Regulation by Organic Acids of Polysaccharide-mediated Microbe-plant Interactions

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Received February 23, 2000; Accepted June 5, 2000

A polysaccharide flocculant of Klebsiella pneumoniae H12 has been suggested to mediate microbe-plant interactions with the aid of Ca\(^{2+}\) [K. Nakata et al., Biosci. Biotechnol. Biochem., 64, 459-465, 2000]. Here, two-way regulation of polysaccharide-mediated interactions between K. pneumoniae and Raphanus sativus was studied using organic acids. Namely, 10 mm equivalents of organic acids promoted production of the polysaccharide by the bacterium, but inhibited flocculation of bacterial cells by the polysaccharide. These phenomena were counterbalanced by equi-molar equivalents of Ca\(^{2+}\), suggesting competition for Ca\(^{2+}\) between the carboxylic residues of the polysaccharide and those of the aliphatic acids. By electron microscopy observations, bacterial cell aggregates were sparsely distributed over the main roots and root hairs, had various sizes, and seemed to tightly adhere to root tissues. Their shapes seemed to be distorted and abundant in cavities. In brief, these microscopical observations may be explained by a two-way regulation system of bacterial adhesion to a plant by organic acids.

Key words: flocculant; Klebsiella; organic acid; polysaccharide; symbiosis

Microorganisms often grow on plant surfaces in a symbiotic or parasitic relationship. Although microbial extracellular polysaccharides have been considered to be important in the mediation of these microbe-plant interactions,\(^1,2\) regulation of polysaccharide synthesis and flocculation activity by physiological and environmental factors have scarcely been studied. The topic of the interfacial interaction between microorganisms and plants has also not been elucidated in most cases. Various organic acids are produced in abundance from plant roots and bacterial aggregates, but their roles remain to be discovered, except for the facilitating role in incorporation of metal ions into organisms. Therefore, the effects of organic acids on microbe-plant interactions are an important topic.

A facultative anaerobe, Klebsiella pneumoniae, occurs in soil or plants and has a parasitic or symbiotic relationship with plants. Recently, an extracellular polysaccharide having high flocculation activity was purified from K. pneumoniae H12.\(^3\) This polysaccharide flocculates kaolin clay and some bacterial cells in the presence of cations such as Ca\(^{2+}\). The polysaccharide has an approximately 15% molar ratio of uronic acids and the carboxylic residue have been considered to be important for the flocculation activity. H12 polysaccharide-mediated adherence was reported between Pseudomonas fluorescens and Raphanus sativus, and antibiotic production by the bacterium was suggested to increase plant growth in the adherent state.\(^4\) In this report, polysaccharide production and its flocculation activity are studied physiologically and microscopically, and effects of organic acids on microbe-plant interactions are discussed.

Materials and Methods

Microorganisms. Klebsiella pneumoniae H12 and Pseudomonas fluorescens S272 were described previously.\(^3,5\) The latter strain was used in the experiments on microbe-plant interactions, because of its non-production of polysaccharide flocculant and of its distinct adhesion to root tissues of R. sativus through H12 polysaccharide.

Materials. Seeds of Raphanus sativus (Kaiware radish) were purchased from Taneto Co. (Fujisawa, Japan). H12 polysaccharide was purified as described previously.\(^3\)

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Culture of *K. pneumoniae* H12 and assay of polysaccharide productivity. Strain H12 was cultured for 4 days at 28°C in 250-ml Erlenmeyer flasks containing 30 ml of YPE medium [0.5% yeast extract (Difco), 0.5% polypeptone (Nihon Pharmaceutical Co.), 2% (v/v) ethanol, 0.05% K2HPO4, and 0.05% MgSO4·7H2O, pH was adjusted to 7.0] with a rotary shaker (220 rpm with a 50-mm stroke). YPE media including some concentrations of organic acids and CaCl2·2H2O were also tested. Production of the polysaccharide was measured by the dry weight after ethanol precipitation of the culture supernatant.3"

Flocculation of kaolin. Flocculation activity was assayed by modifying the method of Kurane et al.6 In a test tube, 9 ml of a kaolin clay (Wako Pure Chemical Industries) suspension (5.5 g/l) was mixed with 1 ml of polysaccharide solution, 100 μl of organic acid (ammonium acetate, sodium acetate, sodium succinate, or sodium citrate) solution, and 100 μl CaCl2·2H2O solution in this sequence with a Vortex mixer for 5 sec. After 5 min, OD550 of the supernatant was measured. A control experiment was done in the absence of the polysaccharide and the OD550 was measured. To measure the amount of polysaccharide needed for flocculation at each concentration of the organic acids and CaCl2·2H2O, the concentration of the polysaccharide required to reduce the OD550 to (1/10) × OD550, was recorded in parts per million.

In another flocculation assay, 9 ml of kaolin suspension was mixed with 1 ml of various concentrations of polysaccharide solution and 100 μl of various concentrations of organic acid solution concomitantly with 100 μl of CaCl2·2H2O solution (200 mM). OD550 of the supernatant was measured until OD550 reached (1/10) × OD550, and the elapsed time was recorded.

Bacterial flocculation in a model soil. *P. fluorescens* S272 was cultured for 2 days at 28°C in 250-ml Erlenmeyer flasks containing 30 ml of YMPG medium [0.3% yeast extract, 0.3% malt extract (Difco), 0.5% polypeptone, and 1% glucose, pH was adjusted to 7.0] and cells were collected by centrifugation. After twice washing with deionized water, cells were mixed with 30 ml-volume of 5 g/l suspension of kaolin clay. Ten milliliters were mixed with 100 μl of 1% H12 polysaccharide solution and 100 μl each of several concentrations of sodium acetate and CaCl2·2H2O solutions using a Vortex mixer for 5 sec. This was left for 5 min, then the OD550 of the supernatant was measured. As a blank, kaolin suspension without cells was similarly tested. Cell flocculation was expressed as OD550, subtracting that of the blank.

Volcanic soil was sterilized at 150°C for 2 h and suspended at the ratio of 5 g/l. After this was left for 10 min, the supernatant was collected. Assays were made using this suspension instead of kaolin suspension.

Preparation of root tissues with adherent bacterial cells. Fresh seeds of *R. sativus* were sown on wet layers of gauze in the transparent well (35 mmφ × 18 mm) of a microtitre plate which was placed in a transparent box filled with sufficient water to maintain moderate moisture. The seed was incubated at 25°C under light from a fluorescent lamp (20 W). The initial volume of tap water was 10 ml, and the level was maintained throughout the cultivation period by adding more tap water. At the sprouting phase (2 days later), an overnight culture broth of strain S272 in a YPE medium (10 μl), a 1% aqueous solution of H12 polysaccharide (0, 10, or 100 μl), and 200 mM aqueous solution of CaCl2·2H2O (0 or 100 μl) were added in this order. After a further three days of incubation, root tissues were carefully removed.

Scanning electron microscope observation. Root tissues were observed under low vacuum after freezing in liquid nitrogen.7 Equipment; (JSM-5600; JEOL), applied voltage; 15 kV.

Results

Regulation of polysaccharide synthesis by organic acids

When *K. pneumoniae* H12 was cultured with the addition of several concentrations of organic acids, production of polysaccharide was measured (Fig. 1A). Ammonium acetate and sodium acetate promoted the production of polysaccharide at the concentrations of 10–20 mM, although higher concentrations were repressive. Sodium succinate and sodium citrate, which have higher numbers of carboxyl groups, had lower optimum concentrations.

When *K. pneumoniae* H12 was cultured in the presence of several concentrations of sodium acetate and CaCl2·2H2O, production of polysaccharide was measured (Fig. 1B). Promotion of the production by sodium acetate was inhibited by CaCl2·2H2O. The inhibition was more prominent at low concentrations of sodium acetate.

When *K. pneumoniae* H12 was cultured in the presence of several concentrations of sodium acetate and glucose, production of polysaccharide was measured (Fig. 1C). Promotion of the production by sodium acetate was inhibited by glucose. The inhibition was greater at low concentrations of sodium acetate, suggesting that production of the polysaccharide is regulated by catabolite repression.

Regulation of flocculation activity by organic acids

Flocculation tests using kaolin clay were done at
Fig. 1. Effects of Organic Acid Concentration on Polysaccharide Production in Culture of K. pneumoniae.
(A) Test of four organic acids. Symbols: ○, ammonium acetate; △, sodium acetate; ●, sodium succinate; ■, sodium citrate. (B) Polysaccharide productivity in the presence of several concentrations of sodium acetate and CaCl$_2$·2H$_2$O. Concentration of CaCl$_2$·2H$_2$O: ○, 0 mM; △, 17 mM; ▲, 34 mM. (C) Polysaccharide productivity in the presence of several concentrations of sodium acetate and glucose. Concentration of glucose: ○, 0 mM; □, 10 mM; ▲, 100 mM.

Several concentrations of organic acids in the presence of 2 mM of CaCl$_2$·2H$_2$O and 10 mg/l of H12 polysaccharide (Fig. 2A). Ammonium acetate and sodium acetate at concentrations greater than 1 mM increased the amount of polysaccharide needed for flocculation. Sodium succinate and sodium citrate, which have higher numbers of carboxyl groups, showed greater inhibiting activity on kaolin flocculation.

Fig. 2. Effects of Organic Acid Concentration on Flocculation Activity of the Polysaccharide.
(A) Test of four organic acids. Symbols: ○, ammonium acetate; △, sodium acetate; ●, sodium succinate; ■, sodium citrate. (B) Flocculation activity in the presence of several concentrations of sodium acetate and CaCl$_2$·2H$_2$O. Concentration of sodium acetate: ○, 0 mM; △, 10 mM; ▲, 100 mM. (C) Mechanical analysis of inhibition of flocculation by sodium acetate. Time, at which sufficient flocculation was achieved, was plotted against the reciprocal of flocculant concentration [5]. Concentration of sodium acetate: ○, 0 mM; △, 10 mM; ▲, 100 mM.

Flocculation tests using kaolin clay were made in the presence of several concentrations of sodium acetate and CaCl$_2$·2H$_2$O, and 10 mg/l of H12 polysaccharide (Fig. 2B). At low concentrations of CaCl$_2$·2H$_2$O (0.5–5 mM), sodium acetate inhibited the flocculation at low concentrations (10 mM). On the contrary, at the concentrations of CaCl$_2$·2H$_2$O greater than 5 mM, higher concentrations of sodium acetate (more than 100 mM) were needed for inhibition of flocculation.

Flocculation tests using kaolin clay were made in
the presence of several concentrations of H12 polysaccharide and sodium acetate, and 2 mM of CaCl₂·2H₂O. The time at which sufficient flocculation was reached was plotted against the reciprocal of polysaccharide concentration (Fig. 2C). Sodium acetate increased the slope, indicating that it might be a competitive inhibitor of H12 polysaccharide against CaCl₂·2H₂O.

**Regulation of flocculation in model soil**

To discover what regulates bacterial cell flocculation in soil, assays were done in a kaolin (Fig. 3A) or a soil suspension (Fig. 3B). *P. fluorescens* S272 cells were suspended in a kaolin (or soil) suspension and cell flocculation by H12 polysaccharide was observed at several concentrations of sodium acetate and CaCl₂·2H₂O. In a kaolin suspension, 10 mM of sodium acetate inhibited cell flocculation at low concentrations (2 mM) of Ca²⁺, but the inhibition was small at 50 mM Ca²⁺. In a soil suspension, the degree of flocculation inhibition by sodium acetate was less than in a kaolin suspension. The results indicated that sodium acetate is an inhibitor of cell flocculation in a model soil and Ca²⁺ acts antagonistically towards sodium acetate.

**Scanning electron microscope observation**

*R. sativus* was cultured in water in the presence of H12 polysaccharide (10 or 100 mg/l), *P. fluorescens* S272 culture broth (containing 10⁶/ml living cells), and CaCl₂·2H₂O (2 mM). The root tissues were observed with a scanning electron microscope. At the concentration of 10 mg/l of H12 flocculant, bacterial cell aggregates were sparsely distributed among main roots (Fig. 4-1) and root hairs (Fig. 4-2). Cells formed clusters of various sizes, which appeared to tightly adhere to the root tissues (Fig. 4-3), and distinct numbers were half-buried in the root (Fig. 4-4). At high concentrations of H12 polysaccharide (100 mg/l), their shape seemed more distorted and full of cavities (Fig. 5-1 and 5-2). Distinct number of cells appeared to be crushed, suggesting that the optimum concentration of H12 polysaccharide for bacterial adhesion may be 10 mg/l. When radishes were cultured only in the presence of *P. fluorescens* S272 culture broth, no obvious bacterial cell adhesion was seen (Fig. 6).

Ten millimolar sodium acetate was initially added to the culture of Kaiware radish including 10 mg/l of H12 polysaccharide, *P. fluorescens* S272 culture broth containing 10⁶/ml of living cells, and 2 mM of CaCl₂·2H₂O. When the root tissues were observed with a scanning electron microscope, bacterial cell aggregation was not seen (data not shown).

**Discussion**

Bacteria in a rhizosphere often produce exopolysaccharides that are essential for the interaction with plants. Although synthesis of some exopolysaccharides has already been reported to be stimulated in media which are poor in nutrients, a control mechanism has not been described. Many polysaccharides that participate in cell adhesion are acidic heteropolysaccharides and their anionic residues are considered to be important. As the molar composition of H12 polysaccharide is 56.04% galactose, 25.92% glucose, 10.92% galacturonic acid, 3.71% mannose, and 3.37% glucuronic acid, it is abundant in carboxylic residues. In this report, activity of H12 polysaccharide was shown to be regulated by organic acids in both levels of polysaccharide synthesis and regulation of flocculation activity. Organic acids promoted the polysaccharide synthesis at the concentrations of 1~30 mM, but these concentrations were inhibitory for flocculation activity.

Calcium ions counterbalanced organic acids approximately at an equi-molar concentration. Probably, metal cations suppress the anionic charge of both the polysaccharide and the bacterial (or plant) cell surface. In an aqueous culture of *R. sativus*, 2 mM CaCl₂·2H₂O was initially added, because culture fluid at day5 contained less than 1 mM of acetic acid, which was the main organic acid present (data not
shown). In the soil experiments, analysis of the cationic and anionic compounds present will be necessary for optimizing the process of adherence in soil.

The promotion of polysaccharide synthesis by organic acids may be explained as being due to catabolite repression, because glucose evidently repressed the promotion (Fig. 1C). Inhibition in flocculation activity may represent competitive inhibition by organic acids. A polysaccharide solution at the concentration of 10 mg/l is calculated to contain 7.84 μM of uronic acid. This study shows that more than 1 mM of sodium acetate is needed for the inhibition of flocculation activity of H12 polysaccharide, suggesting that the affinity of H12 polysaccharide for Ca²⁺ would be approximately 1000 times higher than that of sodium acetate.

The adherence of P. fluorescens to R. sativus roots was observed by scanning electron microscopy under low vacuum after rapidly freezing the root tissues in liquid nitrogen. Fixation, crytical-point drying, or metal-shadowing methods gave more unclear portraits for the specimens, and the freeze-low vacuum method was suggested to be outstanding in the exclusion of artifacts. Bacterial cell aggregates were sparsely distributed among main roots and root hairs, and may thus injure the plant body less than when covering the whole root surface, because root tissues have to exchange various molecules with their rhizosphere. Aggregated cells were of various sizes and seemed to be tightly adhering to root tissues. Their shapes seemed distorted and full of cavities, especially in the presence of high concentrations of H12 flocculant (100 mg/l). These features may increase the surface area of bacterial aggregates and promote the molecular exchange between bacterium and plant, although we may be observing a virtual image of the cell shape covered with a polysaccharide layer. Nakata et al. have previously reported that the adhesion of P. fluorescens cells to root tissues of R. sativus increase plant growth at the concentration of 1–100 mg/l of H12 flocculant, but higher concentrations give a worse effect. The optimum concentration of H12 flocculant for bacterial adhesion to plant tissues may be approximately 10 mg/l.

As addition of 10 mM sodium acetate to the culture of R. sativus prominently inhibited the bacterial cell adhesion, microbe-plant interactions with the aid of
H12 polysaccharide might also be under the control of organic acids. Organic acids are usually in rich supply in a rhizosphere, but their biologically availability changes according to many environmental factors, such as the aerobic status and the concentration of cationic metal ions. A hypothetical interpretation of four different microbe-plant situations is presented. In aerobic and catabolite-repressible conditions of K. pneumoniae H12, polysaccharide production is inhibited. When cells encounter the rhizospherical conditions, which are often anaerobic and rich in organic acids, the cells efficiently synthesize the polysaccharide, but the adhesion activity to root tissues may be weak. On the root surface, metal salts of the organic acids are efficiently incorporated into plant roots and the concentration of free organic acids is low. Bacterial cells may adhere strongly to plant tissues and bacterial clumps grow. When plant tissues are heavily covered with bacterial cells, cellular incorporation of organic acids is inhibited and high density of bacterial cells produces a high concentration of organic acids, thus stopping the increase in clump size.

Exogenous addition of exopolysaccharides of other bacterial species often bypasses the defense response of plants against pathogenic microorganisms, may be because of the excess bacterial adherence. In the case of H12 polysaccharide, it is probable that the regulation described in this report might provide moderate microbial-plant interactions.

Acknowledgment

We thank Mrs Minako Fujikado for the technical assistance.

References

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