Research Note

Digalacturonic Acid Uptake in *Erwinia chrysanthemi*

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Erwinia chrysanthemi produces pectolytic hydrolases and lyases that attack the plant cell wall releasing monomers, saturated and unsaturated dimers, trimers, and oligomers of galacturonic acid that are subsequently catabolized by the pathogen. The uptake system for the dimer molecule, digalacturonic acid, in *E. chrysanthemi* EC16 was studied. Uptake was inducible with growth on galacturonic acid, digalacturonic acid, or a mixture of pectin plus polygalacturonic acid. Induction by the monomer was approximately 1.5-fold less than that by the dimer or polymer. Glycerol-grown cells possessed a basal uptake activity several-fold lower than cells grown on pectin or its derivatives while glucose-grown cells had negligible uptake activity. Uptake for the dimer displayed saturation kinetics, and was insensitive to inhibitors of ATP synthesis but sensitive to dissipators of the proton motive force. Unsaturated digalacturonic acid inhibited [^{3}H]digalacturonic acid uptake in a competitive manner while galacturonic acid did not.

Additional keywords: membrane transport, pectate utilization.

Many members of the plant pathogenic *Erwinia* produce pectolytic lyases and hydrolases necessary to break down the pectinaceous plant cell wall (Mount et al. 1970; Starr and Chatterjee 1972; Collmer and Keen 1986). In *E. chrysanthemi*, the importance of these enzymes in virulence and the utilization of pectate polymers has been the subject of much investigation (Kotoujansky 1987; Barras et al. 1994). A number of products are formed as a result of the action of bacterial pectolytic enzymes on polygalacturonic acid (PGA). These include galacturonic acid (GA) and saturated (dGA) and unsaturated digalacturonic acid (udGA), as well as saturated and unsaturated trimers and oligomers of galacturonic acid (Nasuno and Starr 1966; Collmer and Bateman 1981; Preston et al. 1992). These compounds are formed extracellularly and their rapid assimilation by the bacteria may play a crucial role in determining the outcome of either disease or resistance responses by the host plant. A subset of molecules released due to the depolymerization of pectate are taken up by the pathogen, after which they are catabolized to yield energy and inducers for the production of pectolytic enzymes

(Collmer and Bateman 1981; Collmer and Bateman 1982; van Gijsegem and Toussaint 1983; Chatterjee et al. 1985; Reverchon and Robert-Baudouy 1987). The uptake systems for GA and 2-keto-3-deoxygluconate (KDG) have already been studied in this bacterium (Hugouvieux-Cotte-Pattat et al. 1983; Condemine and Robert-Baudouy 1987; San Francisco and Keenan 1993). The transport of these molecules has been shown to be energy dependent and activated by growth of the bacteria in the presence of GA, dGA, or a mixture of pectin plus PGA. The *exuT* gene(s) encode the uptake system for GA and glucuronic acid (GU) (van Gijsegem and Toussaint 1983; Freeman and San Francisco 1994), while the *kdgT* gene encodes the KDG permease (Condemine and Robert-Baudouy 1987; Allen et al. 1989). The critical step in the entry of dGA (or udGA) into the bacterial cell has yet to be characterized. These studies focus on this important component in the multifaceted and dynamic interaction that occurs between plants and soft-rot *Erwinia* species (Beaulieu and van Gijsegem 1990; Gold et al. 1992; Bourson et al. 1993; Barras et al. 1994).

[^{3}H]dGA (15.5 mCi/mmol) was used as a substrate in the uptake studies. [^{3}H]dGA was prepared from saturated dGA (98% pure) purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeling was carried out essentially as described by Evans et al. (1974) with tritium gas in the presence of a metal catalyst. Purification and assay of the resulting [1-^{3}H]dGA was carried out using ion-exchange chromatography with AG-MP-1 resin and a linear gradient of 0 to 100% 1 M sodium formate, pH 4.7, as described by Donner et al. (1988). Prior to assays, cells were grown to exponential phase in M-9 salts medium supplemented with appropriate sources of carbon, amino acids, vitamins, and trace elements (Miller 1972). Carbon sources were added to a final concentration of 0.1%. Typically, *E. chrysanthemi* strains were grown shaken at 28°C. Carbon substrates used for growth were either glycerol, GA, dGA, or pectin plus PGA (0.05% each). Glycerol and GA were purchased from United States Biochemical Corporation (Cleveland, OH). Pectin, PGA, and dGA were purchased from Sigma Chemical Co.

Uptake was measured as described previously (San Francisco and Keenan 1993). Exponentially growing cells were harvested, washed, and starved for 15 min in transport assay buffer (M9 salts containing 5 mM $MgCl_2$, 0.3 mM dithiothreitol, and 40 mM Tris, pH 7.5) prior to uptake measurements. Uptake was measured at 28°C at various substrate concentrations. The final assay volume of 0.5 ml contained 50 μl of exponentially growing cells. In the metabolic inhibitor experi-

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ments, the cell suspension was preincubated for an additional period of 2 min with the inhibitor in the transport assay buffer at the assay temperature prior to the addition of radioactive substrate, (50 nmol [0.015 mCi] of [^{3}H]dGA per mmol). Uptake was terminated at various times by filtration through a nitrocellulose filter (0.45-μm pore size, Micro Separation Systems, Dublin, CA) and the filters immediately washed with 1.5 ml of transport assay buffer at the assay temperature. All competition and inhibitor experiments were carried out with pectin/PGA-grown cells. Competition experiments were carried out by adding the preincubated cell suspension at the assay temperature to a prewarmed mixture of ^{3}H-labeled substrate and the competitor. Filters were air dried, placed in scintillation vials containing 3 ml of Scintisafe 30% (Fisher Scientific, Pittsburgh, PA) and radioactivity measured in a Beckman 7600 scintillation counter.

Comparisons were made to determine the effect of the carbon substrate used for growth on the uptake activity. Glucose-grown cells showed negligible uptake activity, suggesting repression of the dGA uptake system in a manner similar to the repression of the GA uptake system (Hugouvieux-Cotte-Pattat et al. 1983) (Fig. 1). Glycerol-grown cells possessed basal uptake activity that was approximately three- to fivefold lower than cells grown on GA, dGA, or a mixture of pectin plus PGA. Induction of the uptake activity in cells grown on these substrates was reflected in both the initial rates and magnitude of [^{3}H]dGA accumulation. GA-grown cells consistently showed lower uptake activity than cells grown on the dimer or polymer of GA. This might indicate incomplete induction of the uptake system by GA. Indeed, inducers of the pectin degrading enzymes, 5-keto-4-deoxy-uronate (DKI), 2,5-diketo-3-deoxygluconate (DKII), and KDG are generated due to the intracellular catabolism of dGA, (and possibly trimers and oligomers), whereas intracellular catabolism of GA results only in the formation of KDG (Collmer and Bateman 1981; Reverchon et al. 1991). Up-regulation of dGA transport by molecules of pectin degradation or their intracellular metabolites is not unexpected since the uptake of GA and KDG has also been shown to be similarly inducible (Hu-

gouvieux-Cotte-Pattat et al. 1983; Condemine and Robert-Baudouy 1987; San Francisco and Keenan 1993).

The degradation of pectin and pectate by extracellular enzymes produced by soft-rot *Erwinia* spp. and the assimilation of the products is one of several mechanisms employed by these bacteria in sensing their environment (Barras et al. 1994). Among the major products generated due to the action of these enzymes are saturated and unsaturated dGA (Preston et al. 1992). It has been suggested these molecules are taken up by the bacteria prior to their intracellular conversion into inducers for pectinase production (Collmer and Bateman 1981; Collmer and Bateman 1982; Reverchon et al. 1991; Nasser et al. 1992; Nasser, et al. 1994). This study directly demonstrates the uptake of [^{3}H]dGA into *E. chrysanthemi* cells.

Cells grown in the presence of pectin plus PGA were used in studies to determine the kinetic parameters of dGA uptake. Entry of [^{3}H]dGA into cells of *E. chrysanthemi* was saturable with increasing substrate concentrations between 0.001 mM and 0.25 mM, with an apparent K_m of 67 μM and a V_{max} of 115 nmoles/min/mg protein (Fig. 2). The transport system concentrated the substrate approximately 250-fold within cells (1 mg [dry weight] of cells has a cell volume of approximately 2.7 μl (Winkler and Wilson 1966; Hugouvieux-Cotte-Pattat et al. 1983; San Francisco and Keenan 1993)). The dependence of [^{3}H]-dGA uptake on a source of metabolic energy was investigated using a variety of metabolic inhibitors. Uptake was only slightly inhibited by sodium arsenate (5 mM), with more than 80% of the activity retained (Table 1). Potassium cyanide (5 mM) and 2,4-dinitrophenol (1 mM), however, were more potent inhibitors, reducing uptake activity 61 and 85%, respectively. Both these compounds interfere with electron transport and the maintenance of an energized membrane. Thus, the uptake of [^{3}H]dGA appears to be proton motive force–dependent.

udGA, one of the primary products of pectate degradation by pectate lyases, structurally resembles dGA except for 4,5-
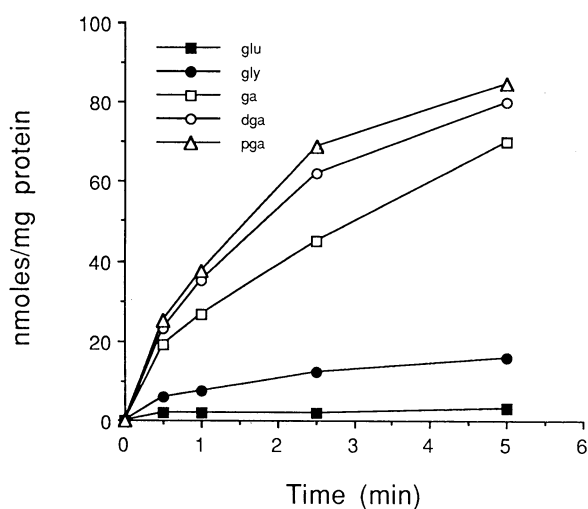

Fig. 1. Induction of [^{3}H]digalacturonic acid uptake in *Erwinia chrysanthemi*. Cells were grown on the indicated carbon source and assayed for uptake of [^{3}H]digalacturonic acid. Values represent averages of at least three independent experiments.
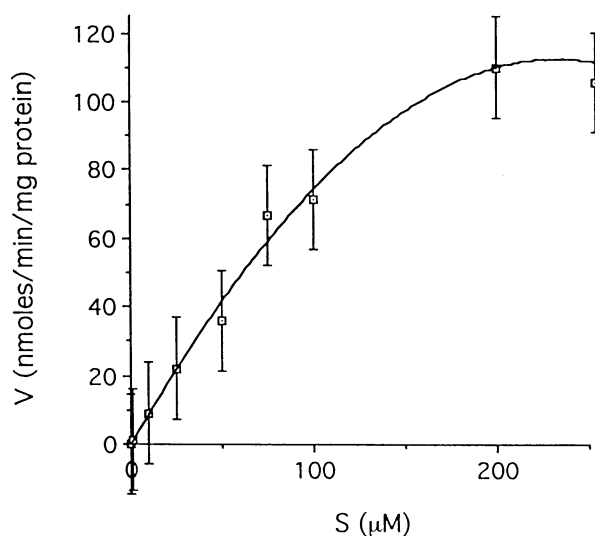

Fig. 2. Uptake of [^{3}H]digalacturonic acid in whole cells of *Erwinia chrysanthemi* EC16. Measurements were made 30 s after the addition of the radioactive substrate. Values represent averages of four independent experiments. Bars represent the standard errors.

unsaturation at the nonreducing end, and thus might utilize the same uptake system as dGA. Uptake of [³H]dGA was measured in the presence of udGA to monitor its effect on the rate and affinity of the dGA permease. Figure 3 shows that uptake of [³H]dGA was competitively inhibited by udGA with an apparent K_i of 300 μM. This suggests that the two molecules are taken up by the same system, or at least share a common component in their uptake systems. Differences between the K_i and the K_m indicate that there may be more than a single route of dGA entry into the bacteria. Similar studies on the uptake of [³H]dGA in the presence of GA showed no effect on the uptake of the dimer, suggesting that GA and dGA do not share the same uptake system. *Escherichia coli* GA uptake mutants containing *E. chrysanthemi exuT* genes to complement the defect have also been shown to be defective in [³H]dGA uptake (*E. coli* cannot grow on or take up dGA (San Francisco and Keenan 1993)). Furthermore, an *E. chrysanthemi* B374 GA uptake mutant (strain ERH 215, (van Gijsegem et al. 1985)) is fully capable of [³H]dGA uptake (M. J. D. San Francisco, unpublished observations). Although these observations suggest that the uptake systems for GA and dGA are distinct, they do not rule out the possibility of shared components in the uptake of the two molecules.

Table 1. Effect of metabolic inhibitors on [³H]dGA uptake by whole cells of *Erwinia chrysanthemi* EC16

Addition	Concentration	Percent uptake
None	...	100 (1.6)
Arsenate	5 mM	82 (0.94)
Cyanide	5 mM	39 (1.0)
2,4-dinitrophenol	1 mM	15 (4.0)

[a] Control uptake rate was 35 nmoles/min/mg protein. Values represent an average of three independent experiments. Numbers in parentheses are the standard errors.
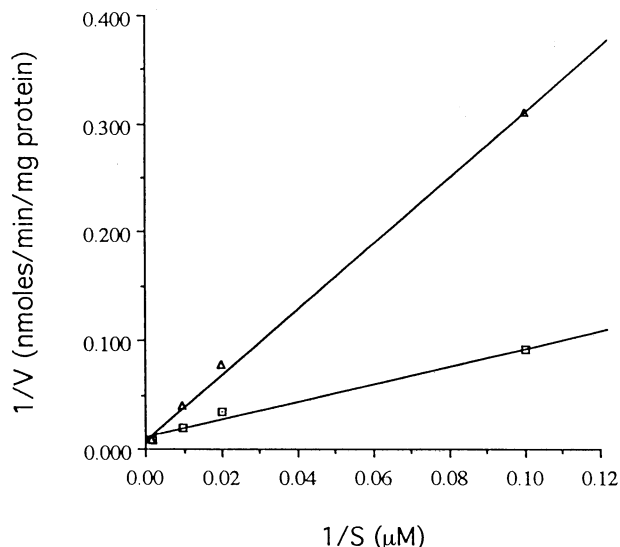
Fig. 3. Inhibition of [³H]digalacturonic acid ([³H]dGA) uptake by unsaturated digalacturonic acid (udGA). Initial rates of [³H]dGA were measured in cells of *Erwinia chrysanthemi* EC16 grown in pectin plus PGA. Concentrations of dGA ranged from 1 to 500 μM either with (△) or without (□) 1,000 μM udGA.

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