Early Responses During Plant-Bacteria Interactions in Tobacco Cell Suspensions

C. Jacyn Baker, Nichole R. O'Neill, L. Dale Keppler, and Elizabeth W. Orlandi

First, third, and fourth authors: Microbiology and Plant Pathology Laboratory, United States Department of Agriculture, ARS, Beltsville, MD 20705.

Second author: Germplasm Quality and Enhancement Laboratory, USDA, ARS, Beltsville, MD 20705.

Correspondence should be addressed to the first author.

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ABSTRACT

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Bacteria that induce a hypersensitive response in tobacco leaves initiate a net uptake of extracellular H^+ and a net increase in extracellular K^+ in tobacco suspension cells. Other studies have shown that these interactions also result in a transient increase in lipid peroxidation and O_2^- production. The relationships between these early plant responses were studied simultaneously using suspension-cultured tobacco cells and compatible, incompatible, and saprophytic bacteria. The bathing medium was monitored during a 6-h period for pH, conductivity, $[K^+]$, and luminol-dependent chemiluminescence. In all bacterial treatments, an immediate increase in chemiluminescence and pH occurred within 30 min after addition of the bacteria. After about 2 h a second increase in pH, conductivity, $[K^+]$, and chemiluminescence occurred with incompatible bacteria. The first response appears nonspecific, while the second response appears to depend on the host/pathogen incompatibility.

We are studying the very early biochemical interactions that occur between plants and bacteria during the hypersensitive reaction (HR). In 1985 Atkinson et al (3,4) reported that one of the earliest detectable responses associated with the HR in tobacco involves an uptake of H^+ by the plant cells and a net K^+ efflux (K^+/H^+ response). Subsequent work with Tn*5* mutants of *Pseudomonas syringae* confirmed the close association between this K^+/H^+ response in cell suspensions and the hypersensitive response in leaves (6). Therefore, our current efforts are focused on understanding the mechanisms leading to this K^+/H^+ response, which is a much more quantifiable phenomenon than the HR itself.

Immediately after the addition of bacteria to tobacco cell suspension cultures, we have observed concurrent increases in both superoxide levels, detected with nitroblue tetrazolium reduction, and lipid peroxidation, detected by ethane production and thiobarbituric acid reactivity of breakdown products (11). Superoxide scavengers, which inhibited these increases in superoxide and lipid peroxidation, also reduced the K^+/H^+ response, suggesting the possible involvement of active oxygen metabolism in the latter. Studies using luminol-dependent chemiluminescence (LDC), which allowed closer monitoring of active oxygen production, supported these findings (12). The term *active oxygen* signifies species of oxygen that oxidize substrates without energy input, which in this situation involves O_2^-, H_2O_2, and $OH\cdot$.

In this study several early events in the tobacco/bacteria interaction were simultaneously monitored on the same cell suspensions using a broader range of bacteria to compare incompatible, compatible, and saprophytic interactions. The objective was to examine more precisely how these responses interrelate chronologically in different plant-bacteria interactions. Preliminary results from this study have been reported (7,13).

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MATERIALS AND METHODS

Bacteria. Bacteria used in this study that cause a hypersensitive reaction on tobacco included *P. s.* pv. *glycinea,* races 4 and 6, and a nalidixic acid-resistant strain of *P. s.* pv. *syringae* 61 NalR (wild type, WT). Bacteria that did not cause a hypersensitive reaction on tobacco included *P. s.* pv. *syringae* B7 (a Tn*5* mutant of the WT strain) (6), *P. s.* pv. *tabaci* (compatible), and *P. fluorescens* (saprophyte). Cultures were maintained on King's B agar at 30 C. The *P. s.* pv. *syringae* strains were maintained on 50 μg/ml of nalidixic acid with 25 μg/ml of kanamycin sulfate added for B7.

Bacterial inocula were prepared from plates that were 20–22 h old. The bacteria were suspended in 0.5 mM MES (2-[N-morpholino]ethanesulfonic acid), pH 6.0, washed one time in buffer by centrifugation, and diluted in either the same buffer for most assays or in 5.0 mM MES, pH 5.60, for chemiluminescence assays (12). Bacterial density was determined turbidimetrically and checked periodically by dilution plating.

HR bioassay. Tobacco plants (*Nicotiana tabacum* L. 'Hicks') were grown under greenhouse conditions and infiltrated with 5×10^8 cfu/ml as previously described (6). All plants were observed 24 h later for hypersensitive necrosis. One leaf on four different plants was inoculated for each bacterial strain.

K^+/H^+ response bioassay. The K^+/H^+ response was monitored in tobacco suspension cells as in previously described procedures (1,2). The standard assay medium contained 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 0.5 mM MES, pH 6.0. Cells were collected on Miracloth (Calbiochem, La Jolla, CA), washed with assay medium, and then transferred, 0.5 g fresh weight, to 50-ml beakers containing 15 ml of assay medium. Cell suspensions were preincubated at 27 C on a rotary shaker at 180 rpm for 1 h. Bacterial inoculum in MES buffer or buffer alone, 1 ml, was added for a final concentration of 1×10^8 cfu/ml, and the pH of the mixture was immediately adjusted to about 6.0. The pH was monitored over a 6-h period and the change in pH converted to μmole H^+ as determined by a titration curve. The differ-

ence between the treatment and buffer controls was then determined. Data represent the average of three experiments with two replicates each.

Periodically, aliquots of the assay mixture were filtered and assayed for K^+ and Cl^- using ion chromatography. The pH and conductivity of the suspension were measured directly using electrodes.

Ion chromatography. Aliquots of 0.4 ml were collected and suspension cells gently removed by filtration through Miracloth. Samples were then passed through 4-mm nylon syringe filters with 0.2-μm pore size to remove bacterial cells. Filtrates were stored at −20 C and later assayed for K^+ and Cl^- by ion chromatography. The latter was monitored to insure cell lysis did not occur.

Ion chromatography was performed using Waters IC-Pak columns and a Waters 430 conductivity detector (Waters Chromatography Division, Milford, MA). Samples were injected onto an IC-PakC column with 2 mM HNO_3 as the eluent to quantify monovalent cation concentrations. Anion chromatography was performed on an IC-PakA column with 8 mM benzoic acid and 3% methanol adjusted to pH 6.71 with NaOH.

LDC determinations. Chemiluminescence measurements were carried out as previously described (12). The suspension cells, 0.033 g fresh weight per milliliter, and bacteria, 1×10^8 cfu/ml, were from the same cultures as used for the K^+/H^+ response assays, except that the assay medium contained 5.0 mM MES buffer, pH 5.6, to maintain constant pH. One-milliliter aliquots of tobacco cell suspension in 5 mM assay buffer were transferred to scintillation vials and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma, St. Louis, MO) was added. Aliquots at time periods 0–1 h received 5 μl from a stock solution, and later time periods received 10 μl, corresponding to final concentrations of 5 and 10 μM, respectively. Vials were immediately counted in a Beckman scintillation counter in the "out-of-coincidence" mode (12).

H_2O_2 scavenging in cell suspensions by endogenous catalase (bacterial or plant cell) was estimated as previously described (12), measuring LDC in the presence of the catalase inhibitor, aminotriazole (3-amino-1,2,4-triazole, Sigma) (10), which is added to aliquots 2 min before determining LDC.

Experimental statistics. Results are the average of three experiments with two replicates each. In most cases the data reflect the difference in response between the treatment and the buffer control. In some cases standard error bars were omitted to avoid confusion on the graph. However, for these graphs the standard error bars shown for *P. s.* pv. *syringae* WT (HR-causing) and B7 (non-HR-causing) are representative of similar bacterial treatments without error bars.

RESULTS

Hypersensitive response bioassay. Tobacco leaf tissue inoculated with *P. s.* pv. *syringae* WT and *P. s.* pv. *glycinea* races 4 and 6 developed a HR within 24 h. Tissue inoculated with *P. s.* pv. *tabaci, P. s.* pv. *syringae* B7, and *P. fluorescens* appeared normal after 24 h. However, after 2–3 days *P. s.* pv. *tabaci* treatments became discolored and, eventually, necrotic.

K^+/H^+ response bioassay. Immediately after bacterial inoculation, 0–1 h, a small increase in extracellular H^+ uptake, 0.5–1.5 μmoles/g of tissue, occurred in the assay medium of tobacco cell suspensions incubated with compatible, incompatible, or saprophytic bacteria (Fig. 1A). Between 2 and 6 h after treatment with HR-causing bacteria, a second, more prolonged, and greater H^+ uptake occurred. This second response represented an additional 10–12 μmoles/g of tissue. The extracellular H^+ uptake of tobacco suspensions incubated with non-HR-causing bacteria was about 4 μmoles/g of tissue during the 6-h period. The changes in conductivity followed very similar patterns to the pH changes for each bacteria (Fig. 1B). Increases in extracellular $[K^+]$ occurred between 2 and 6 h in HR-causing bacterial treatments, about 10–12 μmoles/g of tissue (Fig. 1C). There was no increase in $[K^+]$ immediately after addition of bacteria. Monitoring of $[Cl^-]$ by ion chromatography showed no significant differences between controls and treatments with non-HR- and HR-causing bacteria.

Luminol-dependent chemiluminescence determinations. Increased LDC was detected during two periods after treatments. The first occurred immediately after addition of bacteria, 0–0.5 h, and appeared to be nonspecific, occurring in all bacterial treatments (Fig. 2A). It was characterized by a large increase followed by a rapid decline during the first hour in all treatments except with the saprophyte *P. fluorescens,* which remained sporadic but relatively high throughout the 6-h period. A second subtle increase in LDC began after 2–3 h in treatments with HR-causing bacteria, while chemiluminescence in treatments with *P. s.* pv. *tabaci* and *P. s.* pv. *syringae* B7 continued to decrease (Fig. 2B).

Because H_2O_2 is suspected of being the major form of active oxygen in these interactions (12), we added a catalase inhibitor, aminotriazole, to determine whether catalase was present and

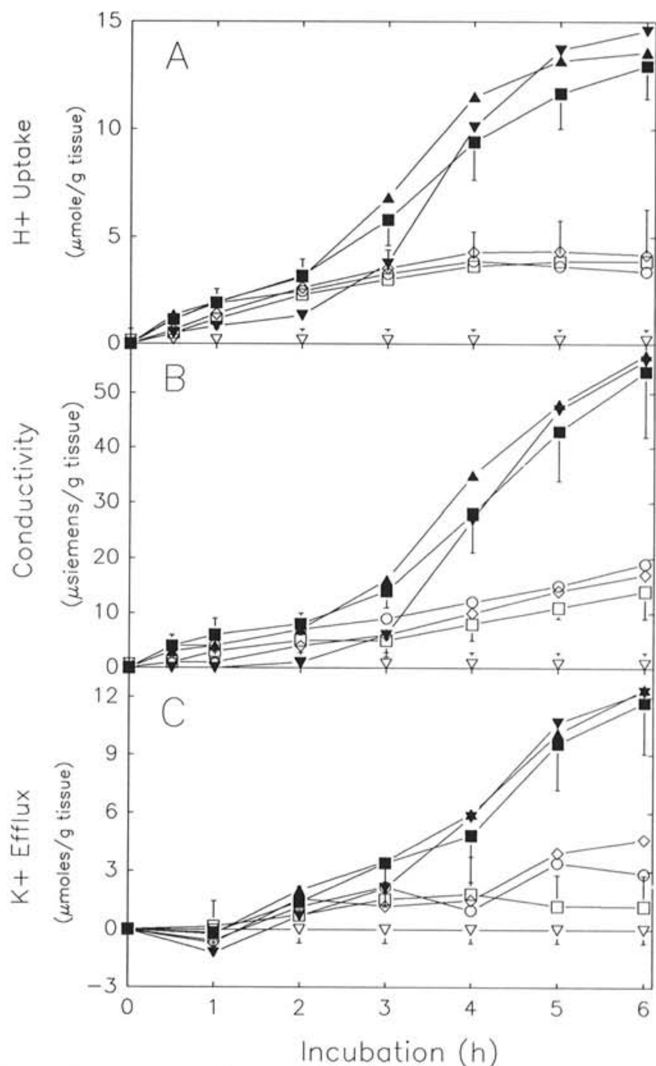


Fig. 1. Monitoring of H^+ uptake, conductivity, and K^+ efflux of tobacco suspension cells incubated with compatible, incompatible, and saprophytic bacteria. **A,** H^+ uptake was monitored by a pH meter and μmoles/g of tissue was calculated using titration curves; **B,** conductivity was monitored by a conductivity meter; **C,** K^+ efflux was determined by ion chromatography. Bacteria that result in a hypersensitive reaction on tobacco are represented by solid symbols (*Pseudomonas syringae* pv. *syringae* wild-type, ■; *P. s.* pv. *glycinea* race 4, ▲, and race 6, ▼); open symbols designate bacteria that do not cause a hypersensitive reaction on tobacco (*P. s.* pv. *syringae* B7, □; *P. s.* pv. *tabaci,* ○; *P. fluorescens,* ◇; buffer control, ▽). The data represent the difference between bacterial treatments and buffer controls and are the average of three experiments each with two replicates. The standard error bar is shown for *P. s.* pv. *syringae* wild-type and B7 and is typical of the standard errors of the other bacterial treatments.

reducing the active oxygen levels detected by our assay. The addition of aminotriazole to aliquots of both non-HR- and HR-causing bacterial treatments for 2 min before LDC readings increased LDC 4–5× and emphasized the differences in active oxygen levels between these treatments during the 2- to 6-h period after bacterial addition (Fig. 3).

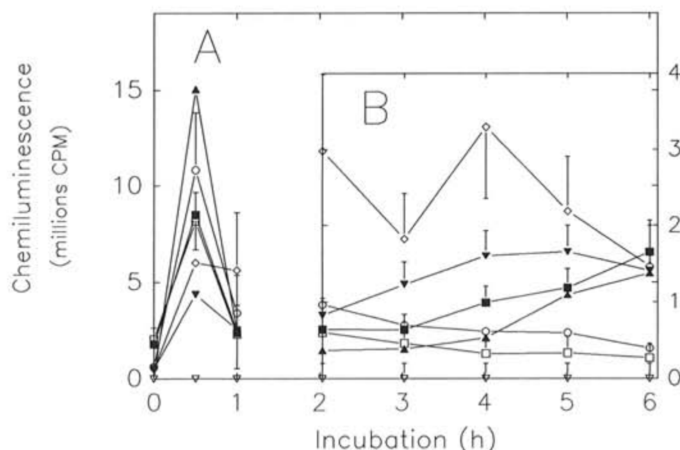


Fig. 2. Luminol-dependent chemiluminescence of tobacco suspension cells incubated with compatible, incompatible, and saprophytic bacteria. Chemiluminescence was measured in the presence of luminol (5 μM, 0–1 h; 10 μM, 2–6 h) and counted with a scintillation counter in "out-of-coincidence" mode. Bacteria that result in a hypersensitive reaction on tobacco are represented by solid symbols (*Pseudomonas syringae* pv. *syringae* wild-type, ■; *P. s.* pv. *glycinea* race 4, ▲, and race 6, ▼); open symbols designate bacteria that do not cause a hypersensitive reaction on tobacco (*P. s.* pv. *syringae* B7, □; *P. s.* pv. *tabaci,* ○; *P. fluorescens,* ◇ ; buffer control, ▽). The data represent the difference between bacterial treatments and buffer controls and are the average of three experiments each with two replicates. The standard error bar is shown for *P. s.* pv. *syringae* wild-type and B7 and is typical of the standard errors of the other bacterial treatments, unless otherwise indicated. The Y scale of **B** is reduced to better observe subtle changes.

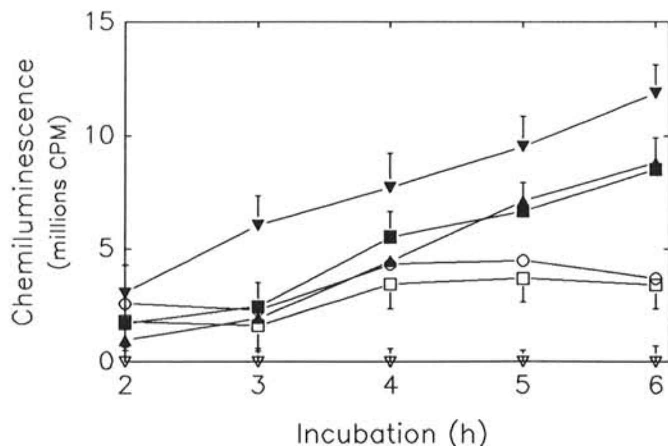


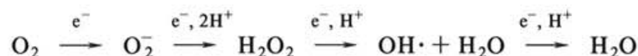
Fig. 3. Luminol-dependent chemiluminescence in the presence of the catalase inhibitor, aminotriazole, in tobacco suspension cells incubated with compatible, incompatible, and saprophytic bacteria. Chemiluminescence was measured in the presence of luminol (10 μM) and aminotriazole (final concentration of 100 μM) and counted with a scintillation counter in "out-of-coincidence" mode. Bacteria that result in a hypersensitive reaction on tobacco are represented by solid symbols (*Pseudomonas syringae* pv. *syringae* wild-type, ■; *P. s.* pv. *glycinea* race 4, ▲, and race 6, ▼); open symbols designate bacteria that do not cause a hypersensitive reaction on tobacco (*P. s.* pv. *syringae* B7, □; *P. s.* pv. *tabaci,* ○; buffer control, ▽). The data represent the difference between bacterial treatments and buffer controls and are the average of three experiments each with two replicates. The standard error bar is shown for *P. s.* pv. *syringae* wild-type and B7 and is typical of the standard errors of the other bacterial treatments.

DISCUSSION

This study monitored several physiological responses that occur early during a plant-bacteria interaction. These responses were examined simultaneously in the same plant tissue treated with several *Pseudomonas* strains to compare more precisely the relationships between responses in compatible, incompatible, and saprophytic interactions. There appear to be two phases in these early interactions. The first phase includes rather nonspecific and rapid responses that occur immediately after addition of the bacteria. The second phase, after about 2 h of incubation, is more prolonged and is specific for HR-causing bacteria.

While carrying out these experiments it was noticed that bacterial treatments that increased most rapidly in pH during the first 30 min consistently increased most rapidly in LDC. Comparison of bacterial treatments showed similar relative changes in chemiluminescence (Fig. 4A) and H^+ uptake (Fig. 4B) during the first 30 min of incubation. *P. s.* pv. *glycinea* race 4 showed the most rapid increases in chemiluminescence and pH, while race 6 was the slowest. The major discrepancy in this comparison was *P. s.* pv. *syringae* B7, which ranked above *P. fluorescens* in chemiluminescence but below in H^+ uptake.

The close correlation between these two responses suggests a causal effect of one on the other. Similar correlations between the increases in pH and luminol-dependent chemiluminescence have been previously observed (12). Increased chemiluminescence affected by increased extracellular pH can be ruled out because all chemiluminescence measurements were adequately buffered (12). However, reduction of molecular oxygen to active oxygen species (species that will oxidize appropriate substrates without energy input) requires an uptake of electrons and protons from the surrounding environment and yields superoxide, hydrogen peroxide, and hydroxyl radical (8,9).



$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-,\,2H^+} H_2O_2 \xrightarrow{e^-,\,H^+} OH\cdot + H_2O \xrightarrow{e^-,\,H^+} H_2O$$

It is feasible that the slight pH increase noticed immediately after addition of the bacteria is due to the uptake of protons in the

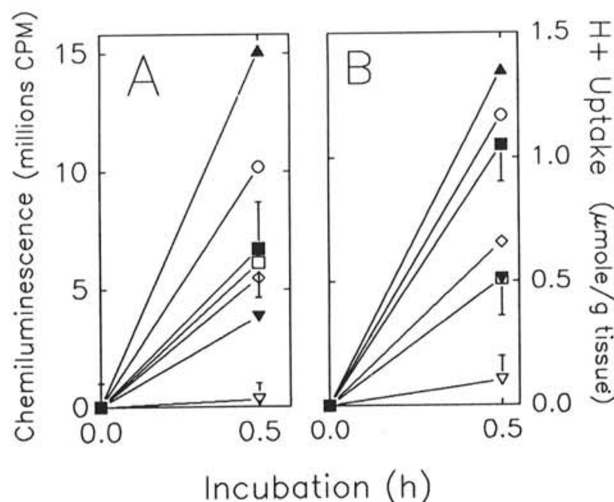


Fig. 4. Comparison of **A**, luminol-dependent chemiluminescence, and **B**, rate of H^+ uptake, immediately after incubation of tobacco suspension cells with bacteria. Bacteria that result in a hypersensitive reaction on tobacco are represented by solid symbols (*Pseudomonas syringae* pv. *syringae* wild-type, ■; *P. s.* pv. *glycinea* race 4, ▲, and race 6, ▼); open symbols designate bacteria that do not cause a hypersensitive reaction on tobacco (*P. s.* pv. *syringae* B7, □; *P. s.* pv. *tabaci,* ○; *P. fluorescens,* ◇; buffer control, ▽). The data represent the difference between bacterial treatments and buffer controls and are the average of three experiments each with two replicates. The standard error bar is shown for *P. s.* pv. *syringae* wild-type and B7 and is typical of the standard errors of the other bacterial treatments.

process of producing hydrogen peroxide and/or superoxide, as well as its further reduction to water.

Another interesting aspect relative to the first phase is the rapid decrease in active oxygen that occurred in all pathogen interactions. The one saprophytic interaction involving *P. fluorescens* did not show this dramatic decrease, and active oxygen levels declined slowly over the course of the experiments. Additional saprophytes will need to be tested to determine if this rapid decrease is an active process unique to pathogenic interactions. The lack of ability to lower extracellular active oxygen levels could significantly reduce the ability of microorganisms to multiply in plant tissue (11).

The second phase of the interaction occurs with HR-causing bacteria after about 2 h and includes a more prolonged increase in extracellular pH and extracellular K^+ as well as a net increase in chemiluminescence. The pH and K^+ changes are a result of the K^+/H^+ response as previously reported (1,5,6). Chemiluminescence indicates active oxygen is also increasing at this phase. However, due to the much greater pH increase during this second phase compared with active oxygen levels, it is unlikely that active oxygen production is directly and solely accountable for this K^+/H^+ response. The increased LDC due to addition of the aminotriazole, a catalase inhibitor, suggested active oxygen is continually produced and degraded during this period (Fig. 3). However, because of possible unknown side effects of the inhibitor on the system, even with brief exposure, caution must be exercised in interpretation of the results.

It is unlikely that the efflux of K^+, the major inorganic ion in the plant cell, is due primarily to nonspecific leakage due to plasmalemma damage caused by active oxygen. Monitoring of extracellular Cl^-, the second major inorganic ion, does not show any release of Cl^- in HR-causing bacterial treatments compared with controls. This is consistent with previous findings (4,6). Additionally, we have found that an intact and active membrane is required for this response to occur (1). If ATPase activity is inhibited, the K^+ efflux/H^+ uptake also is inhibited. If extracellular Ca^{++} is blocked from transport across the membrane, the K^+/H^+ response also is blocked (5). These data are consistent with the hypothesis that the K^+/H^+ response is an active response by the plant cells to HR-causing pathogens and not a direct result of membrane damage by active oxygen or lipid peroxidation.

It is conceivable that the increase in active oxygen may in some way trigger the K^+/H^+ response by perturbing either cell wall or membrane components. However, close examination of these two responses (Figs. 1A, 2B, and 4) in this study indicates that in most interactions the active oxygen response appears slower than the K^+/H^+ response.

This study has allowed us to generate working hypotheses in regard to early responses associated with compatible, incompatible, and saprophytic plant-bacteria interactions. All bacteria stimulate a rapid increase in active oxygen levels on contact with tobacco cells. These levels rapidly decrease within the first hour in interactions involving pathogenic bacteria. Saprophytic interactions may be slower in decreasing these levels; this hypothesis will require work with additional saprophytes. Incompatible (HR) plant-bacteria interactions stimulate a second burst of active oxygen production coincident with the K^+/H^+ response. However, the concentration of active oxygen does not reach the levels of the first increase, possibly due to increased degradative processes such as catalase activity.

We are currently testing these hypotheses. It would be especially interesting to determine which of these early responses associated only with incompatibility appear after treatment with saprophytic or compatible bacteria that have been genetically altered to cause an incompatible response. These studies may help link molecular, genetic, and biochemical studies of host/pathogen recognition mechanisms.

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