25 μM acrylamide-Phos-tag ligand and 50 μM MnCl₂) (25).

For the detection of BfmR and BfmS phosphorylation in DK2 and AU15431 strains or in their derivatives, overnight cultures (LB) were washed twice and diluted 100-fold in fresh PB medium. The liquid cultures were grown in a 20-ml tube with a volume-to medium ratio of 5:1, shaken at 250 rpm for 3 h, of aeration. After collection of the cells by centrifugation, the pellet was washed once with 1 ml of 1 × PBS and suspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl, 1
mM DTT, 5% Glycerinum) supplied with 1 μl protease inhibitor cocktail (ThermoFisher, Cat#: 78430). The mixture was homogenized by mechanical disruption (Fast Prep FP2400 instrument; Qbiogene) and then centrifuging at 2, 300 g for 5 min. The supernatant was collected and followed by addition of 5 × SDS loading buffer, and 15 μl aliquots were loaded onto a Phos-tag gel as described above for electrophoresis.

After 3 h of electrophoresis at 30 mA in Tris-Glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.4) at 4°C, the Phos-tag gel was washed 10 min at room temperature with Transfer Buffer [20%(v/v) methanol, 50 mM Tris, 40 mM glycine] supplied with 1 mM EDTA to remove Mn²⁺ from the gel, then the gel was incubated at room temperature with gentle shaking for another 10 min in Transfer Buffer (20%(v/v) methanol, 50 mM Tris, 40 mM glycine) twice to remove EDTA.

**Co-immunoprecipitation**

Overnight cultures of *P. aeruginosa* were washed twice and diluted 100-fold in fresh M8-glutamate minimal medium supplemented with glucose. The liquid cultures were grown in a 100 ml flask with a volume-to-medium ratio of 3:1, shaking with 250 rpm at 37 °C for 3 h. 30 ml cultures were harvested by centrifugation, washed once and suspended in 1 ml of lysis buffer provided in the CO-IP kit (Pierce) supplied with 1 μl protease inhibitor cocktail (ThermoFisher, Cat#: 78420). The mixture was homogenized by mechanical disruption (Fast Prep FP2400 instrument; Qbiogene) and then the debris was removed by centrifuging and filtrating through 0.44 μm filters. 500 μl clarified lysates were incubated with the resins immobilized with specific antibodies or not for 12 h at 4 °C. The bound materials were washed and eluted following the manufacturer’s recommendation (ThermoFisher, Cat#: 26149). Total samples (clarified lysates) and the immunocomplexes eluted from the resins were analyzed by western blot with specific antibodies, as indicated.

**Western blot analysis**

Samples resolved on gels were transferred to PVDF (Bio-Rad) membranes through semi-dry transfer assembly (Bio-Rad) for 30 min at room temperature. The membrane was incubated with the primary antibody in 10 ml of 5% (wt/vol) skim milk at 4 °C overnight following the blocking step [10 ml of 5% (wt/vol) skim milk] at room temperature for 2 h, and then washed three times at room temperature for 15 min in TBST buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20]. Then, membranes were incubated with the secondary antibody for 2 h at room temperature and washed three times for 15 min in TBST. The chemiluminescent detection reaction
was performed and detected by Tanon-5200 multi, according to the manufacturer’s recommendation.

BfmR-Flag proteins were detected by Western blot analysis using a mouse anti-Flag monoclonal antibody (Cat#: AGM12165, Aogma) followed by a secondary, sheep anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Code#: NA931, GE Healthcare). GtrS-HA proteins were detected with a mouse anti-HA monoclonal antibody (Cat#: 66006-1-Ig, Proteintech) followed by a secondary, sheep anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP). For detection of BfmS protein, anti-BfmS polyclonal antibody prepared by immunizing a rabbit with an N-terminal 6His-tagged periplasmic segment (residues 34-154) of BfmS (Shanghai Immune Biotech CO., Ltd) and an anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Code#: NA934, GE Healthcare) were used. For detection of RNAP protein, anti-RNAP (Neoclone, #WP003) antibody and anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Code#: NA931, GE Healthcare). For detection of ClpP, anti-ClpP polyclonal antibody prepared by immunizing a rabbit with a *P. aeruginosa* N-terminal 6His-tagged full-length ClpP protein (Sangon Biotech Co., Ltd.) and an anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Code#: NA934, GE Healthcare) were used. For detection of PA2480, anti-PA2480 polyclonal antibody prepared by immunizing a rabbit with ploypeptides of *P. aeruginosa* PA2480 (96-112aa) (Wuhan ABclonal Biotechnology Co., Ltd) and an anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Code#: NA934, GE Healthcare) were used.

**Bacterial two-hybrid assays**

The Euromedex bacterial two-hybrid system (Cat #: EUK001), consisting of two compatible plasmids, pKT25 and pUT18C, were designed to investigate GtrS-BfmR interactions in vivo. The pKT25 and pUT18C plasmids allow the creation of in-frame fusions of the T25 and T18 catalytic domains of *Bordetella pertussis* adenylate cyclase with GtrS and BfmR.

For generating pKT25 derivative pKT25-*gtrS*, GtrS gene was amplified by PCR from MPAO1 genomic DNA using primer pair T25-*gtrS*-F/T25-*gtrS*-R (*BamHI/KpnI* sites), and then ligated into the same sites of pKT25. In the resultant plasmid pKT25-*gtrS*, the full length *gtrS* is inserted immediately downstream of the N-terminus of T25 in pKT25, creating a single hybrid gene (*T25-gtrS*) that encodes the target protein at the C-terminus and the T25 domain at the N-terminus. For generating pKT25 derivative pKT25-*gtrS*- *bfmS*Δ*K2*, a ~1.4 kb PCR product covering 20 bp of the *bfmS* upstream region and the *bfmS*Δ*K2* was amplified from DK2 genomic DNA using primers bfmS-comp-2F (with *KpnI* site) and bfmS-comp-2R (with *EcoRI* site), and the PCR products were cloned into plasmid pKT25-*gtrS*. In the resultant plasmid pKT25-*gtrS*- *bfmS*Δ*K2*, the *bfmS*Δ*K2* gene is inserted downstream of the hybrid T25-
gtrS and the direction of its transcription is in the same orientation as the lac on pKT25. For generating pUT18C derivative pUT18C-bfmR, primer pair T18C-bfmR-F/T18C-bfmR-R (BamHI/EcoRI sites) were used to amplify bfmR from MPAO1 genomic DNA, and the PCR products were cloned into pUT18C, creating a single hybrid gene that encodes the BfmR protein at the C-terminus and the T18 domain at the N-terminus. The primers used for PCRs are listed in table S2, and all constructs were sequenced to ensure that no unwanted mutations resulted.

To test the possible interaction between two proteins, a pKT25 derivative and a pUT18C derivative were co-transformed by electroporation into the adenylate cyclase-deficient E. coli strain, BTH101. Transformants were selected using kanamycin (50 µg/ml) and ampicillin (100 µg/ml) on LB agar plates, and were subsequently inoculated onto the M63/maltose agar plates supplemented with kanamycin (25 µg/ml), ampicillin (50 µg/ml), X-gal (40 µg/ml), and IPTG (0.5 mM), incubated at 30°C for up to 6 days. The appearance of (blue) colonies indicated positive interactions between the target proteins. Protein-protein interaction was also detected by measuring the β-galactosidase activities in bacteria grown in LB broth containing kanamycin (50 µg/ml), ampicillin (100 µg/ml), and 1 mM IPTG at 37°C for 6 h and 12 h, according to BACTH system procedure.

**Isolation of cytoplasmic membrane proteins**

Overnight *P. aeruginosa* LB cultures were washed twice and diluted 50-fold in M8-glutamate minimal medium supplemented with 10 mM glucose. The liquid cultures (20 ml) were grown in a 100-ml flask shaking with 250 rpm at 37°C for 6 h (OD$_{600}$ ≈ 0.5), the cultures were harvested by centrifugation at 5, 000 g for 10 min at 4 °C, suspended in lysis buffer (20 mM Tris-Cl, pH 8.0; 50mM NaCl, 1mM DTT) supplied with 1 X protease inhibitor cocktail (ThermoFisher, Cat#: 78430), and lysed by sonication. Unbroken cells were removed by centrifugation at 5, 000 g for 20 min at 4 °C and membranes were pelleted by centrifugation at 100, 000 g for 1 h at 4 °C. The inner membrane was solubilized by adding sodium N-lauroylsarcosinate to the suspension at a final concentration of 1 % and incubating for 30 min at room temperature and the outer membrane was removed by centrifugation at 40, 000 g for 40 min at 4 °C. The whole cell proteins and inner membrane proteins were analysed by 10 % (w/v) SDS-PAGE.

**Monitoring gene expression by lux-based reporters**

The plasmids mini-CTX-lux (carrying a promoterless luxCDABE reporter gene cluster, table S1) was used to construct promoter-luxCDABE reporter fusions *pa4103-lux*. To this end, the *pa4103* promoter region (−659 to +19 of the start codon) was
amplified by PCR using the primers pro-pa4103-F (with XhoI site) and pro-pa4103-R (with BamHI site) and cloned into the XhoI and BamHI sites of the mini-CTX-lux (table S1) to generate mini-CTX-pa4103-lux. The resulting plasmid was conjugated into *P. aeruginosa* strains and the construct was integrated into the attB site as described previously though a diparental mating using *E. coli* S17 λ-pir as the donor. In MPAO1 and its derivatives, parts of the mini-CTX-lux vector containing the tetracycline resistance cassette were deleted using a flippase (FLP) recombinase encoded on the pFLP2 plasmid. The plasmid pMS402 was used to construct promoter-lexCDABE reporter fusions of the gltB as described previously (24). Briefly, the gltB promoter region (~740 to +18 of the start codon) was amplified by PCR using the primers pro-pa3190-F (with XhoI site) and pro-pa3190-R (with BamHI site). All the promoters are oriented in the same direction as luxCDABE, and constructs were sequenced to ensure that no unwanted mutations resulted.

Unless noted otherwise, the expression of promoter fusion genes was measured in M8-glutamate minimal medium supplemented with 2 mM glucose in a 96-well black-wall clear-bottom plate (Corning incorporated, Costar, Code#: 3603). Briefly, overnight LB cultures were washed twice and diluted to an OD$_{600}$ of 0.05 in the minimal medium, and then a 100 µl volume of the sample was added to the wells, and subsequently a 60 µl volume of filter-sterilized mineral oil was added in order to prevent evaporation during the assay. Promoter activities were measured as counts per second (CPS) of light production with a Synergy 2 Multi-Mode Microplate Reader as described previously (24, 50). Additionally, the expression of promoter fusion genes was also carried out using a tube culture method, as indicated. In the tube culture method, bacteria were grown in a 20 ml tube with a flask volume-to-medium volume ratio of 5:1. After incubation at 37°C with shaking (250 rpm) for the indicated times, a 100 µl volume of the sample was added to the well of a 96-well black-wall clear-bottom plate (Corning incorporated, Costar, Code#: 3603) in order to measure the CPS of light production. Each sample was tested in triplicate. Relative light units were calculated by normalizing CPS to OD$_{600}$.

**Analysis of bfmRS transcripts by RT-PCR**

The total DNase-treated RNA (0.1 µg) was reversely transcribed to synthesize cDNA using the PrimeScript RT reagent Kit (Takara) with Random 6 mers primer according to the manufacturer’s protocol. PCR amplified using primers F (anneals 311 bp upstream of the bfmR stop codon) and R (anneals 183 bp downstream of bfmS start codon) or primers F and 2R (anneals 741 bp downstream of bfmS start codon). The reaction was carried out using an OneStep reverse transcription-PCR (RT-PCR) kit (Qiagen, Code#: 210212) according to the manufacturer’s instructions. The RT-PCR products were then analyzed by electrophoresis through 1 % agarose gel.
Analysis of microarray expression data of DK2 isolates

Two microarray expression data sets obtained from the online NCBI Gene Expression Omnibus (GEO) Database with the accession numbers GSE31227 and GSE62970 were used. In data set GSE31227, the transcriptomes of sixteen DK2 isolates, including twelve DK2 late stage isolates (that are, CF30-1979, CF173-1984, CF333-1991, CF66-1992, CF333-1997, CF173-2002, CF243-2002, CF333-2003, CF173-2005, CF333-2005, CF333-2007, and CF66-2008) and four DK2 early stage isolates (CF43-1973, CF66-1973, CF105-1973, and CF114-1973) have been measured at exponential growth phase in LB medium (26, 55). To identify evolved gene expression changes in DK2, we compared the transcriptomes of twelve DK2 late stage isolates (12 triplicate samples) to those of three DK2 early isolates (CF43-1973, CF105-1973, and CF114-1973; 3 triplicate samples), and a fold change ≥ 3 and P < 0.01 by paired Student’s t-test was used as threshold to determine significant differentially expressed genes (DEGs). Meanwhile, we compared the transcriptomes of the twelve DK2 late stage isolates to those of the non-CF-adapted isolate PAO1 strain, respectively. The transcriptome of the early stage isolate CF66-1973 was not used in our analysis, since this isolate may belong to the group of adapted isolates, given that it has two mutations located in the genes rpoN and mucA and these mutations are common to the adapted isolates and they are associated with an adapted phenotype (9, 26, 37, 55).

We also compared the gene expression profiles of DK2-91 and DK2-07 to that of DK2-WT, based on the data set GSE62970, respectively, and a fold change ≥ 3 and P < 0.01 by paired Student’s t-test was used as threshold to determine significant DEGs as described above. Data set GSE62970 contains gene expression data of triplicate analysis of three DK2 isolates (DK2-WT, DK2-91 and DK2-07) grown in vitro in glucose minimal medium. Among them, DK2-91 and DK2-07 are late stage isolates isolated from the same patient in 1991 and 2007, respectively, while DK2-WT resembles a non-adapted isolate.

RNA isolation, RNA-Seq, and RNA-Seq data analysis

Overnight cultures of WT P. aeruginosa MPAO1 strain and its derivatives, including ΔbfmS, ΔbfmSΔgtrS, hfmS^DK2, hfmS^DK2ΔgtrS, and ΔbfmRS mutants, were washed and diluted 50-fold in M8-glutamate minimal medium supplemented with 10 mM glucose. 20 ml liquid cultures were grown in a 100 ml flask at 37 °C, shaking with 250 rpm for 6 h. Total RNA was immediately stabilized with RNA protect Bacteria Reagent (Qiagen, CAS#:76506) and then extracted by using a Qiagen RNasy kit (Cat#:74104) following the manufacturer's instructions. rRNA removal, cDNA library construction, and paired-end sequencing with the Illumina HiSeqTM 2000 were completed by Guangdong Magigene Biotechnology Co., Ltd. The edgeR software package (56) was utilized to detect DEGs. A fold change ≥ 2 and FDR ≤ 0.05 (edgeR, Benjamini-Hochberg’s method) was used as threshold to determine the DEGs. All RNA-seq data (three independent biological replicates for each sample) have been submitted to the NCBI Sequence Read Archive (SRA).
Generating \textit{bfmS} natural alleles


Bioassay of C4-HSL activity

The autoinducer of the \textit{rhl} system, C4-HSL, was measured using pDO100 (pKD-\textit{rhlA}), which is an \textit{rhlA} promoter-based \textit{P. aeruginosa} strain. This detection system was developed by fusing the C4-HSL-responsive \textit{rhlA} promoter upstream of \textit{luxCDABE} and introducing the construct into pDO100, an \textit{rhl} mutant strain (57). For preparation of the sample, \textit{P. aeruginosa} cells were grown in PB medium at 37°C with shaking (250 rpm) in a 20-ml tube (with a flask volume-to-medium volume ratio of 5:1) for indicated time, and then 1 ml bacterial culture was centrifuged and sterilized by using a 0.22 μm pore size filter.

The reporter strain pDO100 (pKD-\textit{rhlA}) was grown in LB medium plus 100
μg/ml kanamycin overnight at 37°C with shaking (250 rpm) and diluted to an OD600 of 0.05 in fresh LB plus kanamycin, and subsequently, 90 µl was added to the wells of a 96-well black-wall clear-bottom plate (Corning incorporated, Costar). A 10 µl portion of the samples or medium control was added to the wells than gently blending, at last 60 µl volume of filter-sterilized mineral oil was added in order to prevent evaporation during the assay. The luminescence value was measured in a Synergy 2 Multi-Mode Microplate Reader, and calculated from the luminescence value minus that of the medium control. In this assay, the growth curves of pDO100 (pKD-rhlA) are identical, and CPS values became an indirect measure of the supernatant C4-HSL.

Measurement of pyocyanin production.

Pyocyanin was extracted from culture supernatants and measured using previously reported methods (24). P. aeruginosa DK2 and its derivatives were grown in PB medium, cultures were diluted down to a final OD600 of 0.05 in fresh PB medium and 3 ml of this suspension was added to 18 mm X 200 mm (18 in width and 200 in length) glass tubes at 37 °C with shaking (250 rpm) for 12 h, then 3 ml culture was centrifuged and filtered (pore size, 0.22 µm). 1.5 ml of chloroform was added to 2.5 ml of culture supernatant. After extraction, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 N HCl. After centrifugation, the top layer (0.2 M HCl) was removed and its absorption measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were determined by multiplying the optical density at 520 nm (OD520) by 17.072.

Biofilm formation assays

The biofilm formation by MPAO1 and its derivatives were assayed by determining the ability of the cells to adhere to the wells of polystyrene Stripwell™ Microplate (1 X 8 Flat Bottom; Corning incorporated, Costar, Code#: 42592) as previously described with some modifications (58). Briefly, an overnight PB culture was diluted down to a final OD600 of 0.05 in fresh PB medium and dispensed at 100 μl per well. Inculcated plates were incubated under static conditions at 37°C for 72 h. Biofilm formation by DK2 and its derivatives was measured in 20 ml glass tubes (Code#: 95-3, Haimen). The overnight PB culture was diluted down to a final OD600 of 0.05 in fresh PB medium and 3 ml of this suspension was added to 18 mm X 200 mm (18 in width and 200 in length) glass tubes, which were incubated statically at 37°C for 72 h. In order to measure the degree of attachment, non-adhered cells were removed and the biofilms rinsed with distilled water. Biofilms were stained by the addition of 150 µl (or 5 ml to the tubes) of 1% crystal violet (Cat#: 3603, Sigma-Aldrich) for 15 min followed by rinsing with distilled water. Photos were taken and the cell-associated
dye was solubilized in 150 μl (or 4.5 ml to the tubes) of 30% acetic acids in water and quantified by measuring the OD$_{550}$ of the resulting solution.

**Lettuce leaf model of infection.**

A lettuce leaf virulence assay was performed as described previously (24, 50). *P. aeruginosa* strains were grown overnight at 37°C with shaking (250 rpm) in PB broth, washed, resuspended and diluted in sterile 10 mM MgSO$_4$ to a bacterial density of $1 \times 10^8$ CFU/ml. Lettuce leaves were prepared by washing with sterile distilled H$_2$O and 0.1% bleach. Samples (10 μl) were then inoculated into the midribs of Romaine lettuce leaves. Containers containing Whatman paper moistened with 10 mM MgSO$_4$ and inoculated leaves were kept in a growth chamber at 37°C for five days. Symptoms were monitored daily.

**Drosophila infection assays**

*Drosophila melanogaster* (male, aged of 5 ± 1 days) were infected by needle pricking according to the protocol previously described (59) with some modifications. Overnight PB cultures of *P. aeruginosa* were diluted 100-fold in fresh PB medium. The liquid cultures were grown in a 20-ml tube with a volume-to medium ratio of 5:1, shaking with 250 rpm at 37°C for 3 h (OD$_{600}$ ≈ 0.7). The cell pellets from 1 ml of culture medium were rinsed once and resuspended in 1 X PBS, at a final OD$_{600}$ of 0.3. The flies were anesthetized using carbon dioxide and prick in the dorsal thorax using a sterilized tungsten needle dipped in the appropriate bacterial suspension. The pricked flies were returned to the fly-food vial, kept at 25°C and 65% humidity. At least twelve control flies were also pricked with a solution of 1 X PBS (survival rates 4 days after wounding were between 92% and 100%). Fly survival was scored and survival curves were processed with GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) to perform a statistical log-rank (Mantel-Cox) test.

**Statistical analysis.**

The log-rank test was used for survival analysis. Student’s *t*-tests (two-tailed) and Mann-Whitney U test were used to compare two data sets.

**Supplementary Materials**
Fig. S1. The promoter activities of \textit{bfmR} in MPAO1 and its derivatives.
Fig. S2. Consequences of either \textit{bfmS} missense mutations or \textit{bfmR} deletion.
Fig. S3. Identification of genes involved in activating BfmR.
Fig. S4. Glucose increases the regulatory activity of GtrS.
Fig. S5. Phosphorylation of BfmR and its variant, and co-immunoprecipitation.
Fig. S6. Bacterial two-hybrid assays showing GtrS-BfmR interactions.
Fig. S7. Sequence alignment of the histidine kinases BfmS, GtrS, EnvZ, and HK853.
Fig. S8. Promoter fusion analysis and the phosphorylation assays.
Fig. S9. Pro-Q Diamond staining and surface plasmon resonance (SPR) assays.
Fig. S10. Role of natural \textit{bfmS} variants in BfmR activation.
Fig. S11. Dephosphorylation of BfmR--P by SUMO-BfmSc and its variants.

Table S1. Plasmids and bacterial strains and used in this study
Table S2. Primers used in this study.
Table S3. \textit{bfmS} variants \textit{P. aeruginosa} CF isolates.

Data file 1. Microarray data analysis of DK2
Data file 2. RNA-seq analysis

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mutagenesis experiments. N.Y, C.X, K.F, F.C, H.L., Y.Z, X.D., Y.F, C-G.Y., and M.W. provided technical assistance. L.L. supervised the study. Q.C., T.B, and L.L. analysed the data. L.L. supervised the study and wrote the manuscript with input from Q.C. and T.B. All authors discussed the results and commented on the manuscript. **Competing interests:** All authors have no competing interests. **Data and Materials Availability:** The RNA-seq data have been deposited to the NCBI Sequence Read Archive (SRA) (https://submit.ncbi.nlm.nih.gov/subs/) under the BioProject accession PRJNA597232, with the following BioSample accession numbers: SAMN13674998 to SAMN13675015. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

**Fig. 1. Consequence of L181P/E376Q missense mutations in bfmS.** (A) The bfmR promoter activity of *P. aeruginosa* strains cultured in 96-well plates containing M8-glutamate minimal medium supplemented with 2 mM glucose. Expression of *bfmR-lux* (CPS, counts per second; closed symbols) were determined at different phases of the cell growth monitored by measuring the optical density at 600 nm (OD600; open symbols). Data from *n* = 6 biological replicates reported as means ± SEM. (B) Western blotting analysis for the production of BfmS and its variant from bacterial whole-cell lysates and purified inner membranes. PA2480 (a predicted membrane-associated histidine kinase of *P. aeruginosa*) and cytoplasmic RNA polymerase (RNAP) alpha subunit are probed as a loading control. Images are representative of two independent experiments. (C) Western blotting analysis for the in vivo
phosphorylation of BfmR analyzed on Phos-tag™ gel. Images are representative of three independent experiments. (D) Relative amounts of C4-HSL produced by *P. aeruginosa* strains grown in pyocyanin production broth (PB medium) at 37°C for 9 h. Data from *n* = 3 biological replicates reported as means ± SEM. (E) Photograph shows lettuce midrib after 3 days of infection with *P. aeruginosa* cells. Both MPAO1 and *bfmS*<sup>DK2</sup>/p-<em>bfmS</em> strains show necrosis and tissue maceration of infection, and the *bfmS*<sup>DK2</sup> mutant shows weak signs of infection. Images are representative of three independent experiments. (F) *D. melanogaster* killing by MPAO1 and its derivatives. *n* indicates the number of flies used. **P < 0.01 (log-rank test; *bfmS*<sup>DK2</sup> compared with either MPAO1 or *bfmS*<sup>DK2</sup>/p-*bfmS*). (G and H) Biofilm formation. Photograph in (G) showing ring-shape biofilms (stained by 1% crystal violet) near the air-liquid interface on the inner surface of polystyrene Stripwell™ Microplate with quantification shown in (H), where data from *n* = 8 biological replicates reported as mean ± SEM (** **P < 0.001, Student's two-tailed *t*-test). No statistically significant difference was observed between MPAO1 and *bfmS*<sup>DK2</sup>/p-*bfmS* strains. In (A, D, E, F, G, and H), MPAO1 and *bfmS*<sup>DK2</sup> carry an empty pAK1900 vector as control, p-*bfmS* denotes pAK1900-*bfmS* (table S1). In (C), MPAO1 and *bfmS*<sup>DK2</sup> carry an empty integration-proficient mini-CTX-lacZ vector (table S1) as control.

Fig. 2. BfmR is highly activated in the CF-adapted isolates of *P. aeruginosa* DK2 lineage. (A) Heatmaps showing the expression profiles of the top 15 upregulated genes in CF-adapted DK2 isolates with respect to the non-CF-adapted isolates of DK2 lineage. Expression level of gene is represented as log<sub>2</sub> expression ratios (Sheet 1 of Data file 1). (B to F) Effect of L181P/E376Q missense mutations in *bfmS* on the amount of C4-HSL (B), the production of pyocyanin (C), the formation of biofilm (D), the phosphorylation level of BfmR (E), and the expression of *pa4103-lux* (F) of the DK2 strain. In (B), data from *n* = 3 biological replicates reported as means ± SEM; in (C), data from *n* = 8 biological replicates reported as mean ± SEM (** **P < 0.001, Student's two-tailed *t*-test), and inserted photographs showing the blue-green pigment of *P. aeruginosa* cultures; in (D), data from *n* = 6 biological replicates.
reported as mean ± SEM (***P < 0.001, Student's two-tailed t-test), and inserted photograph showing ring-shape biofilms (stained by 1% crystal violet) near the air-liquid interface on the inner surface of glass tubes. (E) Western blot analysis of in vivo phosphorylation of BfmR-flag analyzed on Phos-tag™ gel. Protein samples are derived from bacteria carrying pRK415-bfmR-flag plasmid (table S1). Images are representative of two independent experiments. (F) The promoter activities of pa4103 (CPS, counts per second; closed symbols) were determined at different phases of the cell growth monitored by measuring the optical density at 600 nm (OD600; open symbols). Data represent means ± SEM of n = 3 biological replicates.

Fig. 3. GtrS forms a TCS with BfmR. (A and B) Relative expression of bfmR-lux (A) and pa4103-lux (B) when bacteria were grown in tubes containing minimal medium supplemented with 2 mM glucose at 37°C with shaking (250 rpm) for 6 h. The expressions of bfmR-lux and pa4103-lux in MPAO1 were set to 1, respectively, and the other values were adjusted accordingly, as indicated. In (A) and (B), data represent means ± SEM of n = 3 biological replicates (***P < 0.001, Student's two-tailed t-test). In (B), no statistically significant difference was observed between ΔbfmRSΔgltR-gtrS and ΔbfmRSΔgltR-gtrS/p-gtrS strains. P. aeruginosa strains, MPAO1, ΔbfmS, ΔbfmSΔgltR-gtrS, ΔbfmRS, and ΔbfmRSΔgltR-gtrS carrying an empty pAK1900 vector as control; p-gtrS, p-gltR, and p-gltR-gtrS denote pAK1900-gtrS, pAK1900-gltR, and pAK1900-gltR-gtrS (table S1), respectively. (C) Western blot analysis of in vivo phosphorylation of BfmR-flag analyzed on Phos-tag™ gel. Images are representative of two independent experiments. (D) Western blot image
showing the Co-immunoprecipitation of GtrS-HA and BfmR-flag. Whole cell extracts from ΔbfmS mutant expressing gtrS-HA (lanes 1, 3, 4, 6, and 7) and/or bfmR-flag (lanes 2–6) were immunoprecipitated without (lane 4; n.a.) or with (lanes 5–7) anti-HA antibody. Images are representative of two independent experiments. (E) Autoradiogram showing the auto-phosphorylation and trans-phosphorylation activity of His6-GtrSc. After auto-phosphorylation of His6-GtrSc with [γ-32P]ATP for 20 min, His6-BfmR was added to the reaction mixtures and samples were taken as indicated time. M, protein marker. Images are representative of two independent experiments. (F) Phosphoprotein detection with Pro-Q Diamond phosphoprotein gel stains showing the phosphorylation of His6-BfmR by His6-GtrSc. Aliquots were removed at the indicated time points for analysis by SDS-PAGE, followed by Pro-Q Diamond staining to identify phosphorylation and Coomassie blue staining to detect protein levels. Heating, the sample was exposed to the heating at 100°C for 5 min prior to SDS-PAGE. M, protein markers. Images are representative of three independent experiments.

Fig. 4. GtrS activating BfmR when bfmS undergoes L181P/E376Q missense mutations. (A) Expression of pa4103-lux in MPAO1 and its derivatives cultured in M8-glutamate minimal medium supplemented with or without glucose. Data represent means ± SEM of n = 3 biological replicates. (B) Western blot analysis of the production of BfmS in P. aeruginosa strains cultured in tubes containing M8-glutamate minimal medium supplemented with or without glucose at 37°C.
shaking for 6 h. Images are representative of two independent experiments. (C) Relative amounts of C4-HSL. Data represent means ± SEM of n = 3 biological replicates. (D) Photograph shows lettuce midrib after 3 days of infection with P. aeruginosa cells. Images are representative of three independent experiments. (E) D. melanogaster killing by MPAO1 and its derivatives. n indicates the number of flies used. ***P < 0.001 (log-rank test). (F) The adhesion/biofilm formation (A550) by P. aeruginosa strains. Data represent means ± SEM of n = 8 biological replicates (***P < 0.001, Student's two-tailed t-test). (G) Western blot images showing the co-immunoprecipitation of GtrS-HA and BfmR-flag. Whole cell extracts from bfmS<sup>DK2</sup> mutant expressing GtrS-HA and/or BfmR-flag were immunoprecipitated without (n.a.) or with (IP: Flag) anti-Flag antibody. Images are representative of two independent experiments. (H) Expression of pa4103-lux in bfmS<sup>DK2</sup> and WT MPAO1 cultured in M8-glutamate minimal medium supplemented with or without glucose, as indicated. Data represent means ± SEM of n = 3 biological replicates. (I, J, and K) Expression of pa4103-lux (I) and the production of BfmS protein (J and K) in DK2 and its derivatives cultured in PB medium for 6 h. In (I), data represent means ± SEM of n = 3 biological replicates (***P < 0.001, Student's two-tailed t-test). In (J and K), images are representative of two independent experiments. In (A, C, D, E, and F), MPAO1, bfmS<sup>DK2</sup>, and bfmS<sup>DK2</sup>ΔgtrS mutants harbor an empty pAK1900 vector as control; p-gtrS denotes pAK1900-gtrS plasmid (table S1). In (J), DK2 and its isogenic ΔgtrS mutant harbor an empty pRK415 plasmid as control. ΔgtrS-C denotes ΔgtrS mutant carrying a pRK415-gtrS plasmid (table S1).

**Fig. 5. BfmS<sup>DK2</sup> activates BfmR in a direct manner.** (A) Western blot images showing the Co-immunoprecipitation of BfmR-flag and BfmS<sub>L181P/E376Q</sub> (BfmS<sup>DK2</sup>). Whole cell extracts from bfmS<sup>DK2</sup> mutant expressing bfmR-flag were
immunoprecipitated without (lane 3, n.a.) or with (IP: Flag) anti-Flag antibody. Images are representative of two independent experiments. (B) Autoradiogram after SDS-PAGE showing the autophosphorylation and transphosphorylation activity of BfmS\textsuperscript{DK2} and BfmS\textsuperscript{DK2 H238A}. Protein bands stained with Coomassie blue (bottom panel) after autoradiography (top panel) were shown. Images are representative of two independent experiments. (C) Expression of \textit{pa4103-lux}. Values represent means ± SD (n = 3 biological replicates). (D) \textit{D. melanogaster} killing by MPAO1 and its derivatives. n indicates the number of flies used. **p < 0.01 (log-rank test; bfmS\textsuperscript{DK2} compared with either MPAO1 or bfmS\textsuperscript{DK2 H238A}). (E) Photograph shows lettuce midrib after 3 days of infection with \textit{P. aeruginosa} cells. Images are representative of three independent experiments. (F) Photograph showing ring-shape biofilms (stained by 1% crystal violet) near the air-liquid interface on the inner surface of polystyrene Stripwell\textsuperscript{TM} Microplate. Images are representative of three independent experiments. (G, H) Western blot analysis of in vivo phosphorylation of BfmS analyzed on Phos-tag\textsuperscript{TM} gel. Images are representative of two independent experiments. (I, J) Time course of P~BfmR dephosphorylation in the absence (in I) or presence of additional either SUMO-BfmSc or SUMO-BfmSc\textsuperscript{DK2} (in J). Images are representative of two independent experiments. His\textsubscript{6}-BfmR was first transphosphorylated by SUMO-BfmSc for 10 min. After removal of ATP, P~His\textsubscript{6}-BfmR samples were taken at the indicated time points (in I). In (J), SUMO-BfmSc or SUMO-BfmSc\textsuperscript{DK2} was added to the reaction mixtures after removal of ATP. M, protein markers.
Fig. 6. RNA-seq analysis and role of natural bfmS variants in the transcriptional regulatory activity of BfmR. (A) Effect of deletion of gtrS in the ΔbfmS mutant background on the expression of bfmS-activated and -repressed genes. (B) Effect of deletion of gtrS in the bfmSDK2 background on the expression of L181P/E376Q mutations-induced and -reduced genes. (C) Heatmaps showing the relative expression of BfmR-activated and -repressed genes in different P. aeruginosa strains. Lane 1, BfmR-activated and -repressed genes determined by comparing the transcriptome of ΔbfmS to that of ΔbfmRS (n = 3 independent experiments, with a |log2 fold-change| ≥ 1 and FDR ≤ 0.05). (D) The relative expression of pa4103-lux in P. aeruginosa strains cultured in 96-well plates containing minimal medium supplemented with 2 mM glucose for 3 h. Values are relative to ΔbfmS mutant carrying a WT bfmS (set to 1), as indicated. Data represent means ± SD of n = 3 biological replicates (***P < 0.001, Student's two-tailed t-test; also with a 2-fold higher level of pa4103-lux activity compared to that of WT BfmS). (E) Positions of bfmS spontaneous missense mutations involved in the increased transcriptional regulatory activity of BfmR. TM1, aa 10–32; periplasmic domain, aa 33–151; TM2, aa 152–174. HAMP domain, aa 175–227; HisKA, aa 326–434; HisKA with the active site at H238 in BfmS of PAO1. Periplasmic domain was not predicted in SMART but was assumed to be between the TM1 and TM2; aa, amino acid. (F) A proposed mechanism and consequences of mutations in bfmS. In wild-type cells, BfmS prevents the phosphorylation of BfmR; however, BfmS may have a role in promoting the accumulation of P~BfmR under certain conditions. In ΔbfmS mutant, the absence of bfmS leads to the phosphorylation
of BfmR by GtrS. When \textit{bfmS} was mutated to either L181P/E376Q or A42E/G347D, accumulation of P~BfmR also occurs, mainly resulting from two effects: i) increased autophosphorylation of BfmS, and ii) cross-phosphorylation between GtrS and BfmR. The lines show the interaction between the players: arrow, activation; hammerheads, repression; solid line, a direct direct connection; dotted line, a putative connection. The question mark (“?”) denotes a yet-unidentified effect.

Figures and Figure legends

\textbf{Fig. S1.} The promoter activities of \textit{bfmR} in MPAO1 and its derivatives. Expression of \textit{bfmR}-\textit{lux} (CPS, counts per second; closed symbols) were determined at different phases of the cell growth monitored by measuring the optical density at 600 nm (OD\textsubscript{600}; open symbols). Bacteria were cultured in 96-well plates containing M8-glutamate minimal medium supplemented with either 2 mM sodium acetate anhydrous, D-Mannitol, sodium pyruvate, or D-Fructose at a final concentration of 2 mM, as indicated. MPAO1 and \textit{bfmS}\textsuperscript{DK2} carry an empty pAK1900 vector as control, p-\textit{bfmS} denotes pAK1900-\textit{bfmS} (table S1). Values represent means ± SD of \textit{n} = 6 biological replicates.
Fig. S2. Consequences of either bfmS missense mutations or bfmR deletion. (A) The expression of \( pa4103\)-lux in MPAO1 and its derivatives cultured in 96-well plates containing M8-glutamate minimal medium supplemented with 2 mM glucose. CPS, counts per second; OD\(_{600}\), an optical density at 600 nm. Data represent means ± SD (n = 3 biological replicates). (B) Reverse transcription polymerase chain reaction (RT-PCR) analysis. Upper panel, scheme showing the position and orientation of the primers (black arrows) in respect to bfmR and bfmS. Lower panel, ethidium bromide-stained agarose gel (1%) of whole-cell RNA-based RT-PCR (lanes 2, 3, 5, and 6) and genome-based PCR (lanes 4 and 7) products amplified with the following primers: F and R (lanes 2-4) and F and 2R (lanes 5-7); controls without reverse transcriptase (RT) added were included (lanes 2 and 5); DNA size standards (in base pairs) are indicated. Images are representative of two independent experiments. (C) Relative amounts of C4-HSL measured by the pDO100 (pKD-rhlA) system. MPAO1 and its derivatives were grown in tubes containing pyocyanin production broth (PB medium) at 37°C for 12 h with shaking (250 rpm), the supernatants were subsequently prepared and measured for their ability to promote the luminescence values of the C4-HSL.
reporter strain pDO100 (pKD-rhlA). CPS values became an indirect measure of supernatant C4-HSL. Data represent means ± SD (n = 3 biological replicates). (D) Photograph shows lettuce midrib after 3 days of infection with *P. aeruginosa* cells. Both WT MPAO1 and *bfmS*ΔK2-*ΔbfmR* mutant show necrosis and tissue maceration of infection, while the *bfmS*ΔK2 mutant and the complementary strain of *bfmS*ΔK2-*ΔbfmR* mutant (*bfmS*ΔK2-*ΔbfmR/p-bfmR) show weak signs of infection. Images are representative of two independent experiments. (E) *D. melanogaster* killing by MPAO1 and its derivatives. *n* indicates the number of flies used. *P < 0.05, ***P < 0.001, log-rank test. (F) Photograph showing ring-shape biofilms (stained by 1% crystal violet) near the air-liquid interface on the inner surface of polystyrene Stripwell™ Microplate. Images are representative of three independent experiments. (G) The adhesion/biofilm formation (A550) by *P. aeruginosa*. Data represent means ± SD of *n* = 4 independent experiments (***P < 0.001, Student's two-tailed *t*-test). MPAO1, *bfmS*ΔK2, and *bfmS*ΔK2-*ΔbfmR* carry an empty pAK1900 as control; p-*bfmR* denotes pAK1900-*bfmR* (table S2).

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**Fig. S3. Identification of genes involved in activating BfmR.** (A) Schematic representation of genes involved in glucose uptake and metabolism in *P. aeruginosa* PAO1 ([http://www.pseudomonas.com](http://www.pseudomonas.com)). The transposon insertion site is indicated by triangle. (B) Relative expression of *bfmR-lux* in MPAO1 and its derivatives. The expression of *bfmR-lux* in MPAO1 was set to 1, and the other values were adjusted accordingly. Data are from *n* = 3 biological replicates and reported as means ± SD (***P < 0.001, Student's two-tailed *t*-test). MPAO1, Δ*bfmS*, and Δ*bfmS*Δ*gltB* carry an empty pAK1900 plasmid as control; p-*gltB* denote pAK1900-*gltB* (table S1). (C) Relative expression of *pa4103-lux*. The expression of *pa4103-lux* in Δ*bfmS*Δ*gltB* was set to 1, and the other values were adjusted accordingly. Data from *n* = 3 biological
replicates reported as means ± SD (***P < 0.001, Student's two-tailed t-test). No statistically significant difference was observed between ΔhfmSΔgtrS and ΔhfmSΔgtrS/p-gltB strains. ΔhfmSΔgltB and ΔhfmSΔgtrS carry an empty pAK1900 plasmid as control, while p-gtrS and p-gltB respectively denote pAK1900-gtrS and pAK1900-gltB (table S1). (D and E) Effect of H280A mutation on the production of GtrS (D) and on the regulatory activity of GtrS against the expression of pa4103-lux (E). MPAO1, ΔgtrS, or ΔhfmSΔgtrS carrying an empty pAK1900 plasmid as control; p-gtrS-HA and p-gtrSH280A-HA denote pAK1900-gtrS-HA and pAK1900-gtrSH280A-HA (table S1), respectively. In (D), images are representative of two independent experiments; in (E), data from n = 3 biological replicates reported as means ± SD. In (B-D), bacteria were grown in tubes containing M8-glutamate minimal medium supplemented with 2 mM glucose with shaking (250 rpm) at 37 °C for 5 h. In (E), bacteria were cultured in 96-well plates containing M8-glutamate minimal medium supplemented with 2 mM glucose; CPS, counts per second; OD600, an optical density at 600 nm.
Fig. S4. Glucose increases the regulatory activity of GtrS. (A) The expression of \textit{pa4103-lux} in \textit{P. aeruginosa} strains grown in 96-well plates containing M8-glutamate minimal medium supplemented without or with 20 μM of the indicated carbohydrates. Data represent means ± SD (n = 3 biological replicates). MPAO1 and ΔbfmS harbor an empty pAK1900 plasmid as control; p-bfmS denotes pAK1900-bfmS (table S1). (B and C) The expression of \textit{pa4103-lux} in ΔbfmS (B) and ΔbfmSΔgtrS (C) mutants cultured in 96-well plates containing M8-glutamate minimal medium supplemented with indicated concentrations of glucose. Data represent means ± SD (n = 3 biological replicates). (D) The expression of gltB-lux in MPAO1 and its derivatives cultured in 96-well plates containing M8-glutamate minimal medium supplemented with 20 μM
of indicated carbohydrate. Data represent means ± SD (n = 3 biological replicates). MPAO1 and ΔgtrS harbor an empty pAK1900 plasmid as control; p-gtrS denotes pAK1900-gtrS plasmids (table S1). (E) Expression of gltB-lux in MPAO1 cultured in 96-well plates containing minimal medium supplemented with indicated concentrations of glucose. Data represent means ± SD (n = 3 biological replicates). In (F), MPAO1 and ΔgltB harbor an empty pAK1900 plasmid as control; p-gltB, p-gtrS, p-gltR, and p-gltR-gtrS respectively denote pAK1900-gltB, pAK1900-gtrS, pAK1900-gltR, and pAK1900-gltR-gtrS plasmids (table S1).

Fig. S5. Phosphorylation of BfmR and its variant, and co-immunoprecipitation. (A) Western blot analysis of in vivo phosphorylation of BfmR-flag analyzed on Phos-tag™ gel. Bacteria carrying either pAK1900-bfmR-flag or pAK1900-bfmRDSSA-flag plasmid (table S1), as indicated; P~BfmR-flag denotes the phosphorylated BfmR-flag proteins. Images are representative of two independent experiments. (B) Western blot image showing the Co-immunoprecipitation of GtrS-HA and BfmR-flag. Whole cell extracts from ΔbfmS mutant expressing gtrS-HA and/or bfmR-flag (lanes 2, 3, and 5) were immunoprecipitated without (lane 3; n.a.) or with (lanes 4-5, IP: Flag) anti-Flag antibody. Images are representative of two independent experiments. (C) SDS-PAGE and phosphoprotein staining with Pro-Q Diamond showing the effect of ATP (2 mM) on the phosphorylation of His6-BfmR. Phosphorylation of BfmR by acetyl phosphate (Ac-P) (22) was used as a positive control. The intensities of Pro-Q Diamond stained bands of purified proteins were standardized against the intensities of the same bands after gels were restained with Coomassie blue. Images are representative of three independent experiments. (D) Effect of heating on the phosphorylation of His6-BfmR by His6-GtrSc. His6-GtrSc was first auto-phosphorylation with [γ-32P]ATP for 5 min,
then His6-BfmR was added to the reaction mixtures and samples were taken after 10 min, with or without heating at 100°C for 5 min prior to SDS-PAGE. Protein bands stained with Coomassie blue after autoradiography were shown. “P~” denotes the phosphorylated proteins. Images are representative of two independent experiments.

(E) Autoradiogram after SDS-PAGE showing the transphosphorylation activity of His6-GtrSc against the His6-BfmR<sup>Δ55A</sup> proteins. Images are representative of two independent experiments. Protein bands stained with Coomassie blue after autoradiography were shown, and “P~” denotes the phosphorylated proteins.

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**Fig. S6. Bacterial two-hybrid assays showing GtrS-BfmR interactions.** (A) *E. coli* BTH101 recombinants bearing indicated combinations of plasmids were plated on either M63/glucose agar or M63/maltose agar containing 40 µg/ml X-gal and 0.5 mM IPTG as indicated, and incubated at 30°C for 6 days. *E. coli* BTH101 bearing vectors encoding the zip fragment (pUT18C-zip and pKT25-zip) were used as the positive control, while *E. coli* BTH101 co-expressing T18 and T25 tags on vectors (pUT18C and pKT25) were used as negative control. Images are representative of two independent experiments. (B) Expression of β-galactosidase activity cultured in LB broth containing 1mM IPTG for 6 and 12 h. Data from n = 4 biological replicates reported as means ± SD (***P < 0.001, Student's two-tailed t-test). (C) Western blotting analysis for the production of Bfm<sup>S<sub>DK2</sub></sup> protein in *E. coli* BTH101 bearing
pKT25-\textit{gtrS-hfms}^{DK2} and pUT18C-\textit{bfmR} plasmids. RNAP alpha subunit are probed as a loading control. The asterisk indicates a non-specific signal. Images are representative of two independent experiments.

Fig. S7. Sequence alignment of the histidine kinases BfmS, GtrS, EnvZ, and HK853. Asterisks indicate residues exactly conserved across all the sequences, while dots indicate similar amino acids. The phosphoacceptor residues of \textit{P. aeruginosa} BfmS (H238), \textit{P. aeruginosa} GtrS, \textit{Escherichia coli} EnvZ, and \textit{Thermotoga maritima} HK853 are indicated with black arrow. Partner specificity-determining residues inferred from studies on EnvZ (33) and HK853 (34) are shaded in yellow. Highly conserved partner specificity-determining residues between BfmS and GtrS are shown in red.
**Fig. S8. Promoter fusion analysis and the phosphorylation assays.** (A) Expression of *pa4103-lux* in MPAO1 and its derivatives cultured in 96-well plates containing M8-glutamate minimal medium supplemented without or with 2 mM glucose. Data represent means ± SD of n = 3 biological replicates. (B, C) The autophosphorylation and transphosphorylation activity of BfmSc<sup>Dk2</sup> (B) and BfmSc (C). Lane 1, standard protein molecular sizes, in kilodaltons (kd); lanes 2-7, the recombinant protein BfmSc<sup>Dk2</sup> (in B) or BfmSc (in C) was incubated with 10 μCi of [γ-<sup>32</sup>P] ATP. Aliquots were removed at the times indicated for analysis by SDS-PAGE; lanes 8-10, His6-BfmR was added to either phosphorylated BfmSc<sup>Dk2</sup> (in B) or BfmSc (in C) (prepared by pre-incubating with [γ-<sup>32</sup>P] ATP for 5 min) and samples removed for SDS-PAGE after 5 seconds (lane 8), 10 sec (lane 9) and 30 sec (lane 10). Autoradiogram of the gel (top) and the Coomassie blue-stained gel (bottom) are shown. Images are representative of two independent experiments. (D) Expression of *pa4103-lux* in MPAO1 and its derivatives cultured in 96-well plates containing either LB or PB medium. Data represent means ± SD of three biological replicates.
Fig. S9. Pro-Q Diamond staining and surface plasmon resonance (SPR) assays. 
(A) Phosphoprotein detection with Pro-Q Diamond phosphoprotein gel stains showing the phosphorylation level of the purified BfmSc proteins and its variant. Lane 1, standard protein molecular sizes, in kilodaltons (kd); lane 2, the recombinant protein BfmSc; Lane 3, the recombinant protein BfmScDK2; lane 4 and 5, proteins were dephosphorylated by treatment with 0.5U calf intestinal alkaline phosphatase (CIP) for 1 h. Images are representative of two independent experiments. (B) The
level of phosphorylation of purified SUMO-BfmSc proteins and its variant. Lane 1, standard protein molecular sizes, in kilodaltons (kd); lane 2, the recombinant protein SUMO-BfmSc; Lane 3, the recombinant protein SUMO-BfmSc\(^{\text{DK2}}\); lane 4 and 5, proteins were dephosphorylated by treatment with 30U calf intestinal alkaline phosphatase (CIP) for 1 h. Images are representative of two independent experiments. (C) \textit{In vitro} transphosphorylation of His\(_6\)-BfmR by either purified SUMO-BfmSc (lane 3-7) or SUMO-BfmSc\(^{\text{DK2}}\) (lane 8-11) in the absence of exogenous ATP. Aliquots were removed at the indicated time points for analysis by SDS-PAGE, followed by Pro-Q Diamond staining to identify phosphorylation and Coomassie blue staining to detect protein levels. Images are representative of three independent experiments. (D, E) Analysis of protein interactions by OpenSPR showing the effect of L181P/E376Q mutations on the binding affinity of BfmS to BfmR. Analysis performed in TraceDrawer using a 1:1 binding interaction model. \(K_D\), affinity constant. (F and G) Representative images (F) and analysis (G) of the phosphorylation of BfmR by SUMO-BfmSc and its variant SUMO-BfmSc\(^{\text{DK2}}\) in the presence of exogenous ATP (2 mM). The intensities of Pro-Q Diamond stained bands of His\(_6\)-BfmR proteins were standardized against the intensities of the same bands after gels were restained with Coomassie blue and was further normalized to the vehicle group within each experiment. Data from \(n = 3\) independent experiments reported as means ± SEM. No statistically significant difference (by Mann-Whitney U test) was observed between BfmSc and BfmSc\(^{\text{DK2}}\) groups at the same time point.
Fig. S10. Role of natural bfmS variants in BfmR activation. (A) The relative expression of \textit{pa4103-lux} when bacteria were grown in 96-well plates containing minimal medium supplemented with 2 mM glucose for 3 h. Values are relative to ΔbfmRS mutant carrying a wild-type bfmS (set to 1). Data represent means ± SD of \( n = 3 \) biological replicates. (B) Expression of \textit{pa4103-lux} in MPAO1 ΔbfmS mutant carrying a plasmid-borne WT bfmS or its variants. Data from \( n = 3 \) biological replicates reported as means ± SD. (C) Western blot analysis of in vivo phosphorylation of BfmR-flag analyzed on Phos-tag™ gel. Images are representative of two independent experiments. (D) Expression of \textit{pa4103-lux} in AU15431 isolate and its derivatives cultured in cultured in 96-well plates containing PB medium. Data from \( n = 3 \) biological replicates reported as means ± SD. (E) Western blot images showing the effect of \textit{gtrS} deletion on the production of BfmS in AU15431 isolate. Protein samples derived from bacteria grown in tubes containing PB medium.
supplemented with 2 mM glucose at 37°C for 6 h with shaking (250 rpm). RNA polymerase (RNAP) alpha subunit is probed as a loading control. Images are representative of two independent experiments. (F) Western blot analysis of in vivo phosphorylation of BfmS and its variants analyzed on Phos-tag™ gel. Protein samples derived from ΔbfmS mutant carrying p-bfmS, p-bfmST242R, or p-bfmSA42E/G347D plasmid (table S1), as indicated. Images are representative of two independent experiments. (G) Western blot analysis of in vivo phosphorylation of BfmS and its variants analyzed on Phos-tag™ gel. Images are representative of three independent experiments. (H and I) Effect of bfmS missense mutations on the pa4103-lux when bacteria grown in 96-well plates containing minimal medium supplemented without or with glucose (2 mM) for 4 h. Data from n = 3 biological replicates reported as means ± SD (**P < 0.001, Student's two-tailed t-test). (J) Expression of pa4103-lux in MPAO1 and its derivatives cultured in 96-well plates containing M8-glutamate minimal medium supplemented without (-) or with (+) 2 mM glucose. Data represent means ± SEM of three biological replicates. MPAO1, bfmSR393H, bfmSR393HΔgtrS harbor an empty pAK1900 plasmid as control (table S1).

**Fig. S11. Dephosphorylation of BfmR−P by BfmSc and its variants.** (A) Representative images of Pro-Q Diamond phosphoprotein gel stains showing the dephosphorylation of BfmR−P by purified BfmSc and its indicated variants. His6-BfmR was first phosphorylated by His6-GtrSc for 30 min. After removal of ATP, the P−His6-BfmR sample was treated without (left panel) or with purified SUMO-BfmSc and its variants, as indicated. Aliquots were removed at the indicated time points for analysis by SDS-PAGE, followed by Pro-Q Diamond staining to identify
phosphorylation and Coomassie blue staining to detect protein levels. M, protein markers. Heating, the sample was exposed to the heating at 100°C for 5 min prior to SDS-PAGE. (B) Graphic data showing the dephosphorylation of BfmR–P by purified BfmSc and its variants. Data from n = 4 independent experiments reported as means ± SEM. The significant differences were indicated by marks: **P < 0.01 (Mann-Whitney U test) when compared to the same time point of the BfmSc group. No statistically significant difference was observed between BfmScR393H and BfmSc at the time point of 20 min. The intensities of Pro-Q Diamond stained bands of His6-BfmR proteins were standardized against the intensities of the same bands after gels were restained with Coomassie blue, and the normalized data were further corrected by subtracting the background signal detected from the heated samples. The results are reported as percentage relative to the control sample (at the time point of 0 min), respectively.

**Table S1. Plasmids and bacterial strains and used in this study**

<table>
<thead>
<tr>
<th>Plasmids or strains</th>
<th>Relevant characteristicsa</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>mini-CTX-lacZ</td>
<td>Gene delivery vector for inserting genes at the CTX phage att site on P. aeruginosa chromosome, Tc; lacZ-based promoter reporter</td>
<td>(48)</td>
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<tr>
<td>mini-CTX-lux</td>
<td>Gene delivery vector for inserting genes at the CTX phage att site on P. aeruginosa chromosome, Tc; lux-based promoter reporter</td>
<td>(48)</td>
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<td>pAK1900</td>
<td><em>E. coli</em>–<em>P. aeruginosa</em> shuttle cloning vector, Ap/Cb</td>
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<td>pBT20</td>
<td>Mini-TnM delivery vector; Ap/Gm</td>
<td>(47)</td>
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<td>pET28a</td>
<td>T7 lac promoter–operator, N-terminal His tag, kan</td>
<td>Novagen</td>
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<tr>
<td>pET28a(+)-sumo</td>
<td>SUMO fusion protein expression vector; Kan</td>
<td>Novagen</td>
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<td>Gene replacement vector, mob*sacB, Ap</td>
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<tr>
<td>pEX18Tc</td>
<td>Gene replacement vector, mob*sacB, Tc</td>
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<td>Source of Flp recombinase; Ap/Cb</td>
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<tr>
<td>p-bfmST120K/L164F/G179D/Y280H</td>
<td>p-bfmS derivative carrying T120K, L164F, G179D, and Y280H substitutions</td>
<td>This study</td>
</tr>
<tr>
<td>p-bfmST242R</td>
<td>p-bfmS derivative carrying arginine substitution mutant at the site threonine 242</td>
<td>This study</td>
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<tr>
<td>p-bfmST242R/H238A</td>
<td>p-bfmS derivative carrying T242R and H238A substitutions</td>
<td>This study</td>
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<tr>
<td>p-bfmSV278M</td>
<td>p-bfmS derivative carrying methionine substitution mutant at the site valine 278</td>
<td>This study</td>
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<tr>
<td>pET28a(+)-sumo-BfmSc</td>
<td>pET28a(+)-sumo derivative carrying BfmSc for the expression of an N-terminal 6His-SUMO tagged cytosolic segment of BfmS (residues 175-435)</td>
<td>This study</td>
</tr>
<tr>
<td>pET28a(+)-sumo-BfmSc&lt;sub&gt;DK2&lt;/sub&gt;</td>
<td>pET28a(+)-sumo-BfmSc derivative, with missense mutations (L181P and E376Q) in bfmS</td>
<td>This study</td>
</tr>
<tr>
<td>pET28a(+)-sumo-BfmSc&lt;sub&gt;R393H&lt;/sub&gt;</td>
<td>pET28a(+)-sumo-BfmSc derivative, with</td>
<td>This study</td>
</tr>
<tr>
<td>pET28a(+)-sumo-(BfmSc^{T242R})</td>
<td>R393H missense mutation in (bfmS)</td>
<td>This study</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td>pET28a(-)-sumo-(BfmSc) derivative, with T242R missense mutation in (bfmS)</td>
<td>pET28a derivative carrying (bfmR) ((pa4101))</td>
<td>(24)</td>
</tr>
<tr>
<td>pET28a-6His-Bfmr</td>
<td>pET28a derivative carrying (bfmR) ((pa4101)) which has alanine substitution mutant at the site of aspartate residue 55</td>
<td>(24)</td>
</tr>
<tr>
<td>pET28a-6His-Bfmr(^{D55A})</td>
<td>pET28a derivative carrying (GtrSc) for the expression of an N-terminal His-tagged cytosolic segment of GtrS (residues 269-465)</td>
<td>This study</td>
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<tr>
<td>pEX18Ap:accaptaUTD</td>
<td>pEX18Ap derivative, for replacing (acca)-(pta) loci with a tetracycline resistance cassette from plasmid mini-CTX-lacZ</td>
<td>(24)</td>
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<tr>
<td>pEX18Ap:bfmRSUGD</td>
<td>pEX18Ap derivative, for replacing (bfmRS) loci with a gentamicin resistance cassette from plasmid pPS858</td>
<td>(24)</td>
</tr>
<tr>
<td>pEX18Ap:bfmUGD</td>
<td>pEX18Ap derivative, for replacing (bfmR) with a gentamicin resistance cassette from plasmid pPS858</td>
<td>This study</td>
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<tr>
<td>pEX18Ap:bfmUGD</td>
<td>pEX18Ap derivative, for replacing (bfmS) gene with a gentamicin resistance cassette from plasmid pPS858</td>
<td>(24)</td>
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<tr>
<td>pEX18Ap:gltR-gtrSUGD</td>
<td>pEX18Ap derivative, for replacing (gltR)-(gtrS) loci with a gentamicin resistance cassette from plasmid pPS858</td>
<td>This study</td>
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<td>pEX18Ap:gtrSUGD</td>
<td>pEX18Ap derivative, for replacing (gtrS) with a gentamicin resistance cassette from plasmid pPS858</td>
<td>This study</td>
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<tr>
<td>pEX18Ap:pa3190UGD</td>
<td>pEX18Ap derivative, for replacing (gltB) with a gentamicin resistance cassette from plasmid pPS858</td>
<td>This study</td>
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<tr>
<td>pEX18Tc-For-<em>bfmS</em>&lt;sup&gt;DK2&lt;/sup&gt; &amp; pEX18Tc derivative, for replacing WT <em>bfmS</em> with <em>bfmS</em>&lt;sup&gt;DK2&lt;/sup&gt; (<em>bfmS</em>L&lt;sup&gt;181P/E376Q&lt;/sup&gt;) &amp; This study</td>
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<tr>
<td>pEX18Tc-For-<em>bfmS</em>&lt;sup&gt;DK2&lt;/sup&gt; H238A &amp; pEX18Tc derivative, for replacing WT <em>bfmS</em> with <em>bfmS</em>&lt;sup&gt;DK2&lt;/sup&gt; H238A (<em>bfmS</em>L&lt;sup&gt;181P/E376Q/H238A&lt;/sup&gt;) &amp; This study</td>
<td></td>
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</tr>
<tr>
<td>pEX18Tc-For-<em>bfmS</em>&lt;sup&gt;PAO1&lt;/sup&gt; &amp; pEX18Tc derivative, for replacing mutated <em>bfmS</em> allele with WT <em>bfmS</em> &amp; This study</td>
<td></td>
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<tr>
<td>pEX18Tc-For-<em>bfmS</em>&lt;sup&gt;R393H&lt;/sup&gt; &amp; pEX18Tc derivative, for replacing WT <em>bfmS</em> with <em>bfmS</em>&lt;sup&gt;R393H&lt;/sup&gt; &amp; This study</td>
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<tr>
<td>pGEX-6p-1-<em>BfmSc</em> &amp; pGEX-6p-1-6His derivative carrying <em>BfmSc</em> for the expression of an N-terminal 6His- and GST-tagged cytosolic segment of BfmS (residues 175-435) &amp; This study</td>
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<tr>
<td>pGEX-6p-1-<em>BfmSc</em>&lt;sup&gt;DK2&lt;/sup&gt; &amp; pGEX-6p-1-<em>BfmSc</em> derivative carrying both L181P and E376Q substitutions &amp; This study</td>
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<tr>
<td>pGEX-6p-1-<em>BfmSc</em>&lt;sup&gt;DK2&lt;/sup&gt; H238A&lt;sub&gt;Cter&lt;/sub&gt; &amp; pGEX-6p-1-<em>BfmSc</em>&lt;sup&gt;DK2&lt;/sup&gt; derivative carrying H238A substitution &amp; This study</td>
<td></td>
<td></td>
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<tr>
<td>p-<em>gltB</em> &amp; pAK1900 derivative carrying <em>gltB</em> on a ~1.3 kb HindIII/KpnI fragment in same orientation as <em>plac</em> &amp; This study</td>
<td></td>
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<tr>
<td>p-<em>gltR</em> &amp; pAK1900 derivative carrying <em>gltR</em> on a ~0.77 kb HindIII/KpnI fragment in same orientation as <em>plac</em> &amp; This study</td>
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<tr>
<td>p-<em>gltR-gtrS</em> &amp; pAK1900 derivative carrying <em>gltR-gtrS</em> on a ~2.3 kb HindIII/BamHI fragment in same orientation as <em>plac</em> &amp; This study</td>
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<tr>
<td>p-<em>gtrS</em> &amp; pAK1900 derivative carrying <em>gtrS</em> on a ~1.5 kb HindIII/BamHI fragment in same orientation as <em>plac</em> &amp; This study</td>
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<tr>
<td>p-<em>gtrS</em>&lt;sup&gt;H280A-HA&lt;/sup&gt; &amp; p-<em>gtrS-HA</em> carrying <em>gtrS</em> which has alanine substitution mutant at the site of histidine 280 &amp; This study</td>
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<tr>
<td>p-<em>gtrS-HA</em> &amp; pAK1900 derivative carrying a <em>gtrS-HA</em> &amp; This study</td>
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<td></td>
</tr>
<tr>
<td>Fusion Gene Fusion</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>-------------------</td>
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</tr>
<tr>
<td>pHK25-gtrS</td>
<td>gtrS cloned into pKT25</td>
<td>Euromedex</td>
</tr>
<tr>
<td>pHK25-gtrS- hfmSDK2</td>
<td>gtrS and hfmSDK2 cloned into pKT25</td>
<td>Euromedex</td>
</tr>
<tr>
<td>pRK415-bfmR-flag</td>
<td>pAK415 derivative carrying a bfmR-flag fusion gene for the expression of C-terminal Flag-tagged BfmR proteins</td>
<td>This study</td>
</tr>
</tbody>
</table>
| pRK415-bfmS       | pAK415 derivative carrying bfmS 
\((pa4102)\) on a ~1.4 kb HindIII/BamHI fragment in same orientation as plac | This study |
| pRK415-gtrS       | pAK415 derivative carrying gtrS 
\((pa3191)\) on a ~1.5 kb HindIII/BamHI fragment in same orientation as plac | This study |
| pUT18C-bfmR       | bfmR cloned into pUT18C | Euromedex |

**P. aeruginosa strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU15431</td>
<td>CF isolate, bfmSA42E/G347D</td>
<td>(35)</td>
</tr>
<tr>
<td>DK2</td>
<td>CF isolate, bfmSL181P/E376Q</td>
<td>(27)</td>
</tr>
<tr>
<td>MPAO1</td>
<td>Wild type, WT bfmS</td>
<td>(61)</td>
</tr>
<tr>
<td>pDO100 (pKD-rhlA)</td>
<td>rhlI mutant of PAO1 harboring plasmid pKD-rhlA</td>
<td>(57)</td>
</tr>
</tbody>
</table>
| AU15431-bfmSPAO1 | AU15431 derivative with a WT bfmS allele replaced the mutated bfmS 
\((bfmSA42E/G347D)\) | This study |
| bfmSDK2 | MPAO1 derivative with a mutated bfmS allele 
\((bfmSL181P/E376Q)\) replaced the WT bfmS | This study |
<p>| bfmSDK2 H238A | MPAO1-bfmSDK2 derivative carrying H238A mutation in the bfmSDK2 allele | This study |
| bfmSDK2ΔbfmR (MPAO1) | MPAO1-bfmSDK2 derivative with a gentamicin resistance cassette replaced the bfmR | This study |
| bfmSDK2ΔgtrS (MPAO1) | MPAO1-bfmSDK2 derivative with a gentamicin resistance cassette replaced | This study |</p>
<table>
<thead>
<tr>
<th>Mutant/Strain Description</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$bfmS^{R393H}$</td>
<td>MPAO1 derivative with a mutated $bfmS$ allele ($bfmS^{R393H}$) replaced the WT $bfmS$</td>
<td>This study</td>
</tr>
<tr>
<td>$bfmS^{R393H} \Delta gtrS$</td>
<td>$bfmS^{R393H}$ derivative with a gentamicin resistance cassette replaced the $gtrS$</td>
<td>This study</td>
</tr>
<tr>
<td>$DK2-bfmS^{^{\text{PAO1}}}$</td>
<td>DK2 derivative with a WT $bfmS$ allele replaced the mutated $bfmS$ ($bfmS^{L181P/E376Q}$)</td>
<td>This study</td>
</tr>
<tr>
<td>$\Delta bfmR$</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the $bfmR$ gene</td>
<td>This study</td>
</tr>
<tr>
<td>$\Delta bfmRS$</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the $bfmRS$ locus</td>
<td>(24)</td>
</tr>
<tr>
<td>$\Delta bfmRS$</td>
<td>DK2 derivative with a gentamicin resistance cassette replaced the $bfmRS$ locus, by using the pEX18Ap::$bfmRS$UGD plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>$\Delta bfmRS\Delta pta-acka$</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the $bfmRS$ locus, and a tetracycline resistance cassette replaced the $pta-acka$ locus</td>
<td>This study</td>
</tr>
<tr>
<td>$\Delta bfmRS\Delta gltR-gtrS$</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the $bfmRS$ locus, and a deletion of $gltR-gtrS$ locus</td>
<td>This study</td>
</tr>
<tr>
<td>$\Delta bfmS$</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the $bfmS$ gene</td>
<td>(24)</td>
</tr>
<tr>
<td>$\Delta bfmS$ (AU15431)</td>
<td>AU15431 derivative with a gentamicin resistance cassette replaced the $bfmS$, by using the pEX18Ap::$bfmS$UGD plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>$\Delta bfmS$ (DK2)</td>
<td>DK2 derivative with a gentamicin resistance cassette replaced the $bfmS$, by</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Strain</strong></td>
<td><strong>Description and Details</strong></td>
<td><strong>Study</strong></td>
</tr>
<tr>
<td>------------</td>
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<tr>
<td>ΔbfmS::bfmR-lacZ</td>
<td>ΔbfmS mutant carrying bfmR-lacZ reporter fusion at the CTX phage att site on <em>P. aeruginosa</em> chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>ΔbfmSΔgltB</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the bfmS, and a deletion of gltB</td>
<td>This study</td>
</tr>
<tr>
<td>ΔbfmSΔgltR-gtrS</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the bfmS, and a deletion of gltR-gtrS locus</td>
<td>This study</td>
</tr>
<tr>
<td>ΔbfmSΔgtrS</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the bfmS, and a deletion of gtrS</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgltB</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the gltB gene</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgltR-gtrS</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the gltR-gtrS locus</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgtrS</td>
<td>MPAO1 derivative with a gentamicin resistance cassette replaced the gtrS gene</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgtrS (AU15431)</td>
<td>AU15431 derivative with a gentamicin resistance cassette replaced the gtrS, by using the pEX18Ap::gtrSUGD plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgtrS (DK2)</td>
<td>DK2 derivative with a gentamicin resistance cassette replaced the gtrS, by using the pEX18Ap::gtrSUGD plasmid</td>
<td>This study</td>
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<tr>
<td><strong>E. coli strains</strong></td>
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<td></td>
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<tr>
<td>BL21</td>
<td>F<em>ompT hsdS</em>B (rB− mB−) gal dcm met (DE3)</td>
<td>Lab stock</td>
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<tr>
<td>DH5a</td>
<td>endA hsdR17 supE44 thi-1 recA1 gyrA relA1Δ(lacZYA-argF)U169 deoR (φ80d lacΔ(lacZ)M15)</td>
<td>Lab stock</td>
</tr>
<tr>
<td><em>E. coli</em> BTH101</td>
<td>Cya-BACTH expression strain; F− cya-99</td>
<td>Euromedex</td>
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<td>Details</td>
<td>Source</td>
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<tr>
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<tr>
<td>araD139 galE15 galK16 rpsL1 (Str⁺) hsdR2 mcrA1 mcrB1</td>
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<td></td>
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<tr>
<td>S17 λ-pir</td>
<td>recA thi pro hsdR M⁺ RP4-2-Tc::Mu Km::Tn7 λpir (Tp⁺ Str⁺)</td>
<td>Lab stock</td>
</tr>
<tr>
<td>S17 λ-pir (pFLP2)</td>
<td>S17 λ-pir containing pFLP2</td>
<td>This study</td>
</tr>
<tr>
<td>SM10-λ. pir (pBT20)</td>
<td>E. coli SM10-λ. pir containing pBT20</td>
<td>(47)</td>
</tr>
</tbody>
</table>

ᵃAp⁺, ampicillin resistance; Cb⁺, carbenicillin resistance; Kan⁺, kanamycin resistance; Tc⁺, tetracycline resistance; Tp⁺, trimethoprim resistance; Str⁺, Streptomycin resistance; Gm⁺, gentamycin resistance.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>pro-bfmR-F</td>
<td>5'-TTTCTCGAGCAACCTGGGACCGTGT-3'</td>
<td>Construction the plasmid of mini-bfmR-lacZ</td>
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<tr>
<td>pro-bfmR-R</td>
<td>5'-TTTGGATCCAGGATGTCAGCATGCTC-3'</td>
<td>Construction the plasmid of mini-bfmR-lacZ</td>
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<tr>
<td>AD2</td>
<td>5’-CANGCTWSGTNTSCAA-3’</td>
<td>For localization of the transposon</td>
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<tr>
<td>Gm447</td>
<td>5'-GTGCAAGCAGATTACGGTGACGAT-3'</td>
<td>For localization of the transposon</td>
</tr>
<tr>
<td>Gm464</td>
<td>5'-TGACGATCCCCAGTGCTCTC-3’</td>
<td>For localization of the transposon</td>
</tr>
<tr>
<td>Gm487</td>
<td>5'-ATACAAAGTTGGGACACAG-3’</td>
<td>For localization of the transposon</td>
</tr>
<tr>
<td>gtrS-comp-F</td>
<td>5’-CCCCAGCTTCAGTGGAACCCTGCTG-3’</td>
<td>Construction of plasmids p-gtrS, pRK415-gtrS, and p-gtrS-HA</td>
</tr>
<tr>
<td>gtrS-comp-R</td>
<td>5’-TTTTGGATCCGCCAGCTCACATCACTCA-3’</td>
<td>Construction of plasmids p-gtrS, pRK415-gtrS, and p-gtrS-HA</td>
</tr>
<tr>
<td>gltR-comp-F</td>
<td>5’-CCCCAGCTTTCTCAGTGACGCTCGAGCA-3’</td>
<td>Construction of p-gltR plasmid</td>
</tr>
<tr>
<td>gltR-comp-R</td>
<td>5’-TTTGGTACCCGCTGCTGCTGCA-3’</td>
<td>Construction of p-gltR plasmid</td>
</tr>
<tr>
<td>pa3190-comp-F</td>
<td>5’-GGGAAGCTTTGCAGAATTGCTCCGAA-3’</td>
<td>Construction of plasmid p-gltB</td>
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<td>pa3190-comp-R</td>
<td>5’-TTTGGATCCACTCCGCGAGCATTCA-3’</td>
<td>Construction of plasmid p-gltB</td>
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<tr>
<td>bfmS-comp-F</td>
<td>5’-TGGAAGCTTCGACTTGGTCG AGGCCCATCC-3’</td>
<td>Construction of plasmids pRK415-bfmS</td>
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<tr>
<td>bfmS-comp-R</td>
<td>5’-TTTGGATCCCTCTGTACGG GCAGGATCC-3’</td>
<td>Construction of plasmids pRK415-bfmS</td>
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<tr>
<td>D-gtrS-up-F</td>
<td>5’-CCCCCAATTCGTCCCGCGCTCAGTTGACGCA-3’</td>
<td>Deletion of gtrS</td>
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<tr>
<td>D-gtrS-up-R</td>
<td>5’-CCGCGGATCCCTAGTGACGCGGATC-3’</td>
<td>Deletion of gtrS</td>
</tr>
<tr>
<td>D-gtrS-down-F</td>
<td>5’-CGCGGATCCCTAGTGACGCGGATC-3’</td>
<td>Deletion of gtrS</td>
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<td>D-gtrS-down-R</td>
<td>5’-CCCCCAATTCGTCCCGCGCTCAGTTGACGCA-3’</td>
<td>Deletion of gtrS</td>
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<tr>
<td>D-gltR-up-F</td>
<td>5’-GGTGAAATTCGTGGGAGGACGCGAGTT-3’</td>
<td>For deletion of gltR-gtrS</td>
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<tr>
<td>D-gltR-up-R</td>
<td>5’-TTTGGATCCCTACGAGGCTCTTAT-3’</td>
<td>For deletion of gltR-gtrS</td>
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<td>D-pa3190-up-F</td>
<td>5’-GGTGAAATTCGTCCCGCGCTCAGTTGACGCA-3’</td>
<td>Deletion of gltB</td>
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<td>D-pa3190-up-R</td>
<td>5’-TTTGGATCCCTACGAGGCTCTTAT-3’</td>
<td>Deletion of gltB</td>
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<tr>
<td>D-pa3190-down-F</td>
<td>5’-GGTGAAATTCGTCCCGCGCTCAGTTGACGCA-3’</td>
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<td>D-pa3190-down-R</td>
<td>5’-TTTGGATCCCTACGAGGCTCTTAT-3’</td>
<td>Deletion of gltB</td>
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</table>
D-bfmR-up-F  5'-TTTGAATTCACCTGGGCAC CGTGTTCCAT-3'  Deletion of bfmR
D-bfmR-up-R  5'-TTTGGATCCGTCACCGTGTTGCTCGA-3'  Deletion of bfmR
D-bfmR-down-R  5'-TTGAAGCTTTTGTCCACGACTTGATCG-3'  Deletion of bfmR
D-bfmR-down-F  5'-TTTGGATCCGACCGGAGCTACATACAGC-3'  Deletion of bfmR
pAK1900-mini-F  5'-TTTTCGAGTATCAGGAGGCCCTT-3'  For generating mini-bfmS, -gltR-gtrS, -gltR, and -gtrS
pAK1900-mini-R  5'-TGGTCTGAGACGCGCAATGATCC-3'  For generating mini-bfmS, -gltR-gtrS, -gltR, and -gtrS
bfmS-allelic-DK2-F  5'-TTTGAATTCCTGGATCTTCGACGATCG-3'  Generating bfmS allelic exchange mutants
bfmS-allelic-DK2-R  5'-TTTAAAGCTCGACTGATCGACGATCGAACC-3'  Generating bfmS allelic exchange mutants
gtrS-KD-F  5'-TTTGGATCCATGGCAGAGCCGTCC-3'  Construction of pET28a-GtrSc plasmid
gtrS-KD-R  5'-TTTTAGCTCAACGGATGTGTGAGGCAGCAG-3'  Construction of pET28a-GtrSc plasmid
gtrS-HA-R  5'-TTTGGATCCAGCCCGCATCGGTC-3'  Construction of p-gtrS-HA plasmid
bfmR-comp-F  5'-TTTAAAGCTCAACGGGAGCAGCAGCAGCAGA-3'  Construction of p-bfmR-flag and pRK415-bfmR-flag
bfmR-flag-R  5'-TTTGGATCCCTGATCGTCGTATCGCAGG-3'  Construction of p-bfmR-flag and pRK415-bfmR-flag
bfmS-KD-F  5'-TTTGGATTCGCCGACCGCATCGGTC-3'  Construction of pGEX-6p-1-BfmSc
bfmS-KD-R  5'-CACACTGACGGTACGCTCGAGCTGATG-3'  Construction of pGEX-6p-1-BfmSc
bfmS-KD-2F  5'-TTTGGATTCGCCGACCGCATCGGTC-3'  Construction of pET28a(+)sumo-BfmSc
bfmS-KD-2R  5'-TGGAAAGCTCAACGGGAGCAGCAGCAGCAG-3'  Construction of pET28a(+)sumo-BfmSc
BfmR(D55A)-F  5'-GTCGCCGCCATCGATGGGGAGACGAT
             ACCTGACCAC-3'  Generation of bfmR
BfmR(D55A)-R  5'-GTCGCCGCCATCGATGGGGAGACGAT
             ACCTGACCAC-3'  Generation of bfmR
GtrS (H280A)-F  5'-TGTTCAAGCGCCATCTCCGCACGACGGAGCAG-3'  Generation of gtrS
GtrS (H280A)-R
D-pa3190-up-F
D-pa3190-up-R
D-pa3190-down-F
D-pa3190-down-R
pro-pa4103-F
pro-pa4103-R
pro-pa3190-F
pro-pa3190-R
F
R
2R
T242R-F
T242R-R
L184P-F
L184P-R
Q92E-F
Q92E-R
D295N-F
D295N-R
F31L-F
F31L-R
R393H-F
R393H-R
H238A-F

3’
5’-
TGCGCAGGTCGCGGAGATGGCGCTGAACA-3’
5’-GGTGAACTCCCCCCACCATGCACT-3’
5’-TTTGGTACCACGATCACTCAT-3’
5’-GGTTGGAACCTCTCCTTCAACCGACC-3’
5’-TTAAGCTTGAGCCTGACCCACCTT-3’
5’-TTTCTCGAGACAAGGACAAGCTGTGGAAC-3’
5’-TTTGGATCCCTTCAACCATGCATG-3’
5’-TTTGGTACCGCGACGGATCGCATTCAT-3’
5’-TTTGGATCCCGCAACTTCTAAGCACC-3’
5’-TTTCTCGAGACAGGGACAAGCTGTGGAAC-3’
5’-TTTCTCGAGACAGGGACAAGCTGTGGAAC-3’
5’-TTTGAATTTCCCTTCAACACCATGCACT-3’
5’-TTTGGTACCGCGACGGATCGCATTCAT-3’
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5’-TTTGTACCGCGCAACTTCTTCAACCGACC-3’
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Generation of gtrS$^{H280A}$
Deletion of gltB
Deletion of gltB
Deletion of gltB
Deletion of gltB
Construction of mini-pa4103-lux
Construction of mini-pa4103-lux
Construction of pMS402-gltB-lux
Construction of pMS402-gltB-lux
For analysis of bfmRS transcripts
For analysis of bfmRS transcripts
For analysis of bfmRS transcripts
Generation of bfmS$^{T242R}$
Generation of bfmS$^{T242R}$
Generation of bfmS$^{L184P}$
Generation of bfmS$^{L184P}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{D295N}$
Generation of bfmS$^{D295N}$
Generation of bfmS$^{F31L}$
Generation of bfmS$^{F31L}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{L184P}$
Generation of bfmS$^{L184P}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{L184P}$
Generation of bfmS$^{L184P}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{R393H}$
Generation of bfmS$^{H238A}$
Generation of bfmS$^{H238A}$

H238A- R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS H238A
BfmS(I178N)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS I178N
BfmS(I178N)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS I178N
BfmS(L181Q)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L181Q
BfmS(L181Q)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L181Q
BfmS(V287M)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS V287M
BfmS(V287M)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS V287M
BfmS(A21G)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS A21G
BfmS(A21G)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS A21G
BfmS(G179D)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS G179D
BfmS(G179D)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS G179D
BfmS(L164F)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L164F
BfmS(L164F)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L164F
BfmS(A42E)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS A42E
BfmS(A42E)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS A42E
BfmS(G347D)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS G347D
BfmS(G347D)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS G347D
BfmS(A4T)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS A4T
BfmS(A4T)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS A4T
BfmS(L168-L)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L168-L
BfmS(L168-L)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L168-L
BfmS(L168-D)-F 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L168-d
BfmS(L168-D)-R 5'-TGCGACCGCCGCTCGGCGGGCTGAG-3' Generation of bfmS L168-d
BfmS(V380A)-F 5'-CTCGACGAGGCGCTCAAGCCCTTC-3’ Generation of bfmS\textsuperscript{V380A}
BfmS(V380A)-R 5’-GGCTTGAGCCGCTCAGTTTCC-3’ Generation of bfmS\textsuperscript{V380A}
BfmS(A281T)-F 5’-AAGGCCTGGCTTATACCCGACATG-3’ Generation of p-bfmS\textsuperscript{T120K L168F A281T}
BfmS(A281T)-R 5’-CCGTGCATGCTGCGGACTGCAC-3’ Generation of p-bfmS\textsuperscript{T120K L168F A281T}
T25-gtrS-F 5’-TTTGGATCCATGAGCGAACGGCGCT-3’ For bacterial two-hybrid assays
T25-gtrS-R 5’-TTTGGTACCTCACTCCAGCCCCAGCT-3’ For bacterial two-hybrid assays
T18C-bfmR-F 5’-TTTGGATCCATGAGCGAACGGCGCT-3’ For bacterial two-hybrid assays
T18C-bfmR-R 5’-GGTGAATTCTCATGGATGGGCCTCGACCA-3’ For bacterial two-hybrid assays
bfmS-comp-2F 5’-TTTGGTACCCGACTGGTCGAGGCCCAT-3’ For bacterial two-hybrid assays
bfmS-comp-2R 5’-GGTGAATTCTCAGGGGTCCAGCTCGATGC-3’ For bacterial two-hybrid assays

Table S3. bfmS variants in P. aeruginosa CF isolates

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<th>Isolate or Strain</th>
<th>Accession\textsuperscript{a}</th>
<th>Amino acid change\textsuperscript{b}</th>
<th>Geographic location</th>
<th>Source or Reference\textsuperscript{c}</th>
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<td><strong>Prevalent or epidemic clones</strong></td>
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<tr>
<td><strong>DK2 lineage (50 isolates)</strong></td>
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<td>39 DK2 isolates</td>
<td>NA</td>
<td>L181P</td>
<td>Denmark: Copenhagen</td>
<td>(9, 12, 27)</td>
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<td>11 DK2 isolates</td>
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**DK1 lineage (4 isolates)**

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<td>WP_003159306</td>
<td>T120K L164F</td>
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<td>Direct submission</td>
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**M3L7 sub-lineage (26 isolates)**

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<td>AUS952</td>
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**Liverpool epidemic strain (LES) and LES-like strain (48 isolates)**

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**Australia epidemic strain-1 (AUS-1) (1 isolate)**

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**Manchester epidemic strain (MES) (1 isolate)**

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**ST406 clone in the Netherlands (1 isolate)**

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33 out of 63 CF isolates from a single CF care center in USA in a study by Spilker et al.
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14 out of 24 isolates from Ontario, Canada in a study by Dettman et al.

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20 out of 35 CF isolates in a study by Kos VN et al.
AZPAE12152  WP_034046584  A281T  USA: New York  (68)
AZPAE12138  WP_034008880  A4T R393H  USA: New York  (68)
AZPAE12409  WP_004365749  D295N  USA: Cleveland  (68)
AZPAE12422  WP_023123707  F31L  USA: Cleveland  (68)
AZPAE12149  WP_033876756  F31L D295N  USA: New York  (68)
AZPAE12413  WP_034043645  L163-d H272Y D340N  USA: Cleveland  (68)
AZPAE12414  WP_034043645  L163-d H272Y D340N  USA: Cleveland  (68)
AZPAE12415  WP_034043645  L163-d H272Y D340N  USA: Cleveland  (68)
AZPAE12417  WP_034041871  L168-L  USA: Cleveland  (68)
AZPAE12156  WP_034054432  L181Q  USA: New York  (68)
AZPAE12416  WP_033970808  P383S  USA: Cleveland  (68)
AZPAE12418  WP_033970808  P383S  USA: Cleveland  (68)
AZPAE12423  WP_033970808  P383S  USA: Cleveland  (68)
AZPAE12154  WP_034056531  P6S G112D L164F  USA: New York  (68)
AZPAE12145  WP_034052127  Q40E V149A  USA: New York  (68)
AZPAE12421  WP_043108044  R78Q T120K L164F  USA: Cleveland  (68)
AZPAE12151  WP_03403409  T120K K262R S302R S310N  USA: New York  (68)
AZPAE12152  WP_034065491  R355S  USA: New York  (68)
AZPAE12142  WP_033987210  T242R  USA: New York  (68)
AZPAE12153  WP_034032702  V149A V161A D433A  USA: New York  (68)
AZPAE12140  WP_034065491  Y27H D340N  USA: New York  (68)

24 out of 40 isolates from Trentino Regional Support Cystic Fibrosis Centre (Italy) in a study by Bianconi et al.

TNCF_3  WP_070329877  L184P  Italy  (69)
TNCF_101  WP_070329877  L184P  Italy  (69)
TNCF_105  WP_070329877  L184P  Italy  (69)
| TNCF_106 | WP_070329877 | L184P | Italy | (69) |
| TNCF_109 | WP_070329877 | L184P | Italy | (69) |
| TNCF_130 | WP_070329877 | L184P | Italy | (69) |
| TNCF_133 | WP_070329877 | L184P | Italy | (69) |
| TNCF_14  | WP_070329877 | L184P | Italy | (69) |
| TNCF_151 | WP_070329877 | L184P | Italy | (69) |
| TNCF_154 | WP_070329877 | L184P | Italy | (69) |
| TNCF_155 | WP_070329877 | L184P | Italy | (69) |
| TNCF_155_1| WP_070329877 | L184P | Italy | (69) |
| TNCF_165 | WP_070329877 | L184P | Italy | (69) |
| TNCF_167 | WP_070329877 | L184P | Italy | (69) |
| TNCF_167_1| WP_070329877 | L184P | Italy | (69) |
| TNCF_174 | WP_070329877 | L184P | Italy | (69) |
| TNCF_175 | WP_070329877 | L184P | Italy | (69) |
| TNCF_6   | WP_070329877 | L184P | Italy | (69) |
| TNCF_68  | WP_070329877 | L184P | Italy | (69) |
| TNCF_69  | WP_070329877 | L184P | Italy | (69) |

| TNCF_176 | WP_070332227 | L184P G399D | Italy | (69) |
| TNCF_12  | WP_070341526 | Q92E L184P | Italy | (69) |
| TNCF_10M | WP_070336931 | E77D P150L | Italy | (69) |
| TNCF_76  | WP_070335766 | A21G L184P | Italy | (69) |

141 out of 474 CF isolates from Denmark in a study by Marvig et al.

36 isolates (76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 123, 14)
Denmark

WP_033957058 A122V L164F Denmark (14)

21 isolates (95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 193, 194, 195, LRJ18, LRJ19, LRJ20)

WP_003159306 T120K L164F Denmark (14)

15 isolates (196, 197, 198, 199, 206, 207, 208, 209, 210, 211, 212, 213, 215, 293, 340)

WP_034001102 K262R S310N R355S Denmark (14)

11 isolates (418, 422, 423.1, 426, 428, 430, 431, 434, 437, 437.1, 439)

WP_060961570 T120K L164F A281T Denmark (14)
| 10 isolates (134, 135, 148, 423, 427, 436, 138.1, 439.1, LRJ16, LRJ17) | WP_073649900 | Q113R K186E V294L A422P | Denmark | (14) |
| 9 isolates (419, 420, 421, 425, 429, 432, 433, 435, 438) | WP_073670735 | T120K L164F L233F | Denmark | (14) |
| 8 isolates (201, 203, 204, 205, 214, 223, 201.1, 203.1) | WP_004365749 | D295N | Denmark | (14) |
| 1 isolate (245) | WP_073653557 | Deletion of H210, A211, A212, and A213 | Denmark | (14) |
| 1 isolate (349) | WP_034054976 | A422T | Denmark | (14) |
| 1 isolate (427.1) | WP_074227112 | T120K L164F L233F G425E | Denmark | (14) |

**Other strains**

| SCH_ABX18 | WP_034046584 | A281T | USA: Seattle | PRJNA369567 |
| CIGI | WP_003119101 | A4T | NA | (70) |
| SCH_ABX01 | WP_003119101 | A4T | USA: Seattle | PRJNA369567 |
| SCH_ABX02 | WP_003119101 | A4T | USA: Seattle | PRJNA369567 |
| CF5 | WP_004365749 | D295N | USA | Direct submission |
| CI 1913C | WP_023518026 | D340N | Canada: Vancouver, BC | Direct submission |
| WH-SGI-V-07282 | WP_034001102 | K262R S310N R355S | Portugal | (71) |
| PASS4 | WP_019681168 | L164F | Australia: Sydney | (72) |
| C7447m | WP_019681168 | L164F | Canada | (73) |
| CF614 | WP_023081578 | P6S L164F | USA | Direct submission |
| RP73 | WP_016562269 | R393H | Germany: Hannover | (74) |
The BfmS variants were identified by using BLASTP analysis against the NCBI protein database and literature search.
Data file 1. Microarray data analysis of DK2

Data file 2. RNA-seq analysis