Surface functional group chemistry of intact Gram-positive and Gram-negative bacterial cells and their isolated cell walls was examined as a function of pH, growth phase, and growth media (for intact cells only) using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Infrared spectra of aqueous model organic molecules, representatives of the common functional groups found in bacterial cell walls (i.e., hydroxyl, carboxyl, phosphoryl, and amide groups), were also examined in order to assist the interpretation of the infrared spectra of bacterial samples. The surface sensitivity of the ATR-FTIR spectroscopic technique was evaluated using diatom cells, which possess a several-nanometers-thick layer of glycoprotein on their silica shells. The ATR-FTIR spectra of bacterial surfaces exhibit carboxyl, amide, phosphate, and carbohydrate related features, and these are identical for both Gram-positive and Gram-negative cells. These results provide direct evidence to the previously held conviction that the negative charge of bacterial surfaces is derived from the deprotonation of both carboxylates and phosphates. Variation in solution pH has only a minor effect on the secondary structure of the cell wall proteins. The cell surface functional group chemistry is altered neither by the growth phase nor by the growth medium of bacteria. This study reveals the universality of the functional group chemistry of bacterial cell surfaces.

1. Introduction

Bacteria are ubiquitous in near-surface geological systems, and are known to play important role in different biogeochemical processes, including contaminant transport and degradation,\textsuperscript{1,2} mineral dissolution and precipitation,\textsuperscript{3} and metal sorption by minerals and their redox transformations.\textsuperscript{4,5} Several recent investigations examined the surface chemistry of intact bacterial cells and their cell walls using both macroscopic (e.g., potentiometric titration, ion adsorption) and molecular tools (microscopy and...
spectroscopy. This research led to the identification of major proton-active functional groups on bacterial surfaces and the development of a series of thermodynamic metal binding constants for them. However, molecular information to support the loci of protonation and complex formation remains limited. Structural probes, such as transmission and scanning electron microscopes, are often used to study microbial cell surfaces. These methods require subjecting the specimen to high vacuum, thereby affecting natural hydration levels of the biopolymers, and possibly altering the cell morphological characteristics. The cells are also susceptible to alterations in the presence of water, which can affect natural hydration levels of the biopolymers, and possibly altering the cell morphological characteristics. In addition, the electron microscopic methods cannot provide in situ information on the functional group characteristics of cell walls. More recently, researchers have begun using in situ methods, such as infrared and Raman spectroscopies, to study the behavior of bacteria in water. While these studies have shown the dominant vibrational modes of lipopolysaccharide, phospholipid, and protein components in the cell wall, identification of different functional groups from the infrared spectra and their variation as a function of solution composition is still not complete. Researchers also argue that isolated cell envelopes may not provide complete information on intact bacterial cells, since the chemical environment of the cell wall is significantly altered during the isolation process and in addition it is not adjacent to an energized membrane.

The objective of our research was to obtain functional group specific information on bacterial surfaces in aqueous solutions, and to observe their variation as a function of cell structure (Gram-positive versus Gram-negative cells) and solution composition. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was used to examine these interfacial properties of cells. To assist the interpretation of spectral features of bacterial cells, small chain organic molecules of known structure and functional groups, similar to those found in bacterial cell walls, were also examined. This is important to establish the sensitivity of ATR-FTIR spectra in the unequivocal identification of different functional groups and their changes with pH. Since it has been shown that surface reactivity of bacterial cells depends on the metabolic state of cells (active, inactive, or dead), experiments were also conducted to evaluate the variations in cell wall chemistry as a function of growth stage and growth medium. Cell walls were also isolated from both Gram-positive and Gram-negative cells to help evaluate the sensitivity of the technique to differences in surfaces of bacteria.

Sensitivity of ATR-FTIR to Bacterial Surfaces. In ATR-FTIR spectroscopy, the infrared beam is allowed to go through the ATR crystal and to be totally reflected at the ATR crystal—sample interface. An evanescent wave, generated at the reflecting surface, penetrates the rarer medium. This wave has the same frequency as that of the incident photons, while its amplitude decreases exponentially away from the interface according to the equation

$$E = E_0 e^{-Z/d_p}$$  \hspace{1cm} (1)

where $E_0$ and $E$ are the electric field amplitudes at the interface and at a depth $Z$ away from the interface, respectively, and $d_p$ is the penetration depth, defined as the $e^{-1}$ depth. $d_p$ is given by

$$d_p = \frac{\lambda}{2\pi n_s^2 \sin^2 \theta - n_e^2}^{1/2}$$  \hspace{1cm} (2)

where $\theta$ is the angle of incidence, $\lambda$ is the wavelength of the infrared radiation, and $n_s$ and $n_e$ are refractive indices of sample and the ATR crystal, respectively. For example, the penetration depth for bacterial cells ($n_e \sim 1.39$) in contact with a Ge ATR crystal ($n_s = 4.0$) that accepts the infrared beam at $188$ nm at $1800$ cm$^{-1}$ is $339$ nm at $1000$ cm$^{-1}$ and $188$ nm at $1800$ cm$^{-1}$. It should be noted that this is $1/e$ depth, and the absolute penetration of the evanescent wave can be greater than these estimates. The refractive index of bacterial cells in the visible region is estimated to be around $1.39$, and it is assumed that the refractive indices of bacterial cells in the infrared and visible regions are similar. Several small chain organic molecules are also reported to have refractive indices close to $1.40$. In addition, there are several other ATR crystals with lower refractive indices that accept an infrared beam at different wavelengths. For example, the refractive index of bacterial cells in the visible region is estimated to be around $1.39$, and it is assumed that the refractive indices of bacterial cells in the infrared and visible regions are similar. Several small chain organic molecules are also reported to have refractive indices close to $1.40$. In addition, there are several other ATR crystals with lower refractive indices that accept an infrared beam at different wavelengths.
incident angles. However, of all commercially available crystals, Ge crystal with a 60° incident angle gives the shallowest penetration depth. The average thickness of the bacterial cell wall ranges from 20 to 50 nm. On the basis of the above calculations, it is apparent that the evanescent wave should penetrate the interior of the bacterial cells, provided they are attached to the ATR crystal surface.

It should be noted that there are uncertainties associated with these penetration depth estimates. When cell suspension is placed on the ATR crystal surface, some cells may be in direct contact with the crystal, while water film separates others. The penetration depth estimates shown above are valid for the cells in direct contact with the ATR crystal, while these estimates do not represent the cells that do not attach to the crystal. This is because the evanescent wave penetrates the cell through an intermediate water film of lower refractive index (when compared to the refractive index of the cell) and unknown thickness, and because of the uncertainty of the behavior of the wave at the water—cell wall interface. In addition, the uncertainty of refractive indices of the bacterial cell walls in the infrared region, and the macromolecular structure of its components (open versus condensed) and their variation with solution composition, may significantly alter the penetration depth estimates. Hence, an ATR-FTIR spectrum of bacterial suspension represents these sum effects, and the spectrum cannot be attributed to the surfaces alone. In this study, the ATR-FTIR spectra of live diatom cells, which contain a coating of a few nanometers of glycocalyx on their surfaces alone, are used to estimate the penetration depth of the evanescent wave. In addition, spectral differences between the live intact cells and lysed cells were used to identify the surface sensitivity of the infrared spectroscopic technique.

2. Materials and Methods

2.1. Model Molecules. The chemicals used in this study were reagent grade or better, and they were obtained from Sigma-Aldrich. Model organic molecules selected for this investigation were d-glucuronic acid (sodium salt monohydrate), phytic acid (sodium salt), tripolyphosphate, and l-rhamnose (monohydrate). About 50 mM aqueous solutions of these compounds were prepared for obtaining their infrared spectra. Solution pH was adjusted between 2 and 10 using appropriate quantities of 0.1 M HCl and 0.1 M NaOH. Samples examined at extreme pH values (2 and 10) were back-titrated to pH 7 to ensure that the alteration of sample chemical structure during pH titration was absent. All solutions were prepared using deionized water.

2.2. Bacterial Cell Cultures. Bacterial species examined in this study were Bacillus subtilis 168, Bacillus licheniformis, Pseudomonas stutzeri 17588, and Pseudomonas aeruginosa PAO1. All these species were cultured in Tryptic Soy broth (TSB) medium for 24 h at 25 °C. P. stutzeri cells were in addition grown in Luria-Bertani (LB) broth medium to evaluate the influence of growth medium on the cell surface chemistry. The cells were collected and averaged for each sample, and the samples were spread carefully so as to cover the entire ATR crystal surface. Appropriate sample spectra extract, and 0.5 g of NaCl per liter of solution, whereas TSB consisted of 17.0 g of pancreatic digest of casein, 3.0 g of papaic digest of soybean meal, 2.5 g of dextrose, 5.0 g of NaCl, and 2.5 g of potassium phosphate in 1 L of solution. Lysed bacterial cell suspensions were prepared by repeatedly freezing and thawing the rinsed live cells (discussed above). Cytoplasm and water-soluble portions of cell walls were separated from insoluble cell walls of lysed cells by centrifugation and examined separately using ATR-FTIR spectroscopy.

2.3. Diatom Cells. Live diatom cells of Thalassiosira weissflogii, and their cleaned silica frustules (after removal of the outer organic layer), were examined using infrared spectroscopy. An axenic culture of this marine diatom (clone Actin, CCMP 1336) was obtained from the National Center for the Culture of Marine Phytoplankton (CCMP). The cultures were grown at 20 °C under a continuous saturating irradiance (provided by fluorescent bulbs below the cultures; flux density ~ 160 μmol m−2 s−1). Cultures were maintained using natural seawater enriched with nutrients. For collecting the infrared spectra of live diatoms, a centrifuged cell suspension was spread on the ATR crystal surface and progressively dried by gently blowing air on the wet sample paste. Infrared spectra of wet diatoms (initial wet paste) and air-dried diatoms were collected.

Cleaved diatom silica cell walls were prepared by initially collecting live cells on a polycarbonate filter with a 0.5 μm porosity and suspending them in 10% (w/v) sodium dodecyl sulfate (SDS) solution in deionized water. These samples were sonicated for three 15 s pulses in a Branson sonicator fitted with a microprobe tip (setting 6, 50% duty cycle), and centrifuged at 2500g for 10 min. The supernatant was discarded, and cell walls were resuspended, vortexed, and centrifuged as discussed above until the supernatant appeared colorless. A pelleted portion of this preparation was oxidized to remove surface glycoprotein by incubation in a 70% perchlorate solution at 70 °C for 2 h.

2.4. Isolation of Cell Wall Fragments. Bacteria were grown in TSB to an exponential growth phase as monitored by OD 600. These measurements. Each culture was washed three times in 1 mM HEPES buffer, pH 6.8, and then passed through a French press operating at 20 000 psi to shear the cells. RNase, DNase (both from Sigma), and 1 mM MgCl2 were added to the lysate to digest nucleic acids, and the large particulate fragments (cell envelopes) were removed by centrifugation at 15000g for 30 min. Gram-positive cell walls were boiled in 2% SDS to remove contaminating protein, and the residual SDS was removed by washing and centrifugation. The same procedure was used for the Gram-negative envelopes, and in this case, it removed the outer membrane, the periplasm, and contaminating proteins so that the peptidoglycan sacculi could be recovered. To avoid confusion, these Gram-negative peptidoglycan (or murein) sacculi are called Gram-negative cell wall fragments in this article.

2.5. Infrared Spectroscopy Studies of Model Molecules and Cell Cultures. ATR-FTIR spectra were obtained on a Bruker IFS66v/S (Billerica, MA) instrument using a narrow band mercury cadmium telluride (MCT) detector. Over 2000 scans with a 2 cm−1 resolution were collected and averaged for each sample. The slit opening for the incident beam was set at 4 or 5 mm. Either AMTIR (refractive index = 2.5 and incident angle = 45° for bacterial and diatom suspensions at low pH and model molecules), Ge-60 (refractive index = 4.0 and incident angle = 60° for all cell suspensions, excepting P. stutzeri), or diamond (refractive index = 2.4 and incident angle = 60° for cell wall fragments and P. stutzeri cell suspension) ATR crystals were used for collecting infrared spectra. Although the penetration depths are different for these different ATR crystals, the spectra of bacterial cells collected on different crystals were similar. However, a multireflection Ge-60 crystal was used for samples in the pH range of 4–10, and a single-reflection diamond crystal was used at all pH values when the sample volume was small. The ATR crystal surface was cleaned with water before running each sample, and the samples were spread carefully so as to cover the entire ATR crystal surface. Appropriate sample spectra

were used to remove spectral background, i.e., water for solution spectra of model compounds and supernatant of centrifuged cell suspensions for bacteria. Air was used as a background for Gram-negative cell wall fragments because the supernatant could not be easily centrifuged because their small sample size required high “g” forces. Back-titrations of model molecules and cells were performed to identify permanent chemical changes, if any, occurring during pH titrations. These studies indicated that the spectra of all model molecules were reproducible, but the cell suspensions showed minor variations when they were titrated to extreme pH values (pH <3.0 or >9.0).

3. Results and Discussion

The cell walls of bacteria consist of several polymers and macromolecules, which possess carboxyl, hydroxyl, phosphate, and amide functional groups. Small model molecules containing one or more different functional groups with structural environments similar to those predicted for biomacromolecules in bacterial cell walls were chosen to evaluate vibrational bands of different functional groups. Infrared spectral variations of these model molecules as a function of pH were used to obtain information on the changes in vibrational states of different functional groups during protonation or deprotonation. Detailed spectral analyses of these model molecules were useful in identifying the functional groups of bacterial surfaces from their infrared spectra. It is likely that the coordination environments of these selected moieties in bacteria are different from those of the selected model molecules mainly because of variations due to H-bonding and substitution adjacent to the reactive functional groups. However, studies on model molecules can provide important information on the infrared absorption bands of different moieties in the bacterial cell and, as shown below, the infrared spectra of these model molecules and their variation as a function of pH resembled those of bacterial cells closely.

3.1. Infrared Spectroscopy of Model Molecules.

3.1.1. Alcohol Groups. L-Rhamnose, a methyl pentose sugar commonly found in glycosides and in P. aeruginosa LPS, was used as a representative of molecules containing alcoholic hydroxyl groups. Although the infrared spectrum of aqueous rhamnose showed several absorption bands corresponding to C–O bonds in the range of 950–1200 cm$^{-1}$, it was difficult to assign each peak to vibrations of a specific bond in the molecule (Figure 1). Identification of these bands was complicated by the strong vibrational coupling of C–O with the C–C and C–H bonds, which distributes its infrared absorption over several bands. The hydroxyl groups in this molecule do not undergo deprotonation at pH values lower than 10, and their infrared spectra do not exhibit any spectral variations in this pH range (Figure 1). The absence of spectral variations of hydroxyl group as a function of pH and vibrational coupling made their identification in bacteria difficult. As discussed later, the symmetric and asymmetric stretching vibrations of phosphate also absorb in this energy range, which complicated the unequivocal identification of both phosphate and hydroxyl groups. However, the vibrational bands of phosphate groups changed significantly with protonation and deprotonation in the pH range of 2–10.

3.1.2. Carboxylic Acid Groups. D-Glucuronic acid was used as a representative of organic molecules that contain both carboxylic acid and hydroxyl groups and is analogous to the teichuronic acids of Gram-positive walls (Figure 2). The structure of glucuronic acid is similar to that of rhamnose (discussed above), except for the addition of a carboxylic group in place of the methyl group in the former. Glucuronic acid exhibits spectral features that correspond to both carboxylic and alcoholic groups in the frequency ranges of 1200–1800 and 900–1200 cm$^{-1}$, respectively (Figure 2). The C=O and C–OH of –COOH exhibited vibrational bands at 1728 and 1268 cm$^{-1}$, respectively, in acid solutions. As the pH increased, the intensity of these bands decreased and new bands corresponding to the asymmetric and symmetric stretching of COO$^-$ occurred at 1589 and 1413 cm$^{-1}$, respectively (Figure 2). On the


basis of these spectral changes the pKₐ (protonation or deprotonation constant) of the carboxylic acid group in glucuronic acid is expected to be between 3 and 4.²²

In general, carboxylic acids exhibit similar spectral changes as they deprotonate and form carboxylate anions (see refs 46 and 47 and references therein). The asymmetric stretching vibrations of COO⁻ are also sensitive to the coordination environment of carboxylic anion, and these energies have been used to identify the type of carboxylate, such as aromatic and aliphatic, in bacterial walls.⁴⁷,⁴⁸ In contrast, strong coupling of symmetric stretching vibration of carboxylate with the C–H, C–OH, and C–C groups makes it less sensitive to its structural environment.⁵⁵,⁶³

The spectral features of glucuronic acid in the energy range of 900–1200 cm⁻¹ were caused by the vibrations of C–OH (alcohol), C–C, and C–H groups, and these were similar to those seen with rhamnose, as discussed above. Small spectral variations in this energy range in acidic solutions may be due to intramolecular H-bonding between carboxylic and alcohol groups associated with carboxylic group deprotonation.

3.1.3. Phosphate Groups. Phosphate groups in bacteria can exist in several different forms: inorganic forms of phosphate such as orthophosphate and its oligomers, and organic species in the form of phosphate mono- and diesters. In this study, phytic acid (phosphate monoester) and tripolyphosphate were used as models to evaluate the behavior of phosphoryl groups in bacterial walls (Figure 3). Infrared spectroscopy of aqueous phytic acid (50 mM) at pH 2.0, 3.0, 6.0, 7.0, 8.5, 9.0, and 10.0 on AMTIR crystal. The inset shows the structure of the molecule.

![Figure 3. ATR-FTIR spectra of aqueous phytic acid (50 mM) at pH 2.0, 3.0, 6.0, 7.0, 8.5, 9.0, and 10.0 on AMTIR crystal. The inset shows the structure of the molecule.](image)

A summary of infrared spectral changes of model molecules indicates that the carboxylic and phosphate groups exhibit dominant changes around 1720 and 1200 cm⁻¹, respectively, with changes in pH. The hydroxyl, C–H, and C–C groups of carbohydrates exhibited several bands in the energy range of 900–1200 cm⁻¹, but did not exhibit significant changes with pH. Although the amide groups are common in the protein backbone, their models were not examined here. However, previous infrared and Raman spectroscopic studies of molecules containing amides indicated that the C=O of amide (referred to as amide I) and N–H bending (coped to C–N and referred to as amide II) occur in the range of 1600–1700 and 1520–1550 cm⁻¹, respectively.⁵⁴,⁵⁵ The amide II band is noticed.
B. subtilis of corresponding to carbohydrate and phosphate groups below). The presence of carboxylates in the fragments was clearer to 6.0 (pH of cell culture), to 10.0 (Figure 4). The spectral - O of carboxylic acid exhibits a peak at 1726 cm

The energy and spectral variation of these characteristic bands corresponding to different functional groups were used to identify these groups in cell walls of bacteria.

3.2. Infrared Spectroscopy of Cell Wall Fragments.

Isolated cell wall fragments of both Gram-positive and Gram-negative cells were examined using ATR-FTIR spectroscopy (Figure 4). The isolated walls of B. subtilis examined in this study were composed of peptidoglycan and teichoic acid, and those of P. aeruginosa contained peptidoglycan only. Although the infrared spectra of these cell wall fragments and intact bacterial cells exhibit similar bands, differences existed between the walls of B. subtilis and P. aeruginosa (Figures 4 and 5). The wall fragments exhibited distinct bands around 1640 and 1540 cm

alters the energy, width, and intensity of the amide bands, the contributions to spectral variations from changes in protein conformations cannot be ruled out. Minor spectral variations were also found for the phosphate band around 1215 cm

3.3. Infrared Spectroscopy of Cell Suspensions.

3.3.1. Sensitivity Test of ATR-FTIR to Bacteria Surfaces. Live diatom cells with a thin glycoprotein layer over the silica shell and FTIR spectra of lysed and intact cells were used as model systems for estimating the penetration depth of the evanescent wave at the interface of the ATR crystal.

The thickness of the external glycoprotein layer is about 8–10 nm in the examined diatoms.\(^\text{56}\) The diatoms cleaned with perchlorate do not have this organic layer, and their infrared spectrum showed two bands at ~1630 (weak) and 1100 (strong) cm

These infrared spectra of diatoms, with and without the thin glycoprotein layer, indicated that the collected ATR-FTIR spectra could distinguish between the two diatom surfaces even though the glycoprotein layer was only a few nanometers thick. Clearly the infrared signal could penetrate at least to this thickness. Although the estimated penetration depth for the ATR crystal with the highest refractive index and highest angle of incidence (Ge-60) is greater than 190 nm (<1800 cm

bacterial surfaces (Figure 7). These studies indicate that the amide II and carbohydrate bands (around 1520 and 1080 cm\(^{-1}\), respectively) of soluble cytoplasm-rich fluid of the cell interior are different from those of the intact cells. In addition, the ATR-FTIR spectra exhibited different spectral variations for intact and lysed cells as a function of pH. These studies suggest that the ATR-FTIR spectroscopic results presented above for the intact cells may represent the surface macromolecular composition.

Although these ATR-FTIR measurements did not provide an absolute penetration depth, they did indicate that the ATR spectra of aqueous cell suspensions are highly sensitive to surfaces. It is reasonable to assume that the optical properties (e.g., refractive index) of the bacterial surface and those of the organic layer on the diatom cell surface are very similar. Hence, it is understood that the ATR-FTIR spectra of the bacteria presented below mostly reflect the properties of the bacterial cell wall and not the interior of the cell. As discussed later, the similarities in the infrared spectra of cell wall fragments and the intact cells also lend support to this hypothesis.

**3.3.2. Functional Group Chemistry of Bacteria Surfaces.**

The infrared spectra of Gram-positive (B. subtilis and B. licheniformis) and Gram-negative (P. stutzeri and P. aeruginosa) bacteria in aqueous solution as a function of pH and growth stage were examined using ATR-FTIR spectroscopy (Figure 5). Their infrared spectra exhibited distinct peaks corresponding to carboxyls, amides, phosphates, and carbohydrates (Figure 5, Tables 1 and 2). Some of the bacterial species were also grown at least three times to evaluate variations in their infrared spectra, and these studies indicated that the energy and relative intensities of vibrational bands were highly reproducible (energy variation < 2 cm\(^{-1}\)). Interestingly, the infrared spectra of both Gram-positive and Gram-negative bacteria were similar and exhibited similar variations as a function of changes in pH. The similarity is presumably because the Gram-negative walls consisted of only peptidoglycan sacculi and peptidoglycan is a major component of the Gram-positive cell walls. The maximum spectral changes observed were in the pH range of 2–4, and changes above this pH range were minor.

**Carboxyl Groups.** The carboxylic groups of bacterial cells exhibited a distinct band at ~1720 cm\(^{-1}\) corresponding to
Amide Groups. The amide groups exhibited intense vibrations of the deprotonated carboxylate anion, COO$^-$, discussed above. Although the asymmetric stretching vibrations of the deprotonated carboxylate anion, COO$^-$, was expected to appear around 1590 cm$^{-1}$ in alkaline cell suspensions (pH ≥ 4.0), it was obscured by the intense amide bands in the range of 1500–1560 cm$^{-1}$. However, the symmetric stretching of COO$^-$ vibration appeared around 1386 cm$^{-1}$, which was absent in all samples previously examined at acidic pH. The C$\equiv$O of protonated carboxylate band around 1720 cm$^{-1}$ was almost absent or extremely weak in alkaline cell suspensions. This suggests that (i) ATR-FTIR studies are surface sensitive (similar to the diatom surfaces) and the majority of the carboxylate groups are inside the cell wall, (ii) the protons can diffuse rapidly inside the cell wall and protonate the carboxylates present inside the plasma membrane and the cytoplasm.

Amide Groups. The amide groups exhibited intense bands around 1650 and 1550 cm$^{-1}$ corresponding to the amide I and amide II bands, respectively. Although variations in the energies and intensities of these bands were minor with changes in pH, the intensity ratio of amide I to amide II decreased with an increase in pH. These minor variations in the infrared spectra of amide groups may be caused by (i) appearance of new peaks in this energy region (e.g., corresponding to COO$^-$), which may modify the intensity and energy of amide bands (similar to the variations observed in the case of isolated cell wall fragments), and (ii) changes in protein conformations. Although the insensitivity of amide vibrations to pH fluctuations may suggest that a majority of amide groups are inside the cell wall, this seems unlikely. This is because the isolated cell wall fragments showed intense bands corresponding to amides, and their spectral variations with pH, even if they are minor, were similar to those reported for intact cells (Figures 4 and 5).

The spectral intensity of carboxylate and amide groups can give information on the relative concentration of these groups. A comparison of their intensity ratio with those of known molecules can provide information on the relative concentration of these groups in the cell wall. For example, the infrared spectrum of aqueous Lisinopril, which contains an amide and two carboxylic acid groups, exhibits an intensity ratio of 1.61 for amide I and C$\equiv$O of a protonated carboxy1. The solid spectra of several compounds that contain amides and carboxyls exhibit a ratio that ranges from 1 to 1.1 (average from 1-(aminocarbonyl)-1-cyclopropanecarboxylic acid, N-acetylenuraminic acid, 1-acetyl-4-piperidinecarboxylic acid, succinic 2,2-dimethylhydrazide, N-[(tert-butoxycarbonyl)-L-serine, and D-ala-nylglycine). However, the aqueous spectrum of Lisinopril may represent the bacteria sample better than these other solid samples because of its sample state. When the ratio of amides to carboxylic groups are estimated using this ratio from Lisinopril and the measured spectral intensity of carboxylates and amides in bacterial suspensions at pH 2.0, the concentration ratio of amide to carboxylic groups is in the range of 1.5–2.5 (2.5–4.0 when solid references used). Variations in this ratio may be partly attributed to improper subtraction of spectral background from water (water bending vibrations are around 1630 cm$^{-1}$).

Table 1. Absorption Bands of Bacterial Surface Functional Groups

<table>
<thead>
<tr>
<th>Abs bands (cm$^{-1}$)</th>
<th>Vibrational modes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1721</td>
<td>stretching of C$\equiv$O in protonated carboxylic acid group</td>
</tr>
<tr>
<td>1646</td>
<td>stretching of C$\equiv$O in amide (amide I)</td>
</tr>
<tr>
<td>1541</td>
<td>N–H bending and C–N stretching in amide (amide II)</td>
</tr>
<tr>
<td>1450</td>
<td>CH$_2$ scissoring</td>
</tr>
<tr>
<td>1396</td>
<td>symmetric stretching of COO$^-$</td>
</tr>
<tr>
<td>1233</td>
<td>vibrations of –COOH, and C–O–C group in esters</td>
</tr>
<tr>
<td>1211</td>
<td>stretching of P=O bond in phosphate</td>
</tr>
<tr>
<td>950–1150</td>
<td>asymmetric and symmetric stretching of PO$_4^{2-}$ and P(OH)$_2$ in phosphate; vibrations of C–OH and C–C bonds in polysaccharides and alcohols</td>
</tr>
</tbody>
</table>

Several of the bands identified are broad, and the band position represents the center of the band maximum.

Figure 7. ATR-FTIR spectra of intact P. stutzeri cells at pH 6.0 (a) and 2.0 (b), and their lysed cells (c–e). (c) Soluble fraction of lysed cells, (d) soluble fraction and cell walls, and (e) same as (d) but at pH 2.0. Approximate band positions are indicated in the figure.
Table 2. Absorption Bands of the Dominant Surface Functional Groups of Different Bacterial Species and Their Cell Walls at pH ~6.0

<table>
<thead>
<tr>
<th>functional group</th>
<th>B. subtilis</th>
<th>B. licheniformis</th>
<th>P. stutzeri</th>
<th>P. aeruginosa</th>
<th>cell wall of B. subtilis</th>
<th>cell wall of P. aeruginosa</th>
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<tbody>
<tr>
<td>amide I</td>
<td>1651</td>
<td>1651</td>
<td>1643</td>
<td>1652</td>
<td>1640</td>
<td>1647</td>
</tr>
<tr>
<td>amide II</td>
<td>1545</td>
<td>1542</td>
<td>1542</td>
<td>1544</td>
<td>1539</td>
<td>1555</td>
</tr>
<tr>
<td>CH₂ scissoring</td>
<td>1455</td>
<td>1455</td>
<td>1457</td>
<td>1456</td>
<td>1452</td>
<td>1452</td>
</tr>
<tr>
<td>ν₃ COO⁻</td>
<td>1401</td>
<td>1400</td>
<td>1402</td>
<td>1402</td>
<td>1400</td>
<td>1397</td>
</tr>
<tr>
<td>COOH, P=O</td>
<td>~1230</td>
<td>~1230</td>
<td>~1240</td>
<td>~1230</td>
<td>~1220</td>
<td>~1240³</td>
</tr>
<tr>
<td>–P(OH)₂, –C=O</td>
<td>~1100</td>
<td>~1100</td>
<td>~1100</td>
<td>~1100</td>
<td>~1012</td>
<td>~1050</td>
</tr>
</tbody>
</table>

³ Weak.

cm⁻¹). When this ratio is compared with the ratio of carboxyl to amide in structurally well-characterized biomacromolecules, it is around 1:5 and 1:3 for peptidoglycan in Gram-positive and Gram-negative cells, respectively. Although several major constituents of cell walls are not well characterized to make an accurate estimate of this ratio, this ratio obtained from infrared spectra may represent the average ratio of carboxyls and amides present in bacterial surface membranes.

Phosphate Groups. As discussed earlier, it is difficult to identify different phosphate groups unambiguously from the infrared spectra alone. On the basis of the spectral variations reported for different types of phosphates, the absorption band around 1210 cm⁻¹ was assigned to the stretching of the P=O bonds of phosphate diester or protonated polyphosphate (details discussed above). The relative intensity of this peak also decreased at pH values 4 and above, which is consistent with the trends seen in the case of organophosphates (Figure 3). Although the C–OH bonds of carboxylic acids absorb in the same energy region, the spectral variation around 1210 cm⁻¹ is too low to be attributed to C–OH groups. In addition, spectral variations in this region were also accompanied by variations around 1100 cm⁻¹, where the P–OH bands vibrate (discussed later). Another strong band around 1233 cm⁻¹, which was not affected by pH variations, may have been caused by functional groups, such as esters (C–O–C vibrations) and phosphate groups inside the cell wall.

The infrared spectra of intact Gram-positive and Gram-negative cells are identical in the spectral region corresponding to phosphate groups while their isolated cell wall fragments are significantly different (Figures 4 and 5). It should be noted that the isolated cell wall fragments of Gram-positive cells contain both peptidoglycan and teichoic acids, and the Gram-negative cells contain teichoic acid only. Since the outer membranes of intact Gram-negative cells contain phospholipids, which have phosphate groups in the same local coordination environment as phosphate in teichoic acids, the sum spectra of peptidoglycan + teichoic acids of Gram-positive cells, and peptidoglycan + outer membrane of Gram-negative cells, are expected to be similar.

Polysaccharide and Related Groups. The C–OH (alcohol), C–C, and some of the C–H vibrations exhibited a broad band in the energy region of 950–1150 cm⁻¹. With an increase in pH, the band at 1080 cm⁻¹ became sharp along with sharpening of a weak band around 1115 cm⁻¹ and weakening of the high-energy shoulder around 1050 cm⁻¹. These changes were at a maximum between pH 2.0 and 4.0, and may be caused by variations in the H-bonding interactions associated with the deprotonation of carboxylate groups and the variations in the protonation state of phosphate groups. These changes were consistent in all of the Gram-positive and Gram-negative species examined in this study.

A strong band at 1450 cm⁻¹ was insensitive to pH variation, and this is characteristic of the scissoring motion of CH₂ groups, which are common in peptidoglycan, teichoic acid, LPS, and phospholipids. Kamnev et al. also observed similar peaks in IR spectra of isolated bacterial membranes.

3.3.3. Influence of Growth Phase and Growth Medium on Cell Surface Chemistry. Previous research has shown that metabolically active cells, live nonmetabolically active (or resting) cells, and dead cells possess cell walls of different surface reactivities, possibly due to competition with protons generated by the cell’s energized membrane or because of changes in surface functional groups. To determine whether the functional group chemistry of the bacterial cell surface changes as a function of its growth stage, IR spectra of P. stutzeri were obtained at different growth stages of the organism (Figure 8). These cells at different growth stages showed similar infrared spectral features without significant changes in the energy and intensity of bands. This implies that the dominant functional groups on bacterial cell walls do not change over time with growth.

The influence of growth medium on the surface chemistry of microorganisms was also examined (Figure 9). Infrared spectra of P. stutzeri grown in LB and TSB were identical, indicating that differences in these nutrient-
rich growth media do not alter the dominant functional groups on bacterial surfaces.

4. Summary and Conclusions

This in situ ATR-FTIR study demonstrates the advantages and limitations of infrared spectroscopy in distinguishing different functional groups on bacteria surfaces. Although the ATR-FTIR spectroscopy technique is surface sensitive, the sensitivity of this technique to distinguishing different cell surface membranes cannot be established directly. However, the ATR-FTIR measurements on intact and lysed cells and live and clean diatoms suggest that this technique provides surface-sensitive information.

Results presented in this study also showed that the dominant functional groups of bacterial surfaces are carboxyl, amide, phosphate, hydroxyl, and carbohydrate related moieties. Contrary to the general understanding that Gram-positive bacterial cell walls exhibit significant differences from Gram-negative cell walls in both structure and chemical composition, infrared spectroscopic results presented here suggest that the bulk functional group chemistry of both classes of bacterial surfaces are identical. These findings are in line with the identical macroscale sorption behavior of different metals onto Gram-positive and Gram-negative bacteria and their mixtures. Although these infrared spectroscopic results indicate the universality of the functional group chemistry of bacteria cell surfaces, differences between them may exist for the minor components and their structure. These differences may lead to variations in reactivity when subjected to straining techniques used in distinguishing the Gram-positive and Gram-negative bacteria, and further in-depth studies are necessary to identify the structural differences in functional groups between them.

Among different functional groups, we find that carboxylic, phosphate, and carbohydrate groups are sensitive to changes in solution pH, and the deprotonation of the first two may lead to the net negative surface charge of bacteria surfaces in the ambient pH range of 4–9. Previous potentiometric titrations have also identified them as the primary sites that can deprotonate in the acidic pH range. While a majority of carboxylic acid groups have pKₐ values below 5.0, the phosphate groups have a wide range of pKₐ values, which decrease with phosphate polymerization (e.g., from phosphoric acid to triphosphoric acid). The phosphate mono- and diesters common in bacterial cell walls also exhibit strong acidic behavior. Because of their sensitivity in the acidic pH range, both carboxylic and phosphate groups can act as metal binding sites below a pH of 5.0. The absence of changes in amide groups with pH may suggest that the macromolecular structure of the protein backbone may not change significantly with solution pH.

ATR-FTIR spectroscopic results presented here demonstrate that bacterial surface chemistry does not change significantly with either the growth phase or the nutrient-rich growth media. Although nutrient-rich growth media commonly used by microbiologists are tested in this investigation, it will be important to test the influence of extreme concentration of metals and organic molecules on the cell wall chemistry. This information will be useful in identifying the behavior of bacteria in nature.

Acknowledgment. The authors thank Dr. A. Milligan for providing diatom samples, and Dr. A. Deshmukh for reviewing the manuscript. This work was supported by a NSF (Chemical Sciences) grant to S.C.B.M. and a NSERC-discovery grant to T.J.B.

LA049043+


