# Reports

## Enhancement of Symbiotic Dinitrogen Fixation by a Toxin-Releasing Plant Pathogen

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**An approximate doubling in plant growth, total plant nitrogen, nodulation, and overall dinitrogen fixation of alfalfa are the consequences of the action of a toxin delivered by a *Pseudomonas* infesting the alfalfa rhizosphere. The toxin, tabtoxinine-β-lactam, inactivates selectively one form of glutamine synthetase in the nodules. Thus, normal glutamine synthetase–catalyzed ammonia assimilation is significantly impaired; yet these plants assimilated about twice the normal amount of nitrogen. How plants regulate dinitrogen fixing symbiotic associations is an important and unresolved question; the current results imply that the glutamine synthetase–catalyzed step in ammonia assimilation, a plant function, strongly influences overall dinitrogen fixation in legumes.**

WE HAVE OBSERVED AN UNUSUAL and highly beneficial infestation of nodulating alfalfa plants by a toxin-releasing *Pseudomonas* (Fig. 1). Infestation of alfalfa root and nodule surfaces with this *Pseudomonas* significantly increases plant growth, nitrogenase activity, nodule numbers, total nodule weight, and total plant nitrogen (Tables 1 and 2). This infestation of alfalfa is produced under controlled growth conditions (*1*) and forms a tripartite association composed of alfalfa (*Medicago sativa* L.) and two soil bacteria, one the legume symbiont, *Rhizobium meliloti*, and the other a tobacco leaf pathogen, *Pseudomonas syringae* pv. *tabaci* (*2*). Pathovar *tabaci* lives on the root surfaces of many plants (*3, 4*) and releases tabtoxinine-β-lactam [2-amino-4-(3-hydroxy-2-oxo-azacyclobutan-3-yl)-butanoic acid], which is readily taken up by the roots (*4*). Tabtoxinine-β-lactam is an irreversible inhibitor (*5*) of its target, glutamine synthetase, in plants (*4, 6, 7*); consequently, its action in infested oat roots, for example, is to disable glutamine synthetase–catalyzed ammonia assimilation, and plant death follows (*4*). Thus, the positive effects of infestation of nodulated alfalfa root surfaces with pv. *tabaci* are in striking contrast to the lethal consequences of infestation of nonlegumes.

Nitrogen-fixing nodules form on alfalfa roots after infection by *R. meliloti* (*8*). The bacterial (*Rhizobium*) component of the nodule provides the nitrogen-fixing nitrogenase complex and reduces $N_2$ to ammonia (*8, 9*). The plant fraction of the legume nodule provides energy to the bacterial fraction, regulates the oxygen tension, and assimilates the ammonia released from the bacterial fraction. The initial step of this ammonia assimilation is catalyzed by glutamine synthetase (*9*).

The overall appearance of the pathogen-infested alfalfa plants is strikingly different from that of the control plants (Fig. 1, A and B). The infested alfalfa plants are larger, darker green, and have larger root systems. Plant growth is significantly increased in 30- (Table 1) and 45-day-old (Table 2) alfalfa plants with root surfaces infested with pv. *tabaci*. Fresh and dry weights of whole plants are approximately double the control values (Table 1). The fresh weight of the foliage is markedly increased, but the total fresh weight of roots and nodules is increased to an even greater extent in the infested plants (Table 2). The number of nodules is doubled in infested plants (Tables 1 and 2); the total weight of these nodules is also double the control values (Table 1). The increased number of nodules suggests that the normal regulation of nodule development and nodule numbers has been at least partially overcome. One mechanism regulating nodulation is "feedback inhibition" by older nodules, which inhibits the formation of nodules on young roots (*10*). The possible relation between our observed increase in nodulation on young roots and this regulatory mechanism is unclear. Similar, although less pronounced, increases in growth and nodulation and decreased glutamine synthetase activity have been observed in pv. *tabaci*–infested soybeans. We also tested if alfalfa plants inoculated with strains of *R. meliloti* that nodulate more effectively than the research strain 1021 would also have increased growth and nodulation. We used a commercial, field inoculum for alfalfa (Nitragin, Milwaukee, Wisconsin) and found an approximate doubling of plant growth and nodule numbers when compared with control plants not infested with pv. *tabaci*.

Nitrogenase activity per gram of nodule is also significantly increased in nodules of 30-day-old plants (Table 1) and further increased in the nodules of 45-day-old plants (Table 2). The doubling of nitrogenase ac-

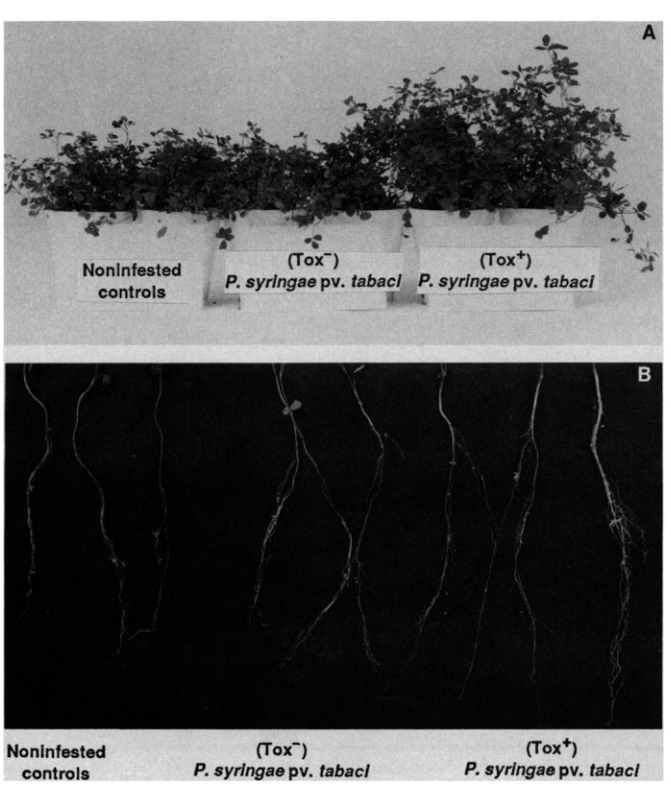


**Fig. 1.** (**A**) Effects on foliar growth of infestation of alfalfa root surfaces with the pathogen *P. syringae* pv. *tabaci*. (**B**) Effects on root and nodule development of infestation with *P. syringae* pv. *tabaci*. Plants were grown and inoculated with pathogen as described (*4*), except that the alfalfa seeds were surface-sterilized before planting. The plants were inoculated at 7 days after planting with *R. meliloti* and inoculated with pv. *tabaci* at day 14. The plants were 30 days old.

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tivity per gram of nodule and the increase in total nodule weight combine to provide the 45-day-old infested plants with about five times the total potential nitrogenase activity when compared with the controls. Total plant nitrogen in infested plants is approximately twice the total nitrogen in control plants after 45 days of growth (Table 2), which suggests that reduced nitrogen is not limiting to growth of these plants.

The increases in plant growth, nodulation, total plant nitrogen, and nitrogenase activity are the result of the *Pseudomonas* toxin, tabtoxinine-β-lactam, a glutamine synthetase inhibitor. These increases as well as the concomitant decrease in glutamine synthetase activity (Table 1) are observed only in plants infested with pv. *tabaci* (toxin producer, $Tox^+$). Plants infested with pv. *tabaci* (nonproducer of toxin, $Tox^-$) grow and nodulate normally and contain normal levels of nitrogenase and glutamine synthetase activities when compared with noninfested controls. We also tested four different spontaneous mutants of the $Tox^+$ strain that no longer produce the toxin because the $Tox^-$ strain is not isogenic to the $Tox^+$ strain; plants inoculated with these mutants grew and nodulated normally and contained normal nitrogenase and glutamine synthetase activities.

In nodulating alfalfa, three sites of glutamine synthetase function are potential targets of tabtoxinine-β-lactam: in the roots, in the plant fraction of the nodule, and in the bacterial fraction of the nodule. To understand the consequences of toxin action in the roots, we tested the effect of infestation of alfalfa plants without nodules (Table 1). Six days after being inoculated with pv. *tabaci*, the non-nodulating plants had no measurable root glutamine synthetase activity; all of these plants died. Thus, nodules were necessary for alfalfa to survive the bacterially delivered toxin.

Glutamine synthetase activity is decreased selectively in infested nodulating plants (Tables 1 and 2). In infested alfalfa roots, no glutamine synthetase activity can be detected (Table 1). In the plant fraction of the infested plants' nodules, where most of a nodule's glutamine synthetase is located, about one-half of the normal amount of glutamine synthetase activity remains (Table 2). In the bacterial fraction, however, the glutamine synthetase activity is normal, and thus the level of this activity is not adversely affected by the toxin (Table 2). The retention of about half of the glutamine synthetase activity in infested alfalfa nodules is in sharp contrast to the rapid and complete inactivation of glutamine synthetase activity in other infested (*6, 7*) or toxin-treated (*4, 7, 11*) plants, for example, oat, tobacco, and pea. Thus, the toxin acts upon glutamine synthetase in the roots and in the plant fraction of the nodule; but, the toxin does not alter the activity of glutamine synthetase in the bacterial fraction of the nodule. To understand the consequences of complete inactivation of all the root and nodule glutamine synthetase activity, we treated 50 nod-

**Table 1.** Comparison of effects on plant growth, nitrogenase, and glutamine synthetase of infestation of nodulated plants with toxin-producing ($Tox^+$) and nonproducing ($Tox^-$) (*21*) strains of *P. syringae* pv. *tabaci* (30 days plant growth). Plants were inoculated with *R. meliloti* (1021) at 7 days after planting and with *P. syringae* (Pt113 or Pa45) at 14 days. Plants were grown in sterilized sand, at 25°C, with 16 hours of light and 8 hours of darkness, and were supplied a modified one-quarter strength Hoagland's solution with the nitrogen omitted. Pathogen growth and inoculation procedures were as described in (*4*). The range of values is shown in parentheses. Plant weights were determined for 50 plants; nodule numbers and weights were determined for 100 plants; all of the determinations were repeated at least three times. Percentages listed are the percentages of the control.

| Inoculation with *P. syringae** | Plant growth parameters | | | | Nitrogenase activity† (U/g of nodule per hour) | Glutamine synthetase activity‡ | |
|---|---|---|---|---|---|---|---|
| | Whole plant fresh weight (mg per plant) | Whole plant dry weight (mg per plant) | Nodules per plant | Nodule weight (mg per plant) | | Nodule plant fraction (U/mg of protein) | Root (U/mg of protein) |
| None | 121 (16) | 22.4 | 3.2 (1.4) | 1.3 | 13.4 | 3.95 | 0.95 |
| | 100% | 100% | 100% | 100% | 100% | 100% | 100% |
| pv. *tabaci* ($Tox^+$) | 240 (21) | 43.7 | 7.1 (1.8) | 2.9 | 19.8 | 2.24 | 0.06 |
| | 198% | 195% | 222% | 223% | 148% | 55% | 6% |
| pv. *tabaci* ($Tox^-$) | 127 (18) | 23.3 | 3.1 (1.3) | 1.4 | 13.3 | 4.06 | 0.88 |
| | 105% | 104% | 99% | 108% | 99% | 103% | 93% |
| pv. *tabaci* ($Tox^+$) non-nodulating plants§ | | | | | | | 0.07 |
| | | | | | | | 7% |

*One week after inoculation with $4 \times 10^9$ bacteria, pv. *tabaci* had increased to the population given in the table, as measured by dilution plating. The identity of pv. *tabaci* was confirmed by bacterial fluorescence and for ($Tox^+$) by toxin production (*4*). The pathogen population per gram of plant roots was $4 \times 10^{14}$ after inoculation with pv. *tabaci* ($Tox^+$) and $6 \times 10^{13}$ after inoculation with pv. *tabaci* ($Tox^-$). †One unit is 1 μmol of acetylene reduced (*22*). ‡One unit is 1 μmol of γ-glutamylhydroxamate formed per minute (*23*). The protein (expressed in milligrams of protein per milligram of nodule) was 0.013 for plants without pathogen, or with the $Tox^+$ pathogen, and 0.014 for plants with the $Tox^-$ pathogen. §All plants died.

**Table 2.** Effects on plant growth, nitrogenase, and glutamine synthetase of infestation of nodulated plants with *P. syringae* pv. *tabaci* (toxin-producing, $Tox^+$) (45 days of plant growth). Plant and bacterial culture procedures, enzyme assays, and data treatment were as described in the legend to Table 1.

| Inoculation with *P. syringae* | Plant growth parameters | | | | | Nitrogenase activity (U/g of nodule per hour) | Glutamine synthetase activity | |
|---|---|---|---|---|---|---|---|---|
| | Fresh weight | | Nodules per plant | Total N* (mg per plant) | N* (mg/g of dry weight) | | Nodule plant fraction (U/g of nodule) | Nodule bacterial fraction (U/g of nodule) |
| | Foliar (mg per plant) | Root and nodule (mg per plant) | | | | | | |
| None | 255 (33) | 58 (12) | 5.1 (2.1) | 1.37 (0.07) | 24.5 (1.4) | 13.6 | 50.4 | 0.45 |
| | 100% | 100% | 100% | 100% | | 100% | 100% | 100% |
| pv. *tabaci* ($Tox^+$) | 383 (47) | 188 (27) | 12.3 (2.8) | 2.85 (0.20) | 27.4 (1.9) | 26.4 | 26.3 | 0.49 |
| | 183% | 324% | 241% | 208% | | 194% | 52% | 109% |

*Total plant nitrogen was determined by Kjeldahl analysis (*24*).

ulated plants with methionine sulfoximine (1 m*M* for 72 hours), another specific, irreversible inhibitor of glutamine synthetase (*12*); all of the plants died. Thus, the apparent selectivity of the toxin's action in the nodule is a characteristic of the toxin and is critically important to the beneficial infestation of the legume with pv. *tabaci*.

The selectivity of the toxin's action, which resulted in the retention of half of the plant fraction and all of the bacterial fraction glutamine synthetase activities, was involved in survival and also in the enhanced plant growth, nodulation, nitrogen fixation, and nitrogen assimilation in the infested plants. Only the nodule-specific form of glutamine synthetase was active in the plant fraction of nodules of infested plants, as shown by chromatographically separating the two glutamine synthetase forms obtained from the nodule plant fraction (Fig. 2A). We examined the biochemical basis for the retention of most of the nodule-specific glutamine synthetase activity in the nodule plant fraction by determining the toxin sensitivity of the two forms of glutamine synthetase present in the nodule plant fraction—the root and nodule-specific forms (Fig. 2B). Purified nodule root form was rapidly inactivated by the toxin (Fig. 2B). In contrast, purified nodule-specific glutamine synthetase was only partially inactivated by the toxin (Fig. 2B). Nodule-specific glutamine synthetase is encoded by a separate gene expressed only in nodules (*13*) and is the only plant glutamine synthetase reported to have any degree of insensitivity to the toxin (*4–7, 11*). The bacterial fraction glutamine synthetase activity, which is sensitive to the toxin in vitro, may be protected from the toxin by a *Rhizobium* β-lactamase found in extracts of the bacterial fraction. This β-lactamase catalyzes the cleavage of the β-lactam ring of tabtoxinine-β-lactam to produce the inactive compound tabtoxinine; we reported a similiar β-lactamase activity functioning as a major part of pv. *tabaci's* self-protection mechanism (*14*).

The infested alfalfa plants are assimilating greater total amounts of nitrogen than that of their counterpart controls; yet these plants are assimilating nitrogen with less than normal amounts of glutamine synthetase in their nodules (Table 2). This finding suggests the operation of alternative routes of ammonia assimilation; certainly, several other enzymes have been shown to catalyze ammonia assimilation in vitro, such as glutamate dehydrogenase and asparagine synthetase (*15*). The greatly elevated ammonia levels in these nodules could approach the high Michaelis constant values for ammonia for these enzymes.

The changes in glutamine synthetase activity in nodules and roots of pv. *tabaci*–infested plants result in altered glutamate and glutamine pools (Fig. 3); these changes may collectively influence nitrogen fixation and assimilation as well as nodulation in these infested plants. Nitrogenous compounds have been suggested as one part of the molecular signaling between *Rhizobium* and its plant host that regulates nitrogen fixation (*16, 17*). Kahn *et al.* have suggested that glutamate, and perhaps other compounds, were imported and oxidized by the bacterial fraction of the nodule (*17*). Kohl *et al.* have proposed that proline, which is derived from glutamate, is taken up into the bacterial portion of the nodule (*18*). The nitrogenous metabolites in the plant fraction of the nodules could be influencing the increase in nitrogenase activity in the nodules of the infested plants. Consistent with this hypothesis are the observations that glutamine synthetase activity and the concentrations of metabolites influenced by this activity are implicated in regulating nitrogenase synthesis through a nitrogen-regulated system (*19*). A similar nitrogen-regulated system is encoded in the *R. meliloti* genome (*20*).

Our initial characterization of this beneficial interaction between pv. *tabaci* and nodulating alfalfa reveals that increased growth, nodulation, and overall $N_2$ fixation and assimilation accompany selective alteration of the glutamine synthetase function within the root and nodule and show that when total $N_2$ fixation is stimulated, the plant has a greater capability to assimilate the increased amounts of ammonia than has been previously appreciated.

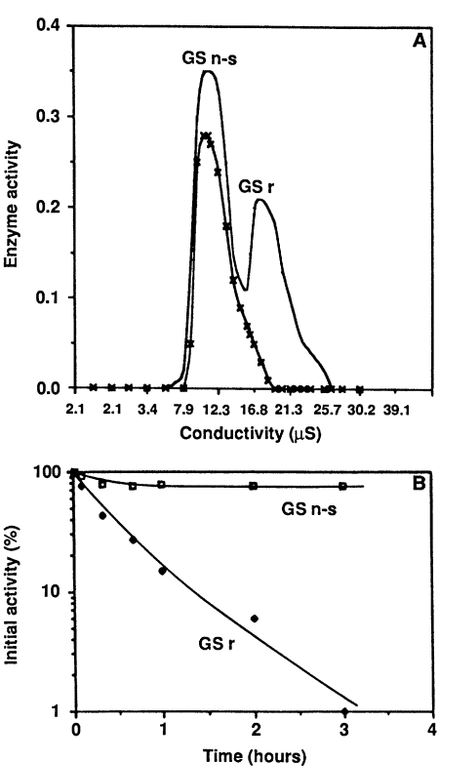


**Fig. 2.** (**A**) Effects of infestation with *P. syringae* pv. *tabaci* on the nodule-specific (GS n-s) and root (GS r) glutamine synthetase activities in the plant fraction of alfalfa nodules. Nodules of infested (x-marked curve) and noninfested (solid curve) plants were extracted, the glutamine synthetase was precipitated with ammonium sulfate, and the two enzyme forms were resolved by ion exchange chromatography by using a gradient of 0 to 0.6 m*M* KCl in 50 m*M* imidazole-HCl, *p*H 7.5, and 10 m*M* $MgCl_2$. The nodule-specific form eluted between 11.3 to 11.5 μS (~0.14 m*M* KCl), and the root form eluted between 17.5 to 17.9 μS (~0.24 m*M* KCl). The data from two representative experiments were plotted as a function of the increasing conductivity of the gradient. The adenosine diphosphate (ADP)–dependent transferase activity of glutamine synthetase is plotted and is expressed as micromoles of γ-glutamylhydroxamate formed per minute (*23*). The data are in good agreement with those of Cullimore *et al.* (*25*), who used a similar procedure to resolve and identify the nodule-specific and root forms of glutamine synthetase from nodules of *Phaseolus vulgaris* L. The data are representative of three separate experiments. (**B**) Sensitivity of purified nodule-specific (GS n-s) and root (GS r) glutamine synthetases to inactivation by pure tabtoxinine-β-lactam. The glutamine synthetases were purified from the plant fraction of nodules of noninfested plants, as described for (A). The inactivation was performed (*5*) by using 100 μ*M* tabtoxinine-β-lactam. The glutamate-dependent biosynthetic and ADP-dependent transferase activities of glutamine synthetase were measured according to the procedures of Shapiro and Stadtmann (*23*). Tabtoxinine-β-lactam was purified as described (*26*). The data are representative of three separate experiments.

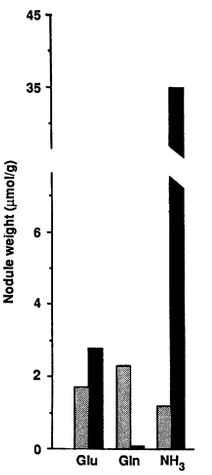


**Fig. 3.** Amino acid and ammonia pools in *P. syringae* pv. *tabaci*–infested (solid bars) and noninfested (shaded bars) nodules at 30 days of plant age. Nodules were extracted into water and immediately analyzed with an automated amino acid analyzer (Beckman 7300). The data are representative of eight separate tissue samples. Because of the high ammonia pools in the pv. *tabaci*–treated nodules, standard ammonia samples were analyzed along with tissue samples to verify that no exogenous ammonia contaminated the sample.

## REFERENCES AND NOTES

1. *Pseudomonas* species can enhance plant growth in native soils by releasing siderophores that act as antibiotics and therefore decrease the populations of

deleterious bacteria in the plant rhizosphere. These mechanisms allow increased plant growth only in native soils, not in sterilized sand cultures such as we have used [J. E. Loper and M. N. Shroth, in *Iron, Siderophores, and Plant Diseases*, T. R. Swinburne, Ed. (Plenum, New York, 1986), pp. 85–98.] Also, some microorganisms have the capability to enhance plant growth by increasing the availability of minerals by increased mineralization processes in native soils. Such a mechanism is ruled out for this system because the effects in our system are specific to the delivery of the toxin and because the plants were provided with an ample amount of available minerals when grown in sand culture [E. A. Curl and B. Truelove, *The Rhizosphere* (Springer-Verlag, Berlin, 1986), pp. 167–176].

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# The Incommensurate Modulation of the 2212 Bi-Sr-Ca-Cu-O Superconductor

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**The incommensurate modulation evident in the diffraction pattern of the superconductor $Bi_2Sr_{3-x}Ca_xCu_2O_{8+y}$ consists of almost sinusoidally varying displacements of up to 0.4 Å of the Bi and Sr atoms in the *a*- and *c*-directions of the unit cell, and of up to 0.3 Å of the Cu atoms in the *c* direction only. Thus, a newly discovered feature of the $Bi_2Sr_{3-x}Ca_xCu_2O_{8+y}$ structure is sizable Cu displacement, which is related to static wave formation in the Cu-O sheets. Reported thermal parameters give evidence that similar distortions occur on cooling of the thallium-containing superconductors.**

THE CRYSTALLOGRAPHY OF THE 2212 phase of the Bi-Sr-Ca-Cu-O system, with stoichiometry $Bi_2Sr_{3-x}Ca_xCu_2O_{8+y}$ has been described in a number of publications (*1–4*). A major feature of the diffraction pattern is the occurrence of satellite reflections, at positions displaced from the main diffraction spots by $q = \pm 0.21a^*$, where $a^*$ is the reciprocal lattice constant. The satellite reflections indicate either a displacive or a substitutional modulation in which position or chemical occupancy vary between unit cells along the *a*-axis. Since $1/q$ is not a simple multiple of *a*, the modulation is incommensurate, and the structure cannot be described on a unit cell that is a multiple of the basic cell. Using recently developed methods (*5*), we have made the first analysis based on the superspace group description of modulated crystals (*6*).

The room-temperature analysis was performed on two single crystals of different origin and slightly differing cell dimensions. Since the results were essentially identical for the two crystals, only one set is reported here. Crystallographic information is summarized in Table 1. The basic space group is *Amaa*, which is a subgroup of space groups reported elsewhere (*2–4*), but in agreement with at least one earlier study (*1*). Atomic parameters, obtained in the refinement of

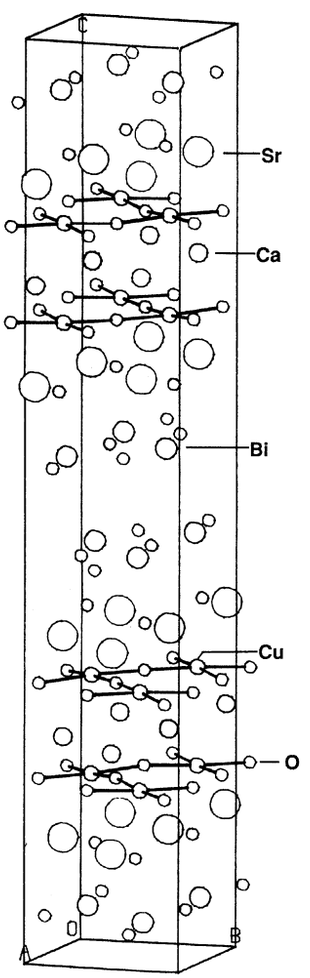


**Fig. 1.** Packing diagram. The large circles represent Sr atoms; successively smaller circles indicate the Bi, Ca, Cu, and O atoms.

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