Inhibitors of two-component signal transduction systems: Inhibition of alginate gene activation in \textit{Pseudomonas aeruginosa}

(bacterial protein kinase inhibitor/inhibition of DNA-protein interaction)

SIDDHARTHA ROYCHOUDHURIY*, NICOLETTE A. ZIELINSKI*, ALEXANDER J. NINFA†, NORRIS E. ALLEN‡, LOUIS N. JUNGHEN‡, THALIA I. NICAS§, and A. M. CHAKRABARTY$$

*Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL 60612; †Department of Biochemistry, Wayne State University School of Medicine, Detroit, MI 48201; and §Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46258

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ABSTRACT \textit{Pseudomonas aeruginosa} strains infecting cystic fibrosis patients often produce copious amounts of the exopolysaccharide alginate. Expression of alginate genes in \textit{P. aeruginosa} is regulated by several proteins including members of the two-component bacterial signal transduction systems. Two of these regulatory proteins are AlgR1, the DNA-binding response regulator that transcriptionally activates alginate gene expression, and AlgR2, the kinase that modifies AlgR1 via phosphorylation to enhance its activity. In this paper, we report the identification of compounds that inhibit alginate gene expression by inhibiting (i) the phosphorylation/dephosphorylation of AlgR2 and (ii) the DNA-binding activity of AlgR1. Compounds with these activities may have potential as components of therapy for eliminating \textit{P. aeruginosa} infection from the cystic fibrosis lung. In addition, we describe the effect of these compounds on the autophosphorylation activity of other known two-component kinases and show the ability of one compound to significantly inhibit the kinase activities of CheA, NRpA, and KinA.

Cystic fibrosis (CF) patients suffer from a major problem of pulmonary infection by mucoid strains of \textit{Pseudomonas aeruginosa} (1). These infections are resistant to antibacterial therapy and persist despite the response of the host immune system. In the CF lung, mucoid \textit{P. aeruginosa} strains synthesize an exopolysaccharide coat, which encapsulates the bacteria and is believed to play a critical role in the pathogenesis of the infection. The exopolysaccharide is alginate, a partially \textit{O}-acylated linear copolymer of \textit{D}-mannurionate and \textit{L}-gulurionate, which are linked via \textit{β}-1-4 glycosidic bonds. The expression of alginate biosynthetic genes in \textit{P. aeruginosa} is primarily associated with the CF-lung environment. Several alginate biosynthetic genes are clustered around the 34-min region of the \textit{P. aeruginosa} chromosome with the \textit{algD} gene at the upstream end. The \textit{algD} promoter is activated by environmental conditions such as high osmolality, nitrogen or phosphate starvation, and ethanol-induced membrane perturbation (2–4). Some of these environmental conditions are similar to those encountered in the CF lung. Several proteins are known to regulate transcription from the \textit{algD} promoter (5). One of these proteins is AlgR1, which belongs to the response regulator family of the bacterial two-component signal transduction proteins. AlgR1 binds DNA, far upstream of the \textit{algD} and \textit{algC} (an alginate biosynthetic gene located outside the 34-min cluster) promoters and activates transcription (6–8). AlgR2 is the kinase that undergoes autophosphorylation in the presence of ATP or GTP and transfers the acquired phosphate to AlgR1 (9, 10). A functional AlgR2 is important for the AlgR1-mediated transcripational activation of the \textit{algD} promoter (11). Thus, phosphorylation of AlgR1 is necessary for its functional activity.

Inhibition of transcriptional activation of alginate-encoding genes by nontoxic inhibitors may provide a way to prevent \textit{P. aeruginosa} from synthesizing alginate in the CF lung, thereby leaving the bacterium susceptible to antibiotic therapy and the immune responses of the host. With this strategy in mind, we screened a collection of synthetic and natural compounds for the ability to inhibit the transcriptional activation of the \textit{algD} promoter. Out of about 25,000 synthetic compounds screened, 15 were identified as inhibitors of \textit{algD} transcription in vivo in whole cells. The majority of these compounds were also found to inhibit alginate synthesis to varying degrees. We therefore chose to further investigate the possible mechanism involved in the inhibition process. In particular, the effect of two types of inhibitors (see Fig. 1) on the activities of AlgR1 and AlgR2 were studied. The inhibitory effect of these compounds on the known kinases of other two-component systems was also examined. This may be especially relevant since kinase-regulated expression of virulence is known in other human infections (12) and in bacterially induced plant tumor formation (13), as well as in important bacterial environmental responses such as chemotaxis (14), nitrogen assimilation (15), and sporulation (16).

MATERIALS AND METHODS

Assays for the \textit{algD} Promoter Activity. A previously constructed transcriptional fusion vector pV2DX (17) was used for studying the transcriptional activity of the \textit{algD} promoter. The reporter gene \textit{xyIE}, which encodes catechol 2,3-dioxygenase, was cloned into the open reading frame of \textit{algD} whose upstream DNA elements necessary for the \textit{AlgR1/AlgR2-mediated transcriptional activation of the algD} promoter were included in the insert. XyIE expression was measured spectrophotometrically at 405 nm as the rate of generation of \textit{α}-hydroxyxymuconic \textit{ε}-semialdehyde from catechol. Alginate-producing \textit{P. aeruginosa} strain 8821 (18), a mucoid strain isolated from the CF lung harboring pV2DX, was grown in the presence and absence of inhibitors. Catechol 2,3-dioxygenase assays were performed after 24 h of growth in tryptone/yeast extract broth supplemented with glucose to 0.3%. \textit{P. aeruginosa} cell growth was unaffected by the inhibitors described at concentrations up to 50 \textmu M.

Isolation of the AlgR1–AlgR2 Protein Complex and Purification of AlgR2. The 80-kDa AlgR1–AlgR2 protein complex was isolated according to a published procedure (9, 10). In brief, the cell-free extract from \textit{P. aeruginosa} strain 8822 (a

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Abbreviation: CF, cystic fibrosis.

*To whom reprint requests should be addressed: Department of Microbiology and Immunology, University of Illinois College of Medicine, 835 South Wolcott Avenue, Chicago, IL 60612.
spontaneous nonmucoid derivative of the mucoid strain 8821) was heated at 57.5°C at pH 5.0 for 30 sec, and the resulting precipitate was removed by centrifugation. The supernatant was subjected to gel-filtration chromatography using a Superose 12 FPLC column. Cell-free extract from *P. aeruginosa* strain 8822 was used as the source of AlgR2, which was also purified according to established procedures (10). The crude extract was heated at 57.5°C for 4 min at pH 5.0. After removing the precipitate by centrifugation, AlgR2 was purified using HPLC hydrophobic interaction, FPLC-anionic interaction, and FPLC gel-filtration chromatography.

**Inhibition of Other Two-Component Kinases.** CheA, NR1, and KinA were the other soluble two-component kinases used to study the effect of the inhibitors. Purified preparations of CheA and KinA were kindly provided by P. Matsuura (University of Illinois at Chicago) and J. Hoch (Scripp's Clinic, La Jolla, CA). Phosphorylation of NR1 was assayed at 37°C for 1 min using a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, and 0.5 mM EDTA. CheA was phosphorylated at room temperature for 1 min using a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 5 mM MgCl₂. KinA was phosphorylated at room temperature for 5 min in the presence of 50 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 0.125 mM EDTA, 5% (vol/vol) glycerol, and 2% (wt/vol) gelatin. In each case, the phosphorylation reaction was initiated by the addition of 16 nM [γ-³²P]ATP or 1 mM unlabeled ATP supplemented with [γ-³²P]ATP. The reaction was terminated by the addition of SDS loading buffer. The effect of the inhibitors was also studied with VirA, a transmembrane protein kinase involved in *Agrobacterium tumefaciens* virulence (13). Purified preparations of VirA were kindly supplied by S. Jin and E. W. Nester (University of Washington, Seattle).

**RESULTS**

**Inhibition of algD Promoter Activation.** Out of about 25,000 compounds screened for inhibition of algD promoter activation, 15 compounds appeared to be effective. Out of the 15 compounds, we tested 4 that exhibited significant inhibition. The inhibition of transcription from the algD promoter by inhibitors A and B (Fig. 1) was determined in *P. aeruginosa* strain 8821 (a mucoid CF isolate). The algD promoter activity was measured by using *xyle* as the reporter gene (17). The concentrations of the inhibitors required to inhibit *xyle* expression by 50% were 0.2 and 0.4 μg/ml for inhibitors A and B, respectively. Over 95% inhibition of algD promoter activity was observed at concentrations of 2.0 and 2.5 μg/ml of inhibitors A and B, respectively.

**Inhibition of AlgR1-AlgR2 Phosphorylation.** We previously reported the isolation of an 80-kDa protein complex containing AlgR1 and AlgR2, in which AlgR1 can be phosphorylated by AlgR2 (9, 10). During this study, we attempted to phosphorylate AlgR1 in the 80-kDa complex when an inhibitor was present at a concentration of 50 μg/ml. The results, presented in Fig. 2, indicate that inhibitors A and A' inhibit the phosphorylation of AlgR1, while inhibitor B does not have such a pronounced effect.

**Inhibition of AlgR2 Kinase Activity.** Since phosphorylation of AlgR2 is a prerequisite for that of AlgR1, we investigated the effects of the inhibitors on the autophosphorylation of AlgR2. AlgR2 was phosphorylated in the presence of [γ-³²P]ATP at various concentrations of the inhibitors. The results show (Fig. 3) that inhibitors A and A' have a significant effect on the autophosphorylation of AlgR2 above a threshold concentration of ~0.5 μg/ml. Inhibitor B, on the other hand, has no significant effect up to a concentration of 50 μg/ml under the experimental conditions used. A time course experiment indicated that in the presence of inhibitor

![Inhibitor A](image)

![Inhibitor A'](image)

![Inhibitor B](image)

![Inhibitor B'](image)

**Fig. 1.** Chemical structures of the inhibitors A and B along with their analogs, A' and B', respectively. A (50 μg/ml), but not of B, the initial rate of phosphorylation was significantly reduced (data not shown).

To determine the mode of inhibition of AlgR2 phosphorylation, the effect of inhibitor A on the initial rate of net phosphorylation was assayed at different ATP and GTP concentrations. Fig. 4 shows the ratio of AlgR2-phosphate (AlgR2-P) in the presence of the inhibitor to that without the inhibitor at various concentrations of ATP or GTP. Results of this experiment indicate that as the ATP or GTP concentration is increased, the inhibitor appears to undergo a transition from being an inhibitor to an activator of net phosphorylation. It should be noted that AlgR2 was previously found capable of spontaneously losing its phosphate due to an autophosphatase activity (10). The level of phosphorylation of AlgR2 is in effect determined by both the kinase and the phosphatase activities. Therefore, a possible explanation of the observation made in Fig. 4 is that, at a higher concen-

![AlgR1](image)

![AlgR2](image)

**Fig. 2.** Inhibition of AlgR1 phosphorylation in the 80-kDa AlgR1-AlgR2 complex. Details of the isolation of this complex and its phosphorylation have been described (9, 10). The complex was incubated with 16 nM [γ-³²P]ATP for 10 min at room temperature with and without inhibitors at 50 μg/ml. The samples were subsequently analyzed by SDS/polyacrylamide gel electrophoresis. The autoradiograph of the electrophoresed gel is shown. Phosphorylation of the samples was carried out in the presence of inhibitor A (lane 1), inhibitor A' (lane 2), and inhibitor B (lane 3). Lane 4 represents the control experiment without inhibitors containing 0.5% (vol/vol) dimethyl sulfoxide in which the inhibitors were dissolved.
Fig. 3. Inhibition of the autophosphorylation activity of AlgR2 at different inhibitor concentrations. In all cases, phosphorylation of AlgR2 was carried out by incubation with 16 nM [$\gamma$-32P]ATP in a buffer containing 50 mM Tris-HCl (pH 8.0) and 200 mM NaCl at room temperature for 10 sec. The level of AlgR2 phosphorylation was measured by 32P incorporation and is shown relative to a control corresponding to a radioactivity of 100%.

Fig. 4. Effect of inhibitor A on the net phosphorylation of AlgR2 at different concentrations of the phosphate donor ATP or GTP. The initial level of net phosphorylation was quantitated from the level of 32P incorporated within the first 10 sec of phosphorylation/desphosphorylation reaction in the presence of [$\gamma$-32P]ATP or [$\gamma$-32P]GTP. The y axis represents the ratio of AlgR2-P formed in the presence of inhibitor A at 50 $\mu$g/ml [AlgR2-P (A)] to that formed in the absence of the inhibitor [AlgR2-P (control)]. Thus when this ratio is <1, the inhibitor has a negative effect on the net phosphorylation of AlgR2, and the inhibitor has a positive effect when this ratio is >1.

Fig. 5. Inhibition of ATP or GTP-induced dephosphorylation of AlgR2. AlgR2 was first phosphorylated in the presence of 16 nM [$\gamma$-32P]ATP, which was subsequently removed by using Bio-Spin 30 size-exclusion chromatography columns. The phosphorylated protein sample was then incubated at room temperature [50 mM Tris-HCl (pH 8.0) and 200 mM NaCl] in the presence of unlabeled ATP or GTP (0.1 or 1 $\mu$M) for 1 min after which the reaction was terminated by the addition of 4 x SDS loading buffer. The samples were analyzed by SDS/PAGE and autoradiography. The level of AlgR2-P was measured by scanning the gel and is reported as percentage of the 32P-labeled AlgR2-P retained in the control experiment (carried out in the absence of ATP, GTP, or the inhibitor). Columns with the "-" sign represent samples without inhibitor A and those with the "+" sign represent samples in which the dephosphorylation reaction was carried out in the presence of inhibitor A at 50 $\mu$g/ml.

Inhibition of AlgR2 Phosphatase Activity. The effect of inhibitor A on dephosphorylation of AlgR2 was ascertained by adding different concentrations of unlabeled ATP or GTP to 32P-labeled AlgR2-P. In this case AlgR2 was first phosphorylated by using [$\gamma$-32P]ATP as the phosphate donor. After the phosphorylation reaction, free [$\gamma$-32P]ATP was removed by using Bio-Spin 30 chromatography columns, and the radiolabeled protein was incubated with 0.1 or 1 $\mu$M unlabeled ATP or GTP to measure the loss of radiolabel due to dephosphorylation of AlgR2-P. As shown in Fig. 5, the results of this experiment indicate that the ATP or GTP-induced dephosphorylation of AlgR2-P is significantly inhibited by inhibitor A, resulting in a much higher level of phosphate retention by AlgR2.

The level of AlgR2-P has been shown to decline after 10 sec of incubation with ATP at a 5 mM MgCl2 concentration (10). However, no such loss of phosphate was observed when the MgCl2 concentration was reduced to negligible levels. Loss of phosphate from AlgR2-P is therefore highly dependent on the MgCl2 concentration. Thus, the phosphatase inhibition experiment was repeated by incubating AlgR2-P with 5 mM MgCl2. After a 1-min incubation with MgCl2, the level of AlgR2-P retained a 2.33-fold higher level of phosphate relative to the control. These results clearly indicate that inhibitor A is not only an inhibitor of AlgR2-autophosphorylation (kinase) activity but also an inhibitor of its autophosphatase activity. It is likely that inhibitor A interacts with AlgR2 in such a way as to inhibit all its activities.

Inhibition of the DNA-Binding Activity of AlgR1. Binding of DNA by AlgR1 at the two specific sites centered at $\pm$ 458 and $\pm$ 382 (considering the position of the transcriptional start site as +1) far upstream of the algD promoter is one of the crucial steps involved in the AlgR1/AlgR2-mediated transcriptional activation process (6, 7). Since the inhibitors were screened on the basis of their ability to inhibit algD promoter activation, it was of interest to study the effect, if any, of the inhibitors on the binding of AlgR1 to its specific sites in the algD upstream DNA. The effect of these inhibitors was studied by monitoring the gel-mobility shift of 32P-labeled algD upstream DNA (containing a 14-mer AlgR1 consensus binding site; see ref. 6) caused by AlgR1 bound to it. As shown in Fig. 6, inhibitor A and its analog did not appear to have any significant effect on the DNA binding properties of AlgR1. Inhibitor B, on the other hand, inhibited AlgR1 from binding any significant amount of the DNA probe, because $\approx$96% of the probe remained unshifted. At higher concentrations of the AlgR1 protein preparation, the algD upstream DNA remained unshifted.
Fig. 6. Effects of the inhibitors on the DNA-binding activity of AlgR1. Gel-mobility shift assay was carried out using an algD upstream DNA probe, a 164-bp Pvu II-Stu I fragment containing the AlgR1-binding site at the −458 position (6). Lane 1, free probe (with an unshifted mobility); lanes 2–6, 13 ng of the AlgR1 protein preparation; lanes 7–11, 39 ng of AlgR1. Lanes 2 and 7, mobility shift control; lanes 3 and 8, control in the presence of 0.5% dimethyl sulfoxide (in which the inhibitors are dissolved); lanes 4 and 9, inhibitor A at 50 μg/ml; lanes 5 and 10, inhibitor A′ at 50 μg/ml; lanes 6 and 11, inhibitor B at 50 μg/ml.

DNA mobility shift due to nonspecific binding of protein remained unaffected by compound B (data not shown). This observation suggests that compound B is not a nonspecific inhibitor of DNA binding activities of protein(s), but rather a specific inhibitor of AlgR1–algD upstream DNA interaction. In fact, compound B has little effect on the binding of a positive regulatory protein CatR to its cognate catBC promoter DNA (M. Parsek and A.M.C., unpublished data). This notion also is supported by the observation that compound B can inhibit transcription from the algD promoter in vivo without inhibiting bacterial cell growth.

Inhibition of Other Two-Component Kinase Activities. Several two-component kinases involved in various bacterial signal transduction processes are known and well characterized (19, 20). All of these kinases are known to autophosphorylate themselves at histidine residues and transfer the phosphate to the conserved aspartate residues of their cognate response regulators. Phosphorylation and phototransfer involved in these processes are therefore believed to share common mechanisms. In fact, a number of these proteins have been shown to be capable of “cross talk” between noncognate kinases and response regulators (21).

Thus we examined the effect of the inhibitor compounds on the kinase activities of CheA, NRt, and KinA, which are the kinases involved in bacterial chemotaxis and nitrogen assimilation in Escherichia coli and sporulation in Bacillus, respectively (Fig. 7). In the case of CheA, inhibitors A and A′ showed partial inhibition (26% and 42%, respectively). However, inhibitors B and B′ were relatively potent inhibitors (70% and 89%, respectively). Inhibitor A′ inhibited NRt kinase activity by 10% while the effect of inhibitor A was insignificant. Interestingly, inhibitors B and B′ exhibited almost complete inhibition of NRt autophosphorylation. Likewise, in the case of KinA autophosphorylation, inhibitors B and B′ showed strong inhibition while A and A′ did not show any significant effect. In addition, the effect of these inhibitors on the transfer of phosphate from NRtP to NRt and KinA-P to SpoOF (the cognate receivers of phosphate) was examined. In both cases, inhibitors B and B′ abolished the phosphate transfer activity (data not shown).

DISCUSSION

Gene regulation in alginate synthesis by P. aeruginosa is a complex process involving several regulatory proteins, not all of which are well characterized. However, the role of AlgR1 as a phosphorylation-dependent transcriptional regulator of alginate gene expression is well established. Furthermore, AlgR2 has been shown to phosphorylate AlgR1, thereby modulating its regulatory activity. Results presented in this paper identify four compounds as inhibitors of these two protein activities involved in activation of the algD promoter. These inhibitors, which strongly inhibit the algD promoter activity in vivo, may help us correlate the phosphorylation/dephosphorylation of the AlgR2–AlgR1 complex and the DNA-binding activity of AlgR1 with their physiological roles in regulating alginate gene expression in P. aeruginosa. A model for the phosphorylation of the AlgR2–AlgR1 complex and the steps leading to the activation of the algD promoter via a DNA-looping mechanism is presented in Fig. 8.

Development of safe, nontoxic inhibitors of the signal transduction pathway regulating alginate synthesis is our eventual goal. Toward this end, one possible approach would be to develop inhibitors to several steps in the signaling pathway for use in combination with appropriate antibiotics. This would have the potential advantage of avoiding rapid selection of mutants resistant to a single inhibitor. It is interesting that the compounds described here appear to act at different steps of the alginate gene-activation pathway and could conceivably be used in combination. Further research may lead to the discovery of additional inhibitors affecting other steps of alginate gene expression.

It should be noted that AlgR1 is known to regulate the expression of the neuraminidase gene in P. aeruginosa and

![Fig. 7](image7.png)  
**Fig. 7.** Inhibition of the autophosphorylation activity of CheA, NRt, and KinA. Purified protein preparations (10, 2, and 2 μg of CheA, NRt, and KinA, respectively) were incubated with [γ-32P]ATP in the presence of inhibitors A, A′, B, and B′ at 50 μg/ml under conditions described in Materials and Methods. In each case, control reactions (CON) were run (shown in the extreme left lane of each gel) in the presence of 0.5% dimethyl sulfoxide in which the inhibitors were dissolved.

![Fig. 8](image8.png)  
**Fig. 8.** A model describing the putative phosphorylation/dephosphorylation of AlgR2 and AlgR1 leading to the transcriptional activation of the algD promoter. The activities inhibited by inhibitors A and B are shown by arrows. The chromosomal DNA showing the alginate gene cluster (5) is not drawn to scale. In addition to AlgR1 and AlgR2, at least two other proteins, AlgR3 (a histone H1-like protein) (22) and a P. aeruginosa analog of the Escherichia coli cyclic AMP receptor protein (CRP) (23), are known to participate in regulating the algD promoter. The E. coli CRP protein binds to the algD upstream region around position −362, which contains a consensus CRP-binding site. The E. coli integration host factor (IHF) is also known to bind the algD upstream region, but its role in the activation of the promoter has not been directly demonstrated (24). The P. aeruginosa analog of CRP and integration host factor as well as AlgR3 may facilitate the contact between the DNA-bound AlgR1 molecules (around positions −382 and −458) and RNA polymerase holoenzyme at the promoter region by a DNA-looping mechanism.
may act as part of a global regulatory network (25). Neuraminidase is believed to facilitate the adherence of \textit{P. aeruginosa} to eukaryotic epithelial cell surfaces. Thus AlgR1-mediated gene activation may affect several important steps in pathogenesis including adherence and encapsulation. The inhibitors described in this paper may therefore play a broader role in preventing infections caused by pathogenic bacteria.

To study the effect of these inhibitors on other members of the kinase family of two-component bacterial signal transducing proteins, we examined the inhibition of autophosphorylation of three soluble kinases, CheA, NR1I, and KinA. Inhibitors A and A', which inhibit AlgR2 kinase activity, do not appear to be general inhibitors of two-component bacterial kinases. This could be attributable to the unusual size and structure of AlgR2, which is not shared by other kinases. Surprisingly however, inhibitor B, which inhibits the DNA-binding activity of AlgR1, strongly inhibits the autophosphorylation activities of CheA, NR1I, and KinA. Inhibitor B, and its analog B', significantly inhibited the kinase activity of a transmembrane protein kinase, VirA (data not shown), suggesting that inhibitor B is active against membrane-associated sensor proteins as well. The low concentration of inhibitor B used in these experiments argues against its effect being nonspecific in nature. Thus, inhibitor B appears to be a general inhibitor of two-component bacterial kinases. The effect of inhibitor B on ortho phosphorylatable substrates may have physiological significance with regard to alginate gene expression in \textit{P. aeruginosa}. It is possible that at least two kinases, AlgR2 and a more ortho phosphorylatable, are required for the AlgR1-mediated regulation of the algD promoter. Inhibitor B might inhibit transcription from the algD promoter in vivo by inhibiting not only the AlgR1-DNA interaction but also the ortho phosphorylatable kinase. It is noteworthy that the algD promoter activity is responsive to diverse signals such as osmolarity, nitrogen or phosphate starvation, dehydration, etc. (2–5). Further investigation may shed light on the exact correlation between the \textit{in vivo} and \textit{in vitro} activities of this compound.

Inhibitors A and B may be potentially useful in further understanding the mechanism of sensory response in a variety of bacterial signal transduction systems. Unlike eukaryotic kinases or phosphatases, no inhibitors of prokaryotic kinases or phosphatases have been reported. Furthermore, since members of the two-component bacterial signal transduction proteins exhibit similarity in their mechanism of action, these inhibitors may provide a generally applicable tool to dissect and study different aspects of the two-component regulatory proteins in greater detail. These inhibitors might also be useful in other cases of bacterial pathogenesis involving two-component signal transduction mechanisms.

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