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EXPERIMENTAL ARTICLES

Transmembrane Adenylate Cyclase Controls the Virulence Factors of Plant Pathogenic *Pseudomonas siringae* and Mutualistic *Rhizobium leguminosarum*

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Abstract—The possible role of transmembrane adenylate cyclase of a plant pathogen *Pseudomonas siringae* pv. *pisi* and of a symbiotroph *Rhizobium leguminosarum* bv. *viceae* in control of the activity of their virulence factors (cellulases and pectinases, the enzymes degrading plant cell walls) was investigated. While transmembrane adenylate cyclase was found to control the activity of virulence factors in both pathogens and symbionts, the strategies employed by these microorganisms in molecular dialogue with plants involving the adenylate cylcase signal system exhibited both similarities and cardinal differences.

Keywords: transmembrane adenylate cyclase, soluble adenylate cyclase, pectinases, cellulase, plant pathogens, mutualists

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The mechanisms of regulating the virulence of phytopathogens are complex and controlled by many factors, including their signal systems [1]. The bacterial enzymes pectinase and cellulase degrading the plant cell wall also belong to the virulence factors. Importantly, mutualistic bacteria, particularly of the genus *Rhizobium*, also actively utilize pectinase and cellulase at the early stages of their interaction with plants [2]. According to the literature data, pectinase synthesis is regulated by a cAMP-dependent receptor protein (CRP) [3, 4]. Thus, the adenylate cyclase signal system controls the activity of certain phytopathogenic virulence factors. Adenylate cyclases (AC) of plant pathogenic bacteria are known to include both transmembrane (tmAC) and soluble enzyme forms (sAC) [5]. However, no data on the role of each AC form in controlling bacterial virulence factors have been available so far.

The goal of the present work was to elucidate and compare the roles of transmembrane and soluble AC forms in the control of the activity of pectinase and cellulase, which are the virulence factors of the phytopathogen *Pseudomonas syringae* pv. *pisi* and of the mutualist *Rhizobium leguminosarum* bv. *viceae*.

MATERIALS AND METHODS

Subjects of research. The following species of bacteria were used in the experiments: *Rhizobium leguminosarum* bv. *viceae*, strain 1060 obtained from the collection of the All-Russia Research Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences (Pushkino), and *Pseudomonas syringae* pv. *pisi*, strain 1845 obtained from the All-Russia Research Institute of Physiology (Bol'shie Vyazemy, Moscow oblast).

Bacterial cultures were grown at 25°C in flasks with the medium containing the following (g/L): dialyzate of yeast extract, 10; glucose, 15; $CaCO_3$, 5; pH 7.0 (control variant) and in the medium of the same composition with the addition of 50 μM of the tmAC inhibitor suramin (experimental variant).

The bacterial titer was determined by the turbidity standard on an Immunochem-2100 tablet spectrophotometer (High Technology Inc., United States) at 655 nm. The studies were conducted with bacterial cultures during the stationary growth phase.

Incubation of pea sprout roots with the bacterial culture. Separated pea sprout roots (25–35 mm) were incubated with the bacterial cultures of *R. leguminosarum* and *P. syringae* for 6 h. After that, the incubation mixture was filtered (with the roots being discarded) and centrifuged at 16000 *g* to separate the bacteria. The supernatant fluid and the cells were used for analysis.

Sample preparation for analysis of the enzyme activity. The sAC, cellulase and pectinase activities were determined in the supernatant fluid obtained after centrifugation. The medium for homogenization, containing 50 mM Tris-HCl, pH 7.2; 0.5 mM phenylmethylsulfonyl fluoride; 0.05 mM

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Table 1. Activity of different forms of AC (cAMP, nM/mg protein) of *Pseudomonas syringae* pv. *pisi* in the presence of suramin (50 μM) and during co-incubation with the roots of pea and potato plants in vitro

AC forms	*P. syringae* pv. p*isi*		*P. syringae* pv. *pisi* + pea		*P. syringae* pv. *pisi* + potato	
	control	suramin	control	suramin	control	suramin
tmAC	11 ± 0.4	0.005 ± 0.0002	294 ± 13	29 ± 1.1	400 ± 15	150 ± 6.3
sAC in bacterial cells	0.3 ± 0.01	0.3 ± 0.02	0.1 ± 0.004	0.1 ± 0.005	0.1 ± 0.004	0.1 ± 0.004
sAC in CL	2 ± 0.1	35 ± 1.3	132 ± 5.8	330 ± 15	166 ± 7.3	700 ± 32

parachlormercuribenzoate; and 1 mM dithiothreitol was added to the cell pellet at a 1 : 3 ratio (wt/vol), the total volume 4–5 mL, and the bacteria were treated with an ultrasonic sonicator (Branson Ultrasonic Corp., United States). The pectinase and cellulase activities were determined in 2 mL of bacterial homogenate. The remaining homogenate was centrifuged at 105000 *g*. The activity of soluble AC (sAC) was determined in the supernatant fluid, and the activity of transmembrane AC (tmAC), as well as that of cellulase and pectinase, was determined in the pellet, representing the membrane fraction.

Determination of the cAMP level in the bacterial growth medium. After removal of bacterial cells by centrifugation, the culture liquid (CL) was boiled and the cAMP level was determined with a modified enzyme immunoassay method [6].

Determination of activities of bacterial cellulase and pectinase. Carboxymethylcellulose (2%, 0.5 mL) and 0.5 mL 0.2 M phosphate buffer, pH 7.0 (when determining cellulase, EC 3.2.1.4) or 2.85 mL 0.05 M Tris-HCl buffer, pH 8.5, 0.03 mL 0.01 M $CaCl_2$ and 0.15 mL 1% sodium polypectate (when determining pectinase, EC 3.2.1.15) was added to 2 mL of the sample containing the enzymes studied. The reaction was carried out in test tubes at 27°C for 3 h and stopped by the addition of 3 mL of the reagent to determine reducing sugars with subsequent heating for 5 min at 100°C. Reducing sugars were determined with a Seignette salt-based reagent on an Immunochem-2100 tablet spectrophotometer (High Technology Inc., United States) at 670 nm [7]. The results were expressed in mg/mL of reducing sugars, and dilution of the bacterial pellet was taken into account when making calculations.

Determination of the activity of the soluble and transmembrane forms of adenylate cyclase. The reaction was initiated by introducing protein at an amount of 100–150 μg/500 μL of the incubation medium containing 50 mM Tris-HCl, pH 7.2, 0.1 mM theophylline (cAMP phosphodiesterase inhibitor), 0.5 mM phenylmethylsulfonyl fluoride, 0.05 mM parachlormercuribenzoate, 1 mM dithiothreitol, and 0.5 mM ATP. $MgSO_4$ and $MnCl_2$ (3 mM each) were used as cofactors for tmAC and sAC, respectively. The reaction was carried out at 27°C for 30 min and was stopped by boiling for 3 min on a water bath. The activities of both forms of adenylate cyclase were determined from the increase in the amount of cAMP in a sample calculated for mg protein.

Purification of the samples from other cyclic nucleotides on aluminum oxide columns was carried out according to the method described in [8]. cAMP was determined using the enzyme immunoassay method [6]. Protein in the sample was determined with the Bradford method.

Statistical data processing. The experiments were carried out in three biological and eight analytical replicates. The results obtained were processed statistically, with the calculation of the error of the mean value.

RESULTS AND DISCUSSION

Activity of *P. syringae* pv. *pisi* transmembrane and soluble forms of adenylate cyclase. Two forms of AC—transmembrane and soluble—were found in the phytopathogen *P. syringae* pv. *pisi*. Activity of the sAC in bacteria was significantly lower than that of tmAC. Moreover, sAC was present in CL where its level of activity was approximately 7 times higher than in bacterial cells (Table 1). Suramin almost completely inhibited tmAC, while the sAC activity remained at the control level in the bacteria and increased significantly in the bacterial CL. cAMP in the CL in the control (without suramin) was present at micromolar concentrations but was considerably lower in the medium with suramin (experiment) (Table 2). This indicates that the synthesis of most of cAMP in the CL was carried out by tmAC.

The virulence of pathogens is known to be most pronounced on contact with a plant [9, 10]. Therefore, in the following series of experiments, the bacteria were incubated with the roots of pea (host plant) sprouts and, in a separate experiment, with the roots of potato plants in vitro (nonhost plant). It is noteworthy that the activity of tmAC in the cells and the activity of sAV in the CL increased drastically by more than an

order of magnitude, whereas the activity of sAC in the cells remained at an invariably low level (Table 1). Interestingly, the activity of these AC forms increased to a larger degree on co-incubation of the phytopathogen with the roots of potato plants in vitro. On addition of suramin to the cultures, tmAC activity decreased more noticeably in the variant with pea sprout roots than in the variant with potato plants. According to the literature data, pea root secretions contain a number of compounds of phenol nature, in particular, *N*-phenyl-2-naphthylamine that inhibits bacterial growth [11]. It is logical to suggest that a decrease in the bacterial growth rate may be associated with a change in the activity of tmAC. Under these conditions, the activity of sAC in CL considerably increased compared to the control (incubation with plant roots without suramin) (Table 1). The cAMP level in the bacterial CL also increased on addition of pea and potato plant roots. It should be noted that the roots of the pea and potato sprouts by themselves secreted cAMP into the growth medium at a very low concentration, which did not exceed the tenth picomole fractions (data not shown).

In our view, the increase in the sAC activity in the CL on inhibition of tmAC can perform the function of a compensatory mechanism for the maintenance of the viability and virulence of this phytopathogen.

The cellulase and pectinase activities of *P. syringae* pv. *pisi*. According to the aim of our study, the cellulolytic and pectolytic activities of *P. syringae* were investigated. It is held by convention that syringotoxin and certain effector proteins inducing hypersensitivity response in resistant plants are the main operating factors of virulence in this bacterium [12, 13]. At the same time, the enzymes hydrolyzing cell walls and acting at the initial steps of pathogenesis remain insufficiently studied. However, the efficiency of their functioning is responsible for successful colonization of a plant by the microorganism when its vascular tissues are affected. In our experiments, the cellulase and pectinase activity in the culture of *P. syringae* pv. *pisi* at the early stationary growth phase was mainly concentrated in the bacterial cells (Fig. 1). The growth of bacteria in the presence of suramin resulted in decreased activity of the hydrolytic enzymes only in the growth medium (Fig. 1). The activity of pectinolytic enzymes of certain phytopathogens is known to be regulated by several transcriptional factors acting in the opposite directions and forming a signal network [14]. They include KdgR responsible for pectinase gene induction by pectin and its metabolites; its ortholog RexZ, an exoenzyme activator; and CRP, a positive pectinase gene regulator. As shown for bacteria of the species *Erwinia carotovora* and *Escherichia coli*, RexZ is controlled by CRP, a cAMP-dependent receptor protein [15, 16], i.e., it depends on the concentration of available free cAMP. As shown by our results, suramin inhibition of the activity of tmAC, the main source of cAMP in a bacterial cell, led to a decrease in the exopectinase and exocellulase activity in the CL.

Table 2. cAMP concentrations in the CL of *Pseudomonas syringae* pv. *pisi*, μM/mg protein

Variant	Control	Suramin
P. syringae pv. p*isi*	50 ± 2.1	3 ± 0.12
P. syringae pv. p*isi* + pea	72.8 ± 3.0	2.6 ± 0.11
P. syringae pv. p*isi* + potato	83.4 ± 3.8	3.2 ± 0.11

Co-incubation of the phytopathogen with the roots of pea and potato sprouts in vitro stimulated the activity of these enzymes. Cellulase in the incubation variant with potatoes was stimulated to a larger degree. According to the literature data, plant extracts are able to increase the activity of pectolytic enzymes in *Erwinia chrysanthemi*, although the chemical nature of these signals remains unknown [14]. However, when bacteria were cultivated with suramin, a tmAC inhibitor, the cellulase and pectinase activities decreased; this was especially pronounced in the CL (Fig. 1). It is noteworthy that the pea and potato roots exhibited almost no hydrolytic activity, and the addition of suramin to the isolated cellulase and pectinase did not inhibit their activity (data not shown).

Activity of the transmembrane and soluble forms of adenylate cyclase of *Rhizobium leguminosarum* bv. *viciae*. Similar experiments conducted with *R. leguminosarum* strain revealed a number of cardinal differences in the activity of tmAC and sAC from the results obtained with *P. syringae* pv. *pisi*.

First and foremost, it should be noted that the activity of all forms of AC in this microorganism was significantly lower than in *P. syringae* (Table 3). Moreover, tmAC was most intensively activated in the presence of the roots of pea sprouts and to a lesser degree in the presence of the roots of potato sprouts (Table 3), which differs from the tmAC activity values of *P. syringae*. In all the variants, suramin inhibited the tmAC activity almost completely but did not influence the activity of sAC in the bacterial cells. However, the CL activity of sAC varied, but not similarly in different experimental variants. For example, addition of suramin resulted in sharply increased activity only in the CL without the plants. On the contrary, co-incubation with pea or potato roots in the presence of suramin resulted in drastically suppressed sAC activity in CL (Table 3). Thus, the functioning of the two forms of adeneylate cyclase in the symbiotrophic microorganism is also interdependent, as in the phytopathogen *P. syringae*, but it has specific features; in particular, it is regulated by plant metabolites in a different way, which is especially manifest in the variants with suramin. The dynamics of cAMP concentration

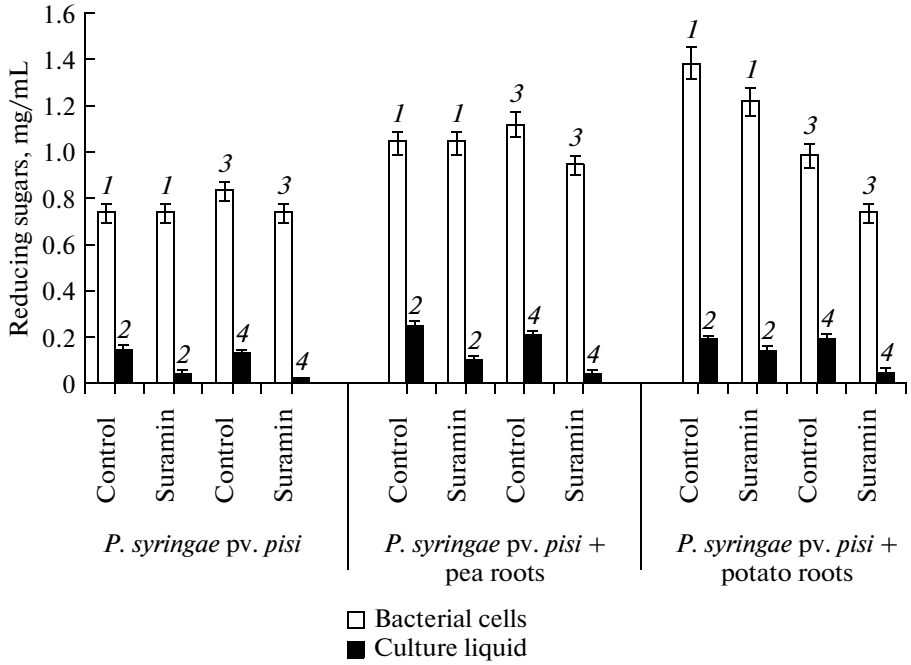

Fig. 1. Activity of cellulase (*1, 2*) and pectinase (*3, 4*) in the cells (light columns) of *Pseudomonas syringae* pv. *pisi* and its culture liquid (dark columns).

in the control (without suramin), as well as in the presence of suramin in the growth medium, was similar to that in *P. syringae*. Incubation with the roots of the pea and potato plants slightly increased the cAMP level in the growth medium of *R. leguminosarum* (Table 4).

Activities of pectinase and cellulase of *Rhizobium leguminosarum* bv. *viciae*. It was interesting to compare the above results with a change in the cellulase and pectinase activity of *R. leguminosarum*. The inhibition of tmAC by suramin was accompanied by a decrease in the activity of these enzymes, especially pectinase (Fig. 2). This pattern was observed not only for the values in the CL, as in the case of *P. syringae*, but also in the bacterial cells. Co-incubation with pea roots partially obviated this effect, which coincides with the results obtained for *P. syringae*. Taking into account a very low level of sAC activity in the bacterial cell, we may suggest that tmAC has the main regulatory role in modulation of the activities of these virulence factors.

Thus, the results of our study showed that the mechanisms of regulation of the exocellulase and

Table 3. Activity of different forms of AC (cAMP, nM/mg protein) of *Rhizobium leguminosarum* bv. *viciae* in the presence of suramin (50 μM) and during incubation with the roots of pea and potato plants in vitro

AC forms	*R. leguminosarum*		*R. leguminosarum* + pea		*R. leguminosarum* + potato	
	control	suramin	control	suramin	control	suramin
tmAC	0.02 ± 0.001	0.008 ± 0.0002	11.8 ± 4.7	0.06 ± 0.002	0.07 ± 0.002	0.06 ± 0.002
sAC in bacterial cells	0.04 ± 0.001	0.04 ± 0.001	0.13 ± 0.04	0.14 ± 0.005	0.01 ± 0.004	0.01 ± 0.003
sAC in CL	1.7 ± 0.12	70 ± 2.8	27 ± 1.2	4 ± 0.012	140 ± 6.2	3 ± 0.12

exopectinase activities with the involvement of the adenylate cyclase signal system have not only a number of similarities but also obvious distinctions in the phytopathogen *P. syringae* and the mutualist *R. leguminosarum*. The similarities include the presence of two interconnected forms of adenylate cyclase, as well as dependence of the cellulase and pectinase activities of both microorganisms on the activity of tmAC as the main source of intracellular cAMP. The fundamental difference concerns the activity of sAC secreted into the bacterial growth medium: in the phytopathogen *R. syringae*, the activity of this enzyme form increased on both inhibition of tmAC and on contact of bacteria with plants. In this case, it may be suggested that sAC acts as an independent virulence factor, possibly with the involvement of type III secretion system (T3SS) [17] present in CL as a low-activity form. Such a suggestion is quite logical, because an artificial construction consisting of the catalytic adenylate cyclase domain of animal pathogens, for example, *Bordetella pertussis*, and the tentative proteinaceous virulence factor of plant pathogens, such as *Pseudomonas syringae* pv. *tomato* or *Erwinia carotovora* sp. *atroseptica*, is used for detection of the virulence factors of plant pathogens. Such a construction easily penetrates into plant cells [12, 13] where AC is activated following the mechanism similar to animal pathogens [13]. Apart from the fact that this approach proves the virulent nature of the protein effector and the mode of its delivery by type III secretion, it demonstrates the common property of adenyate cyclases of animal and plant pathogens to penetrate into a eukaryotic cell.

Table 4. cAMP concentrations in CL of *R. leguminosarum*, μM/mg protein

Variant	Control	Suramin
R. leguminosarum	67 ± 3.1	1.2 ± 0.07
R. leguminosarum + pea	82 ± 3.8	0.9 ± 0.04
R. leguminosarum + potato	79 ± 3.2	0.8 ± 0.03

On the contrary, in the mutualist *R. leguminosarum*, sAC activation in CL occurred only on contact with plants, whereas tmAC inhibition considerably decreased its activity, especially upon incubation with plant roots. The cause of such a decrease in the sAC activity remains to be understood. At present, it is recognized that T3SS is not the prerogative of phytopathogens, but also functions in symbiotic organisms [17], although it might be regulated by the plant in a different way according to the final goals of plant–microbial interactions.

So far our findings do not allow us to assert that sAC is an independent virulence factor of phytopathogens as in animal pathogens, but rather demonstrate that the transmembrane form of AC controls the activity of the virulence factors of both phytopathogens and mutualists.

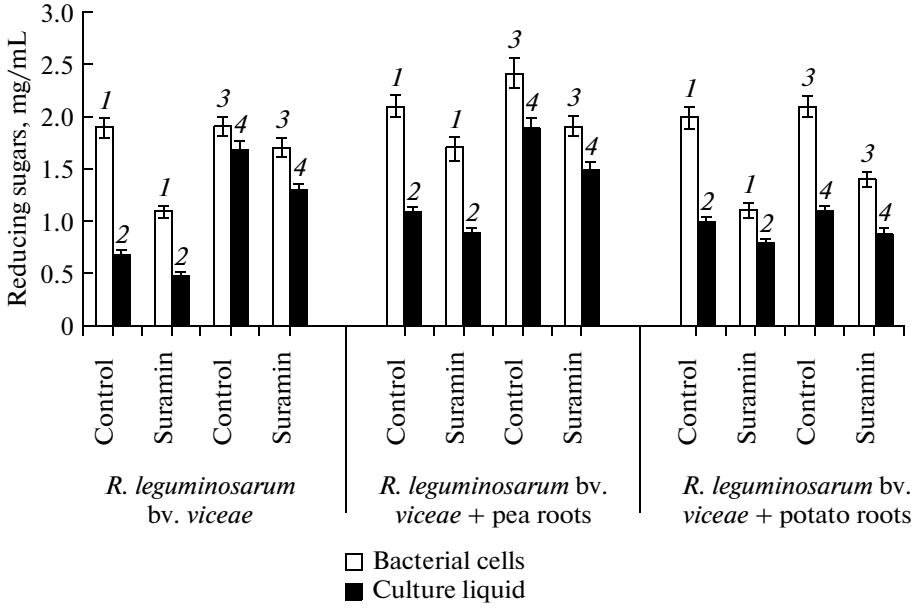

Fig. 2. Activity of cellulase (*1, 2*) and pectinase (*3, 4*) in the cells of *Rhizobium leguminosarum* bv. *viciae* and its culture liquid (see Fig. 1 for designations).

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