bonds, such as flipping of the unbound D atoms, as in the water trimer (*15*), and perhaps is also associated with twisting and bending motions of the oxygen framework, as found in cyclopentane (*24*). Assigning the observed vibration to the flipping mode is consistent with (i) a parallel ($\Delta K = 0$) transition in which the dipole moment change associated with the out-of-plane vibration is parallel to the symmetry axis (perpendicular to the ring) and (ii) the fact that both the ground and the excited state structures are averaged to rigorously symmetric top forms by the flipping dynamics.

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Role of Mutant CFTR in Hypersusceptibility of Cystic Fibrosis Patients to Lung Infections

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Cystic fibrosis (CF) patients are hypersusceptible to chronic *Pseudomonas aeruginosa* lung infections. Cultured human airway epithelial cells expressing the ΔF508 allele of the cystic fibrosis transmembrane conductance regulator (CFTR) were defective in uptake of *P. aeruginosa* compared with cells expressing the wild-type allele. *Pseudomonas aeruginosa* lipopolysaccharide (LPS)–core oligosaccharide was identified as the bacterial ligand for epithelial cell ingestion; exogenous oligosaccharide inhibited bacterial ingestion in a neonatal mouse model, resulting in increased amounts of bacteria in the lungs. CFTR may contribute to a host-defense mechanism that is important for clearance of *P. aeruginosa* from the respiratory tract.

Among the most serious manifestations of CF are chronic pulmonary infections with the bacterium *P. aeruginosa*. The basis for hypersusceptibility of CF patients to this bacterium is not well understood, and the role of mutant CFTR, if any, is not clear. Binding and internalization of respiratory pathogens by epithelial cells followed by desquamation could be an important mechanism for clearing bacteria from the lung. This mechanism has been shown to be important in protecting against bladder infections (*1*).

To investigate whether the most common and severe CFTR mutation (ΔF508) affected uptake of *P. aeruginosa*, we performed bacterial invasion assays (*2*) with four cell lines: CFT1, an airway epithelial cell line derived from a CF patient homozygous for ΔF508 CFTR and that is transformed with human papilloma virus 18 E6/E7; CFT1-ΔF508, which expresses a third copy of ΔF508 CFTR introduced by a retrovirus; CFT1-LC3, which expresses a control gene (β-galactosidase) introduced by the same retrovirus; and CFT1-LCFSN, which expresses a functional wild-type human CFTR gene (*3*). We tested a standard laboratory strain of *P. aeruginosa*, designated PAO1, and two nonmucoid, LPS-smooth clinical isolates from CF patients (*4*). Compared with CFT1-LCFSN cells, the three lines expressing ΔF508 CFTR internalized significantly fewer bacterial cells (Fig. 1A). The ΔF508 mutation causes inefficient processing of CFTR, a defect that is partially corrected if the cells are grown at 26°C (*5*). When epithelial cells were cultured for 72 hours at 26°C there were no longer significant differences in uptake of the *P. aeruginosa* strains by the cells expressing wild-type or mutant CFTR (Fig. 1B). Because the overall uptake of bacteria at 26°C was low, we performed additional experiments with cells grown for 72 hours at 26°C in which the invasion assay was performed at 37°C for 3 hours, conditions under which surface expression of mutant ΔF508 CFTR is maintained (*5*). No significant difference in bacterial cell uptake was measured (Fig. 1C), and overall amounts of internalization approached those of the CFT1-LCFSN cells at 37°C. Returning cells expressing ΔF508 CFTR to 37°C for 24 hours after growth for 72 hours at 26°C removes CFTR from the cell surface (*5*); under these conditions internalization of the bacterial strains was essentially identical to that shown in Fig. 1A (*6*). These data indicate that internalization of *P. aeruginosa* by airway epithelial cells correlated with membrane expression of CFTR.

The effect of ΔF508 CFTR on ingestion appeared to be specific for *P. aeruginosa*; other bacterial pathogens tested (*7*) were internalized equally well by cells expressing mutant ΔF508 or wild-type

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CFTR. Quantitative bacterial binding assays revealed that differences in internalization by the epithelial cells were not simply due to differences in the adherence of *P. aeruginosa*.

Over the course of chronic lung infection in CF patients, variants of *P. aeruginosa* emerge that no longer express complete LPS structures (*8*). To investigate the effect of LPS structure on epithelial cell internalization, we tested eight *P. aeruginosa* strains that differ in LPS structure (*9*) (Fig. 2, A and B). Maximal invasion occurred into the CFT1-LCFSN cells by bacterial strains expressing an intact LPS outer core; in addition, cells expressing wild-type CFTR ingested significantly more *P. aeruginosa*, regardless of LPS phenotype, than did those expressing ΔF508 CFTR. No effect on bacterial ingestion was seen when isogenic *P. aeruginosa* strains differing in production of pili, flagella, or mucoid exopolysaccharide were tested (*6*).

We also compared the internalization of three clinical isolates of *P. aeruginosa* with incomplete LPS structures with that of recombinant strains converted back to expression of complete LPS structures by introduction of portions of the *rfb* locus of *P. aeruginosa* strain PA103 (*10*). All three recombinant LPS-smooth bacterial strains showed the same pattern of ingestion by the cell lines (Fig. 2, C to E) as was depicted in Figs. 1 and 2, A and B, for naturally occurring LPS-smooth strains. Uptake of the recombinant LPS-smooth strains by all of the cell lines was significantly increased compared with the LPS-incomplete clinical isolates carrying only the vector for DNA cloning. These results suggest that the emergence of strains expressing an incomplete LPS structure during chronic infection of CF patients may further reduce epithelial cell uptake and thus contribute to bacterial survival in the airways.

To determine if LPS could inhibit the internalization of *P. aeruginosa*, we isolated LPS and the oligosaccharide fragment lacking lipid A from strains PAC557 and PAC1R *algC*::tet and tested their inhibitory activity in the invasion assay. Strain PAC557 produces an LPS with a complete core but no O side chains, and the isogenic strain PAC1R *algC*::tet expresses an incomplete LPS core (*11*). LPS from the former, but not the latter, strain inhibited ingestion by the CFT1-LCFSN cells (Fig. 3). LPS with a complete core also inhibited bacterial adherence to the CFT1-LCFSN cell line (*12*). These results suggest that the complete LPS outer core is an important bacterial ligand involved in the ingestion

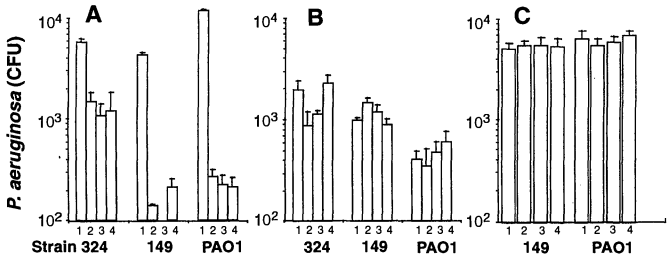

Fig. 1. Internalization of *P. aeruginosa* by 10^5 transformed airway epithelial cells (*2*). Cells were grown for 72 hours and bacteria were then allowed to invade the cells for 3 or 4 hours. Bars indicate the mean of the determinations and error bars the SD. Cell lines are as follows: 1, CFT1-LCFSN; 2, CFT1; 3, CFT1-LC3; and 4, CFT1-ΔF508. (**A**) Cells were grown at 37°C, a temperature that inhibits membrane expression of ΔF508 CFTR (*5*) and the assay was carried out at 37°C. Multiple comparisons for all three bacterial strains were significant ($P < 0.001$, ANOVA); the CFT1-LCFSN line was significantly different from any other cell line ($P < 0.01$, Fisher's PLSD statistic) for all three bacterial strains. (**B**) Cells were grown at 26°C and internalization was assessed at 26°C. (**C**) Cells were grown at 26°C and internalization was assessed at 37°C. When cells were grown at 26°C to promote surface expression of ΔF508 CFTR (*5*), there were no significant ($P > 0.2$, ANOVA) differences in bacterial uptake among the cell lines for any *P. aeruginosa* strain tested.

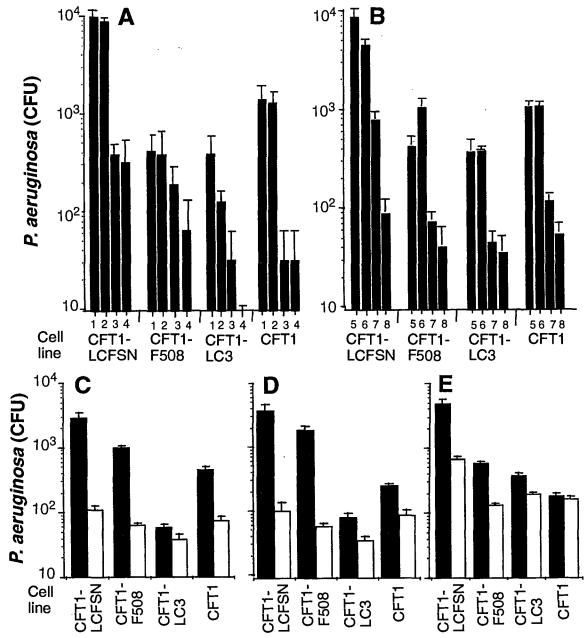

Fig. 2. Role of the complete outer-core region of *P. aeruginosa* LPS in internalization by airway epithelial cells (*2*). Bars indicate the mean of the determinations and error bars the SD. (**A** and **B**) Assays with bacterial strains of defined LPS phenotype (*9*); 1, PAC1R, wild-type, smooth; 2, PAC557, complete core; 3, PAC1R *algC*::tet, incomplete core; 4, PAC605, incomplete core; 5, PAO1, wild-type, smooth; 6, AK44, complete core; 7, PAO1 *algC*::tet, incomplete core; and 8, AK1012, incomplete core. All eight strains were internalized by the CFT1-LCFSN cell line significantly better than by the three cell lines expressing mutant ΔF508 CFTR ($P < 0.001$, ANOVA; $P < 0.05$ for all pair-wise comparisons, Fisher PLSD). Bacterial strains with a smooth or complete-core LPS were internalized by all of the cell lines significantly better than strains expressing an incomplete core ($P < 0.01$, ANOVA; $P < 0.05$ for all pair-wise comparisons, Fisher PLSD). (**C** to **E**) Increased internalization by airway epithelial cells of recombinant LPS-smooth *P. aeruginosa* strains carrying cloned *rfb* genes (solid bars) compared with parental clinical isolates expressing an incomplete LPS structure and carrying only the DNA cloning vector (open bars) (*10*). (C) Strain 2192; (D) strain 332; (E) strain 9125. Ingestion of all of the LPS-smooth strains by all of the cell lines was significantly better than ingestion of the corresponding LPS-incomplete isolate ($P \leq 0.002$, unpaired *t* test), except for strain 9125 by the CFT1 cells. There was also significantly greater ($P < 0.001$, ANOVA) uptake of all of the LPS-smooth bacteria by the CFT1-LCFSN cells compared with the other three cell lines expressing ΔF508 CFTR.

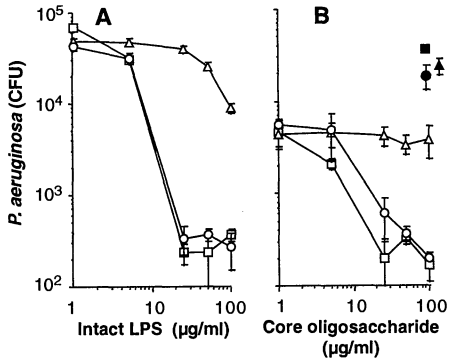

Fig. 3. Effect of intact LPS (**A**) and an oligosaccharide with a lipid A–free core (**B**) on internalization of *P. aeruginosa* into CFT1-LCFSN cells. Each point is the mean of three to seven replicates; error bars are SD. *Pseudomonas aeruginosa* strains: (□), PAO1; (△), 149; and (○), 324. Solid symbols in (B) represent the mean CFU of bacteria internalized in the presence of intact LPS (100 μg/ml) from strain PAC1R *algC*::tet, which has an incomplete-core oligosaccharide. Amounts of internalization $<10^4$ CFU differed significantly ($P < 0.01$, ANOVA and Fisher PLSD for pair-wise comparisons) from amounts of internalization in the presence of incomplete-core LPS (100 μg/ml).

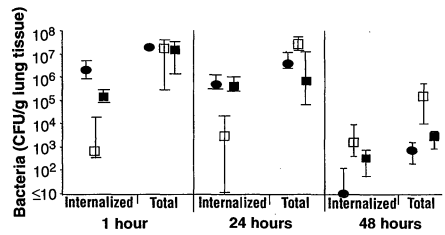

Fig. 4. Effect of complete-core oligosaccharide on *P. aeruginosa* infection in neonatal mouse lungs. Each point indicates the median bacterial CFU for 8 to 10 lungs obtained from each group of mice and the bars indicate the upper and lower quartiles. Inhibitor delivered with inoculum: (●), none; (□), complete-core oligosaccharide; and (■), incomplete-core oligosaccharide. Groups of 7-day-old neonatal BALB/c mice were infected intranasally with ~10^8 CFU of strain PAO1 (*14*) alone or mixed with either complete-core oligosaccharide (50 μg) or incomplete-core oligosaccharide (50 μg) (*11*). After 1, 24, or 48 hours, four to five mice were killed and their lungs were removed, weighed, and dispersed into single-cell suspensions. The total CFU of bacteria present in each lung was determined from a sample treated with Triton X-100 (0.5%) to release intracellular bacteria. The remaining cells were treated with gentamicin (300 μg/ml) for 60 min to kill extracellular *P. aeruginosa*, then the cells were centrifuged, washed twice in tissue-culture medium, and resuspended in 200 μl of 0.5% Triton X-100 to release intracellular bacteria. These suspensions were diluted and plated. Differences among groups were analyzed by nonparametric statistics: $P < 0.0001$, Kruskall-Wallis nonparametric ANOVA; $P < 0.001$, Dunn procedure for individual pair-wise differences between the groups at 1 and 24 hours. Also, at 1 hour the group receiving the incomplete-core oligosaccharide had a reduced amount ($P = 0.05$, Dunn procedure) of intracellular bacteria compared with the group receiving nothing with the inoculum. At 48 hours, the group treated initially with complete-core inhibitor had significantly more bacteria in their lungs ($P = 0.003$, Kruskall-Wallis; $P < 0.05$, Dunn procedure for all pair-wise comparisons) than did the other groups.

by airway epithelial cells expressing wild-type CFTR. LPS has a similar role in the invasion of epithelial cells by *Neisseria gonorrhoeae* (*13*).

To assess the physiological significance of these observations, we used a neonatal mouse model for infection by *P. aeruginosa* wherein epithelial cell internalization of bacteria has been observed (*14*). Inocula containing *P. aeruginosa* PAO1 plus complete-core oligosaccharide produced fewer intracellular *P. aeruginosa* 1 and 24 hours after infection compared with controls inoculated with PAO1 alone or PAO1 plus incomplete-core oligosaccharide (Fig. 4). The total number of bacteria in the lung at 1 hour was the same for all groups, but at 24 hours there were ≥28 million more *P. aeruginosa* per gram of lung tissue when the complete-core oligosaccharide was added to the inoculum. Forty-eight hours after infection, the mice given the complete-core inhibitor still had substantially more bacteria in the lungs compared with controls. Mice given the complete-core inhibitor had more internalized bacteria at 48 hours, although this was not significantly different ($P > 0.05$) from mice given incomplete-core inhibitor; presumably this higher level of internalized bacteria reflected the fact that there were more total bacteria in these mouse lungs and there would be no effect from the inhibitor at this time point. In a separate experiment, addition of intact LPS to the bacterial inoculum produced similar results (6). Thus, addition of an exogenous ligand to block bacterial ingestion by epithelial cells resulted in less bacterial clearance from the lungs of neonatal mice.

Histological analyses of affected lung tissue removed from CF patients at autopsy or for transplantation routinely show that *P. aeruginosa* cannot be seen internalized within airway cells and are rarely attached to epithelial cells. Instead, bacteria are observed as microcolonies encased in the extracellular mucus layer lying in the airway lumen (*15*). Thus, our findings are consistent with the observed histopathology. Some studies have implicated increased receptors for *P. aeruginosa* on epithelial cells from CF patients as the basis for the hypersusceptibility of CF patients to chronic infection (*16*), whereas others have reported no differences in *P. aeruginosa* adherence to nasal polyp epithelial cells cultured from individuals with and without CF (*17*). Further studies are needed to determine the relation between binding of *P. aeruginosa* and internalization by epithelial cells and the pathogenesis of chronic lung infection in CF patients.

Our results suggest that *P. aeruginosa* can initiate infection in CF patients because airway epithelial cells expressing a mutant CFTR are defective in internalization of the bacterium, a process that may be an important host defense. The emergence during chronic infection of *P. aeruginosa* variants with an incomplete LPS structure, which would further impair their internalization, may contribute to the maintenance of the infectious state. Conceivably, genetic or other therapies that promote expression of CFTR on epithelial cell surfaces could enhance elimination of *P. aeruginosa* by cellular uptake and alter the otherwise relentless clinical course of CF.

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2. Cells were released from monolayers in tissue culture flasks by 5-min incubation with a trypsin-versene mixture (BioWhitaker, Walkersville, MD) and washed, counted, and seeded into 96-well tissue culture plates at a concentration of 10^5 cells per well in supplemented F-12 medium (*3*). Plates were incubated at either 26° or 37°C in 5% CO_2. Fresh cultures of *P. aeruginosa* grown overnight at 37°C on a tryptic soy agar plate were suspended in supplemented F-12 medium to prepare the bacterial inoculum; then ~10^6 colony-forming units (CFU; range from 6×10^5 to 2.3×10^6 CFU) were added per well of 10^5 epithelial cells. Bacteria were allowed to be ingested by the epithelial cells for 3 or 4 hours at either 26° or 37°C, after which nonadherent bacteria were removed by washing. The remainder of the assay and controls were as described [S. M. J. Fleiszig, T. S. Zaidi, G. B. Pier, *Infect. Immun.* **63**, 4072 (1995)]. Three to nine replicates were obtained per point and were analyzed by analysis of variance (ANOVA) and the Fisher PLSD statistic to determine pair-wise differences.
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7. Human clinical isolates of bacterial pathogens that failed to be ingested by epithelial cells expressing wild-type CFTR differently from cells expressing ΔF508 mutant CFTR included the following: *Escherichia coli* (two strains), *Burkholderia cepacia* (three strains), *Neisseria meningitidis* (one strain), *Staphylococcus aureus* (two strains), *Staphylococcus epidermidis* (two strains), *Streptococcus pneumoniae* type XIV (one strain), and *Streptococcus agalactiae* (one strain).
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12. Although epithelial cells expressing mutant ΔF508 CFTR ingested the *P. aeruginosa* expressing a complete LPS core better than they ingested bacteria expressing an incomplete LPS core (Fig. 2), there was no statistically significant reduction in bacterial ingestion by mutant CFTR epithelial cells with complete-core LPS as an inhibitor. This could be attrib-

uted either to an inability to measure further reductions in ingestion under circumstances in which the initial uptake is already quite low or to alternative ligand-receptor interactions between LPS-rough *P. aeruginosa* and ΔF508 CFTR–expressing cells. Purified *O* polysaccharides from LPS-smooth wild-type strains did not inhibit bacterial ingestion by the airway epithelial cell lines.
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Control of Aragonite or Calcite Polymorphism by Mollusk Shell Macromolecules

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Many mineralizing organisms selectively form either calcite or aragonite, two polymorphs of calcium carbonate with very similar crystalline structures. Understanding how these organisms achieve this control has represented a major challenge in the field of biomineralization. Macromolecules extracted from the aragonitic shell layers of some mollusks induced aragonite formation in vitro when preadsorbed on a substrate of β-chitin and silk fibroin. Macromolecules from calcitic shell layers induced mainly calcite formation under the same conditions. The results suggest that these macromolecules are responsible for the precipitation of either aragonite or calcite in vivo.

Many organisms are able to exert remarkable control over the minerals they form. Numerous mineralized tissues contain crystals all having a preferred orientation and a mineral composition and morphology different from precipitates that form abiologically under the same environmental conditions (*1*, *2*). A striking example of biological control is the ability of many organisms to determine which polymorph of $CaCO_3$ will precipitate at a given location (*3*). In mineralized tissues a mixture of $CaCO_3$ polymorphs is almost never found at the same site. There are, however, numerous examples of species that precipitate one polymorph type at one location and a second type at an adjacent location (*1*).

The most common biologically formed $CaCO_3$ polymorphs are calcite and aragonite. Vaterite, a less stable polymorph, is not commonly formed by organisms (*1*, *4*). Calcite is thermodynamically more stable than aragonite at ambient temperatures and pressures (*5*). Calcite and aragonite have very similar crystal structures (*6*). The calcium ions are located almost in the same lattice positions in (001) layers, alternating with layers of carbonate ions. The major differences between the two polymorphs occur in the organization and orientation of the carbonate molecules. The presence of other doubly charged ions in $CaCO_3$ solutions, in particular Mg^{2+} as well as a variety of small organic molecules, favors the formation of aragonite (*7*). It has thus been widely believed that the regulation of aragonite-calcite polymorphism in organisms depends on the concentration of ions, primarily magnesium, in the precipitating solution (*8*).

A commonly used strategy in biomineralization is the elaboration of an extracellular organic matrix (*4*). The crystals are induced to form within the matrix voids. This is the strategy used by mollusks to form their shells (*9*). In the matrix sheets of nacre, the core is composed of a layer of β-chitin that is sandwiched between two layers of glycine- and alanine-rich proteins (*10*). The latter proteins have x-ray and electron diffraction patterns remarkably similar to that of silk fibroin and are predominantly in the β sheet conformation (*11*). The surfaces of the matrix are coated with hydrophilic, aspartic acid–rich macromolecules, some of them in contact with the mineral phase (*12*). These hydrophilic macromolecules specifically interact from solution with growing calcite crystals in vitro, suggesting that they are involved in the crystal formation process at the molecular level (*13*). Hare proposed that the organic matrix proteins may be responsible for the polymorph type deposited in mollusk shells (*14*). Experiments testing the ability of organic framework matrices from aragonitic and calcitic mollusk shell layers to induce nucleation of the original phase in a saturated solution met with some success, although calcite formed predominantly, rather than aragonite, irrespective of the origin of the matrix components (*15*). Furthermore, this experimental approach does not guarantee the total absence of undissolved crystal nuclei in the matrix, which could be responsible for seeding the original phase.

We developed an experimental strategy to determine whether components of the matrix, especially the hydrophilic macromolecules, have a primary role in the control of polymorphism and whether the entire matrix assembly is required. We reassembled in vitro a substrate for nucleation composed of the major mollusk shell organic matrix components: β-chitin, silk fibroin, and aspartate-rich soluble macromolecules extracted from individual mollusk shell layers (*10*). The β-chitin and silk were purified from the pen of the squid *Loligo* and the cocoons of the silkworm *Bombyx mori*, respectively (*16*, *17*), where neither are associated with any mineral phase. This approach precluded the possibility that our results were influenced by any residual undissolved mineral phase.

The *Loligo* β-chitin has a complex three-dimensional organization (*18*). The fibers are organized in an interlinked architecture forming a dense net of pores and channels. Purified soluble silk fibroin was allowed to interact with the insoluble chitin and was stabilized in the β conformation by treatment with methanol (*17*). The ensemble of macromolecules was then adsorbed from solution on the β-chitin–silk fibroin assembly. Crystallization was finally induced by incubation of the substrate complex in a saturated solution of $CaCO_3$ (*19*). The macromolecules were extracted from either calcitic or aragonitic layers of four bivalves, a cephalopod, and a gastropod (Table 1). Most of our experiments used the macromolecules from aragonitic layers, as the formation in vitro of the less stable aragonite in the absence of other additives presumably implies an active role for these macromolecules in $CaCO_3$ polymorph determination.

Crystallization on the partial or complete assemblies of chitin, silk fibroin, and mollusk shell macromolecules resulted in the formation of spherulites of $CaCO_3$ on the surface and inside the chitin scaffold (*20*). The spherulites were isolated and characterized by optical and scanning electron microscopy, and their mineralogies were determined by Fourier transform infra-

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