Alteration of Plasmalemma Sucrose Transport in *Phaseolus vulgaris* by *Pseudomonas syringae* pv. *syringae* and its Association with K^+/H^+ Exchange

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ABSTRACT

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Plasmalemma H^+ gradients created by H^+-extruding ATPases are believed to drive the active transport of sucrose, amino acids, and inorganic ions into plant cells. *Pseudomonas syringae* pv. *syringae*, a bean pathogen, activates a host plasma membrane K^+ efflux/H^+ influx exchange, which increases apoplastic pH from approximately 5.5 to 7.5 with consequent reduction or reversal of the H^+ gradient. Because the exchange response is strongly associated with bacterial multiplication in bean leaves, we have investigated the hypothesis that it promotes bacterial multiplication by altering host plasmalemma nutrient transport and thereby increasing availability of nutrients to bacteria. *P. s.* pv. *syringae* induced increased efflux and decreased influx of sucrose in bean tissue within 2–4 hr after inoculation. A bacterial mutant unable to induce the exchange response or multiply in bean leaves had no effect on sucrose transport. Uninoculated leaf tissue was infiltrated with buffers at pH 7–8 or with 25–50 mM K^+ salts to approximate intercellular conditions resulting from the exchange. These treatments induced changes in sucrose transport comparable to those induced by wild-type bacteria. Our findings indicate that the strong association of K^+/H^+ exchange with bacterial multiplication in bean leaves could be partly due to increased availability of sucrose in host intercellular spaces.

Additional key words: brown spot, ion transport, proton gradient.

The molecular basis for pathogenicity of many bacterial plant pathogens is not well understood. Examples include the *Pseudomonas syringae* pathovars, which multiply within leaf intercellular spaces and cause various types of leaf-spotting diseases. It is not known how populations of *P. syringae* reach high levels in leaf tissue. Some pathovars or strains produce toxins, but these are not required for pathogenicity (12,21). Nor do these bacteria produce large quantities of plant cell wall-degrading enzymes, which are pathogenicity determinants for other plant pathogens (5). Consequently, there is no satisfactory explanation for how these bacteria multiply in leaf tissue.

We are specifically studying the ability of *P. syringae* pv. *syringae*, causal agent of brown spot disease (6), to multiply within its host, *Phaseolus vulgaris*. Recently, we have shown that bacterial mutations resulting in the inability of bacteria to multiply within bean leaves are associated with loss of ability to activate a host plasmalemma K^+ efflux/H^+ influx exchange (2), which has been associated with induction of the hypersensitive response (HR) in tobacco (3,4). Induction of the exchange response in bean tissue results in the release within 12 hr of up to 40 μmol K^+/g of fresh weight of leaf tissue into the intercellular spaces. In addition, host intercellular fluid pH rises from approximately 5.5 to 7.5, and this pH change appears to promote bacterial population growth in leaf tissue (2). The association between bacterial multiplication and activation of the exchange is observed in both incompatible and compatible interactions of *P. s.* pv. *syringae* on bean (2). The primary distinctions are that initial rates of exchange are higher and the period of bacterial multiplication shorter in the incompatible interaction.

These results have been interpreted in the context of normal plant plasma membrane H^+ transport. Plant plasma membranes have H^+-extruding ATPases that create a H^+ gradient across the membrane, with external pH typically 5.5 and cytoplasmic pH 7–7.5 (20,25,26). This gradient is believed to drive the active uptake of sucrose, amino acids, and inorganic ions (K^+, PO_4^{2-}) from the apoplast into the mesophyll and phloem (9,10,18,19,22,23). Because K^+/H^+ exchange transports H^+ in the opposite direction as the ATPase, it serves to reduce or destroy the plasmalemma H^+ gradient. We have hypothesized that the resultant interference with active transport leads to a net accumulation of sucrose, amino acids, and inorganic ions within host intercellular spaces where bacteria reside, thus promoting bacterial multiplication. Our hypothesis is supported by reports that increased intercellular pH increases sucrose efflux and decreases sucrose influx in leaf tissue from a variety of plant species (9,13,27). We are also considering the possibility that increased intercellular K^+ may contribute to bacterial multiplication, given that this factor has also been shown to increase sucrose efflux from plant cells (11,24).

To test this hypothesis, we have investigated the effect of *P. s.* pv. *syringae* on sucrose transport in bean leaf tissue. We have also investigated the effects of increased intercellular pH and K^+ concentration, comparable to that resulting from K^+/H^+ exchange, on sucrose transport. Experiments were conducted with the wild-type strain of *P. s.* pv. *syringae*, which induces the exchange response and multiplies in bean (2). A bacterial mutant, strain B7, was included as a control. This strain differs from the wild-type by a single Tn*5* insertion mutation (4) and does not induce the exchange response, multiply, or cause visible symptoms in bean (2).

MATERIALS AND METHODS

Plants. Bean (*Phaseolus vulgaris* 'Pinto 111') plants were grown in a greenhouse in a 1:1:9 (v/v) mixture of peat moss, vermiculite, and soil. Four seeds (obtained from J. R. Stavely, USDA-ARS, Beltsville, MD) per pot were planted in 4-in. pots. The first two primary leaves of two- to three-week-old plants were used for all experiments.

Bacteria. *P. s.* pv. *syringae* strain 61 was isolated from wheat by M. Sasser, University of Delaware, and is avirulent or weakly virulent on bean. This strain has been found to multiply within and activate K^+/H^+ exchange in bean Pinto 111, used in this study (2). Strain B7 was obtained by Tn*5* insertion mutation of the wild-type strain and selection of mutants unable to induce the HR or the exchange response in tobacco. The details of this procedure and the selection of mutants are described elsewhere (4). Strain B7 was shown to have a single Tn*5* insertion and to be indistinguishable from the wild-type strain by growth rate in minimal medium, fatty acid profile, or colony morphology. It does not induce the exchange response or multiply in bean leaves. Bacterial inocula for experiments were prepared as previously described (2). Bacterial suspensions (2.5×10^8 colony-forming units per milliliter) were prepared in 0.5 mM 2-N-morpholino-ethanesulfonic acid (MES)-Tris, pH 6.0.

Effect of bacteria on sucrose uptake by leaf disks. Bacterial suspensions or buffer control were infiltrated into the intercellular spaces of bean leaves with a syringe and hypodermic needle. Excess water was allowed to evaporate for 1 hr. Leaf disks (0.6-cm diameter) were cut under the surface of distilled H_2O with a brass cork borer and transferred in groups of 12, underside down, to 50-ml beakers containing 5 ml of uptake assay medium (0.5 mM MES-Tris, pH 6.0, 0.5 mM $CaCl_2$, and 1 mM unlabeled sucrose). Radiolabeled sucrose (U-^{14}C, 4 mCi/mmol, New England Nuclear, Boston, MA) was added to each beaker to achieve a final level of 0.05 μCi/ml. Beakers were incubated under diffuse room light at 26–28 C on a rotary shaker at 120 rpm. At 2-hr intervals, three-leaf disks were removed from each beaker and given four 5-min washes in unlabeled assay medium to remove free space label. Disks were blotted dry on absorbent paper and transferred to glass scintillation vials with polyethylene cone caps. Disks were digested and decolorized for 24–48 hr in a mixture of 0.2 ml of 70% perchloric acid and 0.4 ml of 30% H_2O_2 at room temperature. Aquasol-2 (New England Nuclear, Boston, MA) scintillation fluid (10 ml) was added to each vial. Vials were counted in a liquid scintillation counter. A counting efficiency of approximately 65% was determined by the external standard-channels ratio method.

Effect of pH on sucrose uptake by leaf disks. Leaf disks were cut from uninfiltrated leaves and then vacuum-infiltrated with assay medium (50 mM MES or N-2-hydroxyethylpiperazine-N-2-ethanesulfonic [HEPES] acid-Tris, pH 5.5–8.0, 0.5 mM $CaCl_2$, and 1 mM sucrose). After infiltration, groups of three disks were immediately transferred to 50-ml beakers containing 5 ml of assay medium. The addition of radiolabeled sucrose and the measurement of its uptake were as described above except that sucrose uptake was measured for a single 2-hr period.

Effect of bacteria on release of [^{14}C] sucrose from leaf disks. Leaf disks were cut from leaves 1 hr after infiltration with bacterial suspensions or buffer. Disks were allowed to take up [^{14}C] sucrose for 1.5 hr under the conditions described for uptake experiments except that [^{14}C] sucrose in the assay medium was increased to 0.4–2.0 μCi/ml and unlabeled sucrose was omitted. Following this uptake period, individual sets of 10 disks were washed with four changes of unlabeled buffer to remove free space label and transferred to 10-ml beakers containing 1 ml of efflux assay medium (0.5 mM MES-Tris, pH 6.0, 0.5 mM $CaCl_2$, and 5 mM sucrose). Disks were incubated under conditions given for uptake experiments. At 1.5- to 2-hr intervals, 0.5-ml aliquots of assay medium were collected for scintillation counting and the remaining 0.5-ml volume was frozen for subsequent analysis by thin-layer chromatography. Fresh assay medium (1 ml) was added to the disks and the incubation was repeated three times. Liquid scintillation samples were mixed with 10 ml of scintillation fluid, with a resultant counting efficiency of approximately 75%.

Analysis of ^{14}C released from leaf disks. Assay medium containing ^{14}C released from wild-type inoculated leaf disks was spotted onto precoated 0.25-mm silica gel 60 plates and chromatographed using 2-propanol-acetone-H_2O) (50:30:20). Plates were divided into 0.5- to 1.0-cm horizontal sections, and silica gel from each was scraped and transferred to liquid scintillation vials. Profiles of ^{14}C were compared to those for sucrose and other standards. Standards were detected by spraying plates with either 5% H_2SO_4 in EtOH (sugars), 0.2% ninhydrin in EtOH (amino acids), or 0.04% bromcresol green in EtOH (organic acids). Plates treated with 5% H_2SO_4 were subsequently heated at 60 C for approximately 1 hr.

Effect of pH and salts on release of [^{14}C] sucrose from leaf disks. Disks were cut from leaves that had not been infiltrated and were allowed to take up [^{14}C] sucrose for 1.5 hr as described above. Disks were then vacuum-infiltrated with assay medium containing 50 mM MES or HEPES-Tris, pH 5.5–8.0, 0.5 mM $CaCl_2$, and 5 mM sucrose. For salt experiments, assay medium contained 0.5 mM MES-Tris, pH 6.0, 0.5 mM $CaCl_2$, 5 mM sucrose, and 25–100 mM salt or mannitol. After infiltration, groups of 10 disks were immediately transferred to 10-ml beakers containing 1.5 ml of the appropriate assay medium and incubated for 2 hr. Aliquots (1 ml) were collected for scintillation counting in 10 ml of scintillation fluid.

RESULTS

Sucrose uptake by leaf disks. The data in Figure 1 show that control bean leaf disks (previously infiltrated with 0.5-mM buffer) took up [^{14}C] sucrose from the external assay medium for at least 8 hr after disks were cut. The quantity of ^{14}C taken up during each 2-hr uptake period increased from approximately 1 μmol/g

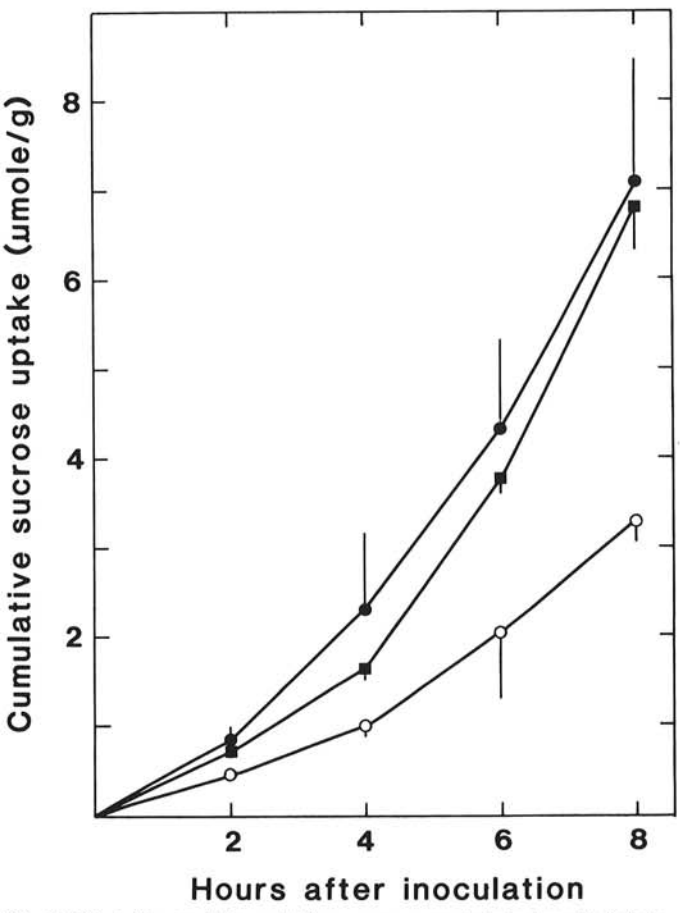

Fig. 1. Effect of bacterial inoculation on sucrose uptake by bean leaf disks. Intact leaves were infiltrated at −1 hr with 0.5 mM 2-N-morpholino-ethanesulfonic acid-Tris, pH 6.0, 2.5×10^8 colony-forming units (cfu) of *Pseudomonas syringae* pv. *syringae* wild-type strain/ml of same buffer, or 2.5×10^8 cfu/ml *P. s.* pv. *syringae* mutant B7, which does not multiply or activate K^+/H^+ exchange in bean. Leaf intercellular spaces were allowed 1 hr to dry, after which leaf disks were cut and transferred to buffer supplemented with 0.5 mM $CaCl_2$, 1 mM sucrose, and 0.04 μCi/ml [^{14}C] sucrose. Each data point represents total accumulation of sucrose at the time given. Data are means ± 1 SD of three replicates. Standard deviation bars are omitted where they are smaller than the symbol. Buffer control, —■—; B7, —•—; wild-type, —o—.

initially to 3 μmol/g between 6 and 8 hr after inoculation. A total of 6–8 μmol sucrose was accumulated within 8 hr. Prior infiltration of leaf tissue with *P. s.* pv. *syringae* strain B7 did not alter sucrose uptake relative to the buffer control (Fig. 1). In contrast, leaf disks inoculated with the wild-type strain exhibited approximately 50% less uptake at all time points relative to the two controls.

To determine whether increased intercellular pH due to K^+/H^+ exchange might contribute to these results, we measured sucrose uptake in leaf tissue infiltrated with 50-mM buffers ranging in pH from 5.5 to 8.0. Tissue infiltrated with pH 5.5 buffers exhibited the greatest uptake (Fig. 2). Small decreases were observed with pH 6.0–6.5 buffers and sharp declines between pH 6.5 and 8.0 to levels that were 45–60% lower than at pH 5.5.

The exchange increases intercellular K^+ concentrations, so we also investigated the effect of intercellular K^+ on sucrose uptake. The infiltration of leaf disks with 0.5-mM, pH 6.0, buffer supplemented with 5–50 mM K-gluconate had little or no effect on sucrose uptake. In a representative experiment, ^{14}C uptake in the presence of 5, 10, 25, and 50 mM K-gluconate was 106 ± 5, 100 ± 6, 98 ± 5, and 97% ± 10, respectively, that of the control (no K-gluconate). Similar results were obtained with KCl and K_2SO_4 (*unpublished data*).

Release of [^{14}C] sucrose from leaf disks. Uninoculated leaf disks, previously allowed to take up [^{14}C] sucrose, released the radiolabel when transferred to a nonradioactive efflux solution. Release of ^{14}C was initially rapid but slowed with time (Fig. 3). Prior inoculation of leaf tissue with mutant strain B7 did not alter the release of ^{14}C relative to the buffer control. Leaf tissue inoculated with the wild-type bacterial strain exhibited an almost constant rate of ^{14}C release for at least 10 hr after inoculation. This rate was approximately 1.5 times that of control rates between 2 and 4 hr after inoculation, increasing to eight times the control rates between 8 and 10 hr. Total release of ^{14}C was more than three times that of either control.

Aliquots of the assay medium were analyzed by thin-layer chromatography to determine how much of the ^{14}C released from wild-type inoculated bean tissue remained as [^{14}C] sucrose. Of the total ^{14}C released from disks between 2 and 4 hr, 4 and 6 hr, 6 and 8 hr, and 8 and 10 hr after inoculation, 85, 77, 74, and 68%, respectively, comigrated with sucrose during thin-layer chromatography on silica gel (Fig. 4). The identities of other labeled compounds were not determined. Of the [^{14}C] sucrose stock solution, 96–98% comigrated with sucrose. With the exception of galactose, amino acid, organic acid, and sugar standards did not comigrate with sucrose.

The effect of intercellular pH on the release of ^{14}C from leaf disks was assessed by measuring ^{14}C efflux from leaf disks infiltrated with 50 mM buffers ranging in pH from 5.5 to 8.0. The quantity of

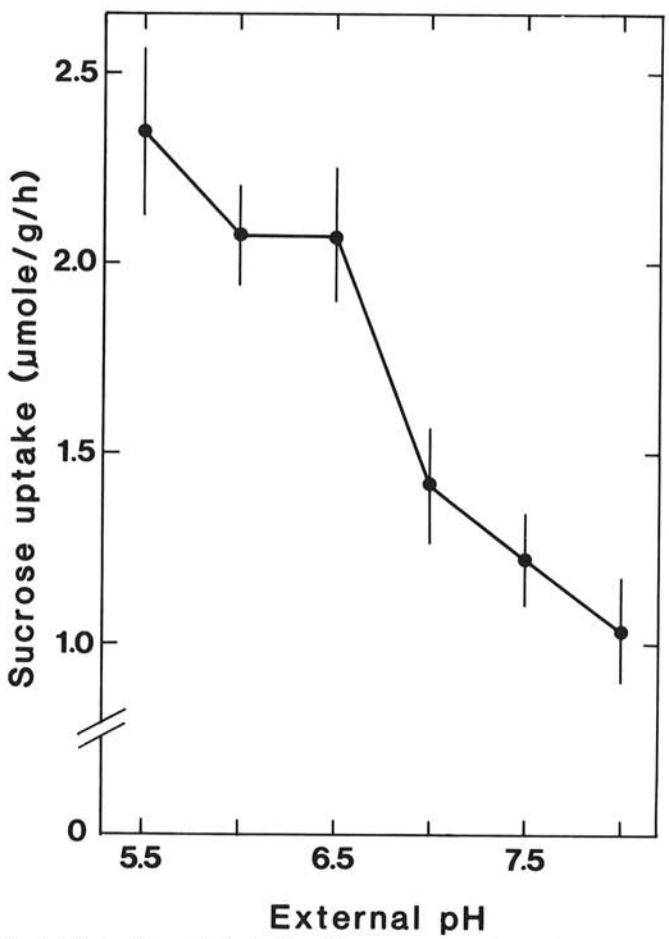

Fig. 2. Effect of intercellular buffer pH on sucrose uptake. Leaf disks from uninoculated leaves were vacuum-infiltrated with 50 mM 2-N-morpholino-ethanesulfonic acid-N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 5.5–8.0. Buffer was then supplemented with 0.5 mM $CaCl_2$, 1 mM sucrose, and 0.05 μCi/ml [^{14}C] sucrose. Uptake of [^{14}C] sucrose during the first 2 hr after infiltration was determined as in Figure 1. Data are means and range of two replicates.

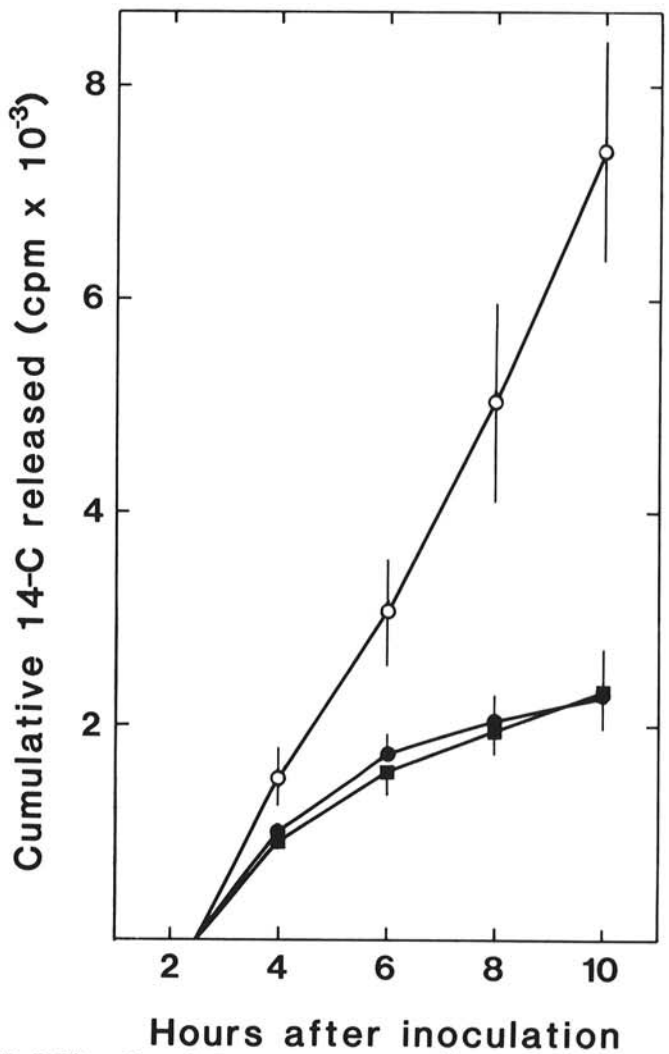

Fig. 3. Effect of bacterial inoculation on release of ^{14}C from bean leaf disks. Leaf disks were prepared as in Figure 1. Disks were incubated in 0.5 mM 2-N-morpholino-ethanesulfonic acid-Tris, pH 6.0, supplemented with 0.5 mM $CaCl_2$ and 0.4–2.0 μCi [^{14}C] sucrose/ml, and allowed to take up [^{14}C] sucrose for 1.5 hr. Disks were washed to remove free space label and transferred into a solution containing 5 mM unlabeled sucrose for measurement of ^{14}C release. Data points represent total ^{14}C released at the time given. Data are means ± 1 SD of three replicates. Standard deviation bars are omitted where they are smaller than the symbol or where overlap would occur. Buffer control, —■—; B7, —•—; wild type, —o—.

^{14}C released from disks was relatively constant between pH 5.5 and 7.0 but increased sharply between pH 7.0 and 8.0 (Fig. 5).

The effect of increased intercellular K^+ concentrations on the release of ^{14}C from leaf disks was also investigated. The infiltration of leaf tissue with 50 mM K^+ in the form of KCl, K_2SO_4, or K-gluconate increased the release of ^{14}C by a factor of 2–3 (Fig. 6). This effect was apparently not specific for K^+, because it was also observed with NaCl and $MgSO_4$. Mannitol at 50–100 mM did not stimulate the release of ^{14}C, indicating that the increase due to salts was not an osmotic effect.

DISCUSSION

Our results indicate that *P. s.* pv. *syringae* can alter the transport of sucrose into and out of host cells. Altered transport developed at approximately the same rate as K^+/H^+ exchange (2) and was not induced by a bacterial mutant unable to activate the exchange. Our results also suggest that changes in sucrose transport are at least partly due to increased intercellular pH and K^+ concentration. These findings provide a reasonable explanation for the strong association between bacterial induction of the exchange response and multiplication in leaf tissue (2).

Because *P. s.* pv. *syringae* (like many bacterial pathogens) does not penetrate the host cell wall or plasma membrane, the ability to alter host nutrient transport may be required for significant multiplication in the intercellular spaces of leaves. It has been reported that intercellular fluid extracted from tobacco leaves supports the growth of the pathogen, *P. s.* pv. *tabaci* (15). However, this fluid was obtained by centrifugation of tobacco leaves cut along the longitudinal axis and therefore must have contained nutrient-rich vascular sap. Accurate determination of apoplastic solute concentrations is extremely difficult. Estimates for sucrose in leaf tissue vary from 20 μM to 1–5 mM (1,10,14,23). These are average concentrations that do not reflect the unequal distribution of sucrose within the apoplast. It is generally believed that apoplastic sucrose concentrations are markedly higher near the phloem where loading occurs than at points distant (10,14). Although these results indicate considerable uncertainty about apoplastic sucrose levels, they suggest that, in at least some cases, carbon availability within host intercellular spaces may limit bacterial multiplication.

By altering host plasmalemma H^+ and K^+ gradients, pathogenic bacteria can draw sucrose from plant cells without causing structural damage to the plasma membrane. In contrast, a structurally damaged membrane would presumably release not only desirable substances, but also toxic substances from plant vacuoles and toxic products of oxidative enzymes. Induction of the exchange response thus appears to be an exceptionally selective method to obtain nutrients without toxic substances caused by cellular damage. Our results are consistent with previous reports that increased extracellular pH increases sucrose efflux and decreases sucrose influx (9,13,24,27,28) and that extracellular K^+ increases sucrose efflux (11,24) in plant cells.

Our results are also consistent with the current hypothesis that sucrose transport across the plasma membrane of plant cells is mediated by a specific carrier protein which cotransports H^+ (9,14,23). Such a carrier should operate in either direction (influx or efflux) with relative rates depending on plasmalemma H^+ and

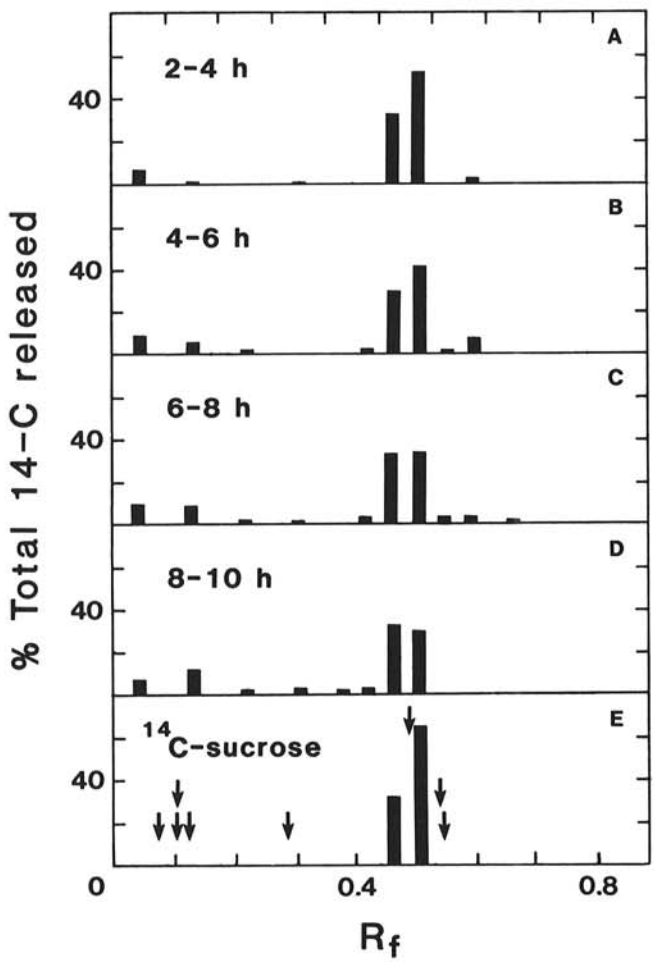

Fig. 4. Thin-layer chromatography of ^{14}C released from bean leaf disks between 2 and 10 hr after inoculation with the wild-type strain of *Pseudomonas syringae* pv. *syringae*. In the upper four panels, bars represent ^{14}C profiles for efflux samples collected at intervals after inoculation. In the lowermost panel, bars represent [^{14}C]sucrose. Arrows from left to right indicate R_f values for galacturonic acid, alanine and citric acid, glycine, malic acid, galactose, fructose, and glucose.

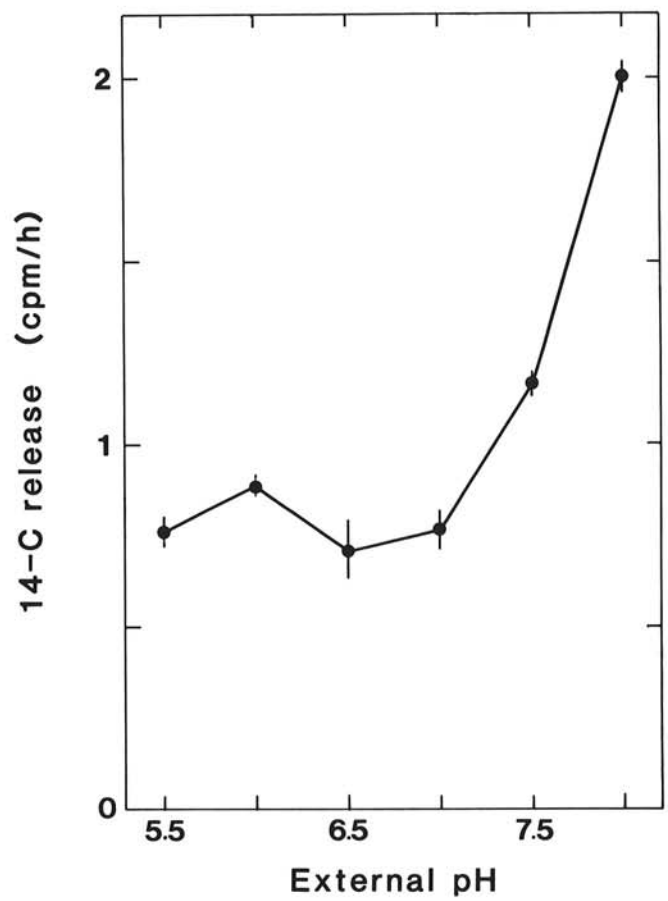

Fig. 5. Effect of intercellular buffer pH on release of ^{14}C from bean tissue. Disks were cut from untreated leaves and allowed to take up [^{14}C]sucrose for 1.5 hr as in Figure 3. Disks were washed to remove free space label and vacuum-infiltrated with 50 mM 2-N-morpholino-ethanesulfonic acid-N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 5.5–8.0. Efflux of ^{14}C into the same buffer was measured. Data represent ^{14}C released during the first 2 hr after infiltration. Data are means and range of two replicates.

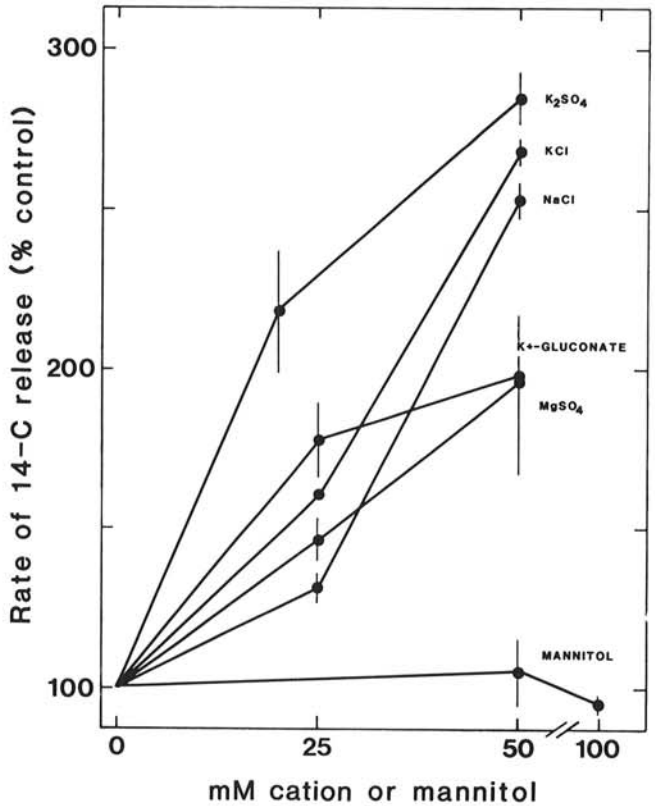

Fig. 6. Effect of K^+, Na^+, and Mg^{2+} salts, and mannitol on release of ^{14}C from bean leaf disks. Experimental details are the same as for Figure 5, except that disks were vacuum-infiltrated with salt or mannitol solutions following the uptake of [^{14}C] sucrose.

sucrose gradients. Under normal conditions, the steep plasmalemma H^+ gradient drives sucrose uptake. With the loss or even a reversal of the H^+ gradient due to K^+/H^+ exchange, sucrose efflux may be driven by high intracellular sucrose and H^+ concentrations. The stimulation of sucrose efflux by external K^+ is less well understood. Secor (24) speculated that K^+ may either directly affect the activity of the sucrose-H^+ carrier or may serve as a counterion to enhance sucrose-H^+ efflux.

Because plasmalemma transport of amino acids and certain inorganic ions, such as PO_4^{2-}, are also believed to depend on H^+ gradients (17,18,22,23), the results shown here may be representative of these other nutrients. However, we have not tested any other nutrients. Although a carbon source and K^+ are necessary for bacterial multiplication, they must be accompanied by adequate levels of nitrogen-containing compounds, PO_4^{2-}, and trace elements. Thus, it would be interesting to investigate free space concentrations of such substances and alterations in transport that might be associated with the exchange response.

We have previously classified the interaction of the wild-type bacterium and bean cultivar used in this study as incompatible or weakly compatible (2). However, because induction of the exchange response is an early step in the development of susceptibility (2) as well as hypersensitivity (3,4) in the host, our present results suggest that alteration of host sucrose transport occurs during both incompatible and compatible bacterium-plant interactions. This is consistent with reports that incompatible bacteria multiply to a significant extent within leaf tissue (2,7,8,16). However, population growth of incompatible bacteria ceases before that of compatible bacteria, resulting in lower maximum population densities. In contrast, saprophytic bacteria such as *P. fluorescens* do not induce the exchange response (*unpublished data*), do not multiply significantly within leaf tissue, and do not induce the HR or cause disease in any host (16). Thus, we propose that the ability to induce the exchange response is a fundamental pathogenicity factor that distinguishes pathogenic from saprophytic pseudomonads. Incompatible and compatible pathogens may be distinguished not by the ability to induce the exchange but rather by the initial severity or latency of induction (2; Atkinson and Hutcheson, *unpublished data*). At equivalent inoculum levels, incompatible interactions are associated with higher exchange rates, whereas compatible interactions are characterized by lower initial exchange rates and/or delayed induction.

We have shown that the association of K^+/H^+ exchange in bean leaves with multiplication of *P. s.* pv. *syringae* may in part be due to alteration of host sucrose transport. Because sucrose and other nutrients are largely compartmentalized within plant cells and vascular elements, the ability to draw nutrients from these sources may contribute to bacterial pathogenicity.

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