Effect of Dissolved Oxygen on Two Bacterial Pathogens Examined using ATR-FTIR Spectroscopy, Microelectrophoresis, and Potentiometric Titration

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The effects of dissolved oxygen tension during bacterial growth and acclimation on the cell surface properties and biochemical composition of the bacterial pathogens Escherichia coli O157:H7 and Yersinia enterocolitica are characterized. Three experimental techniques are used in an effort to understand the influence of bacterial growth and acclimation conditions on cell surface charge and the composition of the bacterial cell: (i) electrophoretic mobility measurements; (ii) potentiometric titration; and (iii) ATR-FTIR spectroscopy. Potentiometric titration data analyzed using chemical speciation software are related to measured electrophoretic mobilities at the pH of interest. Titrations of bacterial cells are used to identify the major proton-active functional groups and the overall concentration of these cell surface ligands at the cell membrane. Analysis of titration data shows notable differences between strains and conditions, confirming the appropriateness of this tool for an overall charge characterization. ATR-FTIR spectroscopy of whole cells is used to further characterize the bacterial biochemical composition and macromolecular structures that might be involved in the development of the net surficial charge of the organisms examined. The evaluation of the integrated intensities of HPO$_4^{2-}$ and carbohydrate absorption bands in the IR spectra reveals clear differences between growth protocols. Taken together, the three techniques seem to indicate that the dissolved oxygen tension during cell growth or acclimation can noticeably influence the expression of cell surface molecules and the measurable cell surface charge, though in a strain-dependent fashion.

Introduction

Several processes such as bacterial transport in the natural subsurface environment, the onset of biofilm formation in water distribution systems, and retention of bacteria in engineered water treatment systems are all dependent on a complex interplay of the fundamental interactions controlling bacterial adhesion to inert surfaces or to each other (i.e., in flocculation processes) (1, 2). Most bacteria-surface interactions are believed to be mediated by the outer membrane of bacterial cells. Previous studies have demonstrated that bacteria growth conditions can influence the density and distribution of cell-surface functional groups on Gram-positive and Gram-negative bacterial surfaces (3–5). Accordingly, bacteria—surface interactions may be affected by variations in the composition of the outer membrane of bacterial cells with changes in environmental conditions (e.g., pH, temperature, substrate availability and composition) (6–9). Nevertheless, the links between changing environmental conditions and bacterial surface properties or bacterial attachment to surfaces are still not well understood.

Bacterial cell walls consist of a wide range of biopolymers and macromolecules which possess carboxyl, hydroxyl, phosphate, and amine functional groups that confer to the cells a net charge. In many aquatic environmental systems, this net charge is determined to be negative (10, 11). Metal adsorption studies indicate the important role of acidic ligands such as carboxyl or phosphate in these processes involving chemical and electrostatic interactions (12, 13). The relative abundance of certain proton-active surface ligands arising from cell wall macromolecules, such as teichoic and teichuronic acids in the case of Gram-positive cells and lipopolysaccharides (LPS) in the case of Gram-negative cells, has been shown to influence the metal sorption process (12, 14) and the extent of electrostatic interactions between bacteria and silica surfaces (15).

Macroscopic acid–base titration has been used increasingly over the past decade to obtain insight into the concentration and acidity of proton-active surface ligands (3, 5). Nevertheless, this technique alone does not provide sufficient information to establish the identity of reactive surface ligands. Recent studies indicate that spectroscopic techniques may be useful complementary tools to study the molecular-scale processes that govern proton–bacteria sorption reactions (7, 16, 17). Infrared (IR) spectroscopy is a well-established analytical technique that can be used to determine the identity and protonation states of organic functional groups (18). Proteins, lipids, and carbohydrates have been widely investigated individually and, more recently, this technique has been applied to more complex systems (17, 19, 20). Because IR radiation is nondestructive to biological materials, it can be used to examine the functional-group chemistry of living cells and isolated cellular components. Hence, IR spectroscopy has been utilized for situ examination of bacterial cells, biofilms, and extracted bacterial surface biomolecules in aqueous systems (19–22). Several recent investigations have examined the surface chemistry of intact bacterial cells and their cell walls using both macroscopic (e.g., potentiometric titration, ion adsorption) and molecular tools (e.g., FTIR and XPS) (7, 14, 16, 17). By combining IR data with information regarding the pK$_a$ values of acid/base functional groups at the cell surface, tentative identifications of the functional groups associated with the pK$_a$ values derived from potentiometric data can be made. Kim et al. (22) conducted potentiometric titrations and electrophoretic mobility measurements to evaluate the variability in cell surface charging behavior for E. coli O157:H7. In their work, FTIR analysis was also used to confirm the effectiveness of enzymatic treatment for removal of extracellular macromolecules from the cell surface.
Changes in the expression of nutrient-specific binding agents and membrane transport proteins, lipopolysaccharides, flagella, etc. have been demonstrated to occur upon switching between metabolic pathways and can therefore alter the ability of microbes to stick to surfaces (23, 24). Ebboigbodin (7) reported that the surface chemistry of E. coli was altered after supplementation of the growth medium with additional glucose at the beginning of the growth phase. Specifically, results obtained using potentiometric titrations and spectroscopic techniques showed that cells displayed a higher concentration of proton-active functional groups in macromolecules present at the cell surface that consequently reduced cell-to-cell aggregation. Haas (3) found sufficient variability by potentiometric titration of the facultative Shewanella putrefaciens, when culture conditions were changed from aerobic to anaerobic, as to confirm two distinct sets of surface complexation parameters. Alternatively, in a study of Bacillus and Pseudomonas spp. by ATR-FTIR spectroscopy, Jiang et al. (19) reported that bacterial surface chemistry did not change significantly with either the growth phase or the composition of the nutrient-rich growth media. Clearly, discrepancies remain regarding metabolic determinants of microbial surface reactivity.

The overall objective of the present study was to evaluate the variations in cell wall chemistry in terms of the identity and abundance of cell surface functional groups as a function of environmental dissolved oxygen (DO) concentration during growth and acclimation of Escherichia coli O157:H7 and Yersinia enterocolitica. These bacterial Gram-negative pathogens are able to grow in a wide range of environments, and understanding the dependence of their physicochemical properties on environmental factors is of interest. Following exposure to different DO tensions in the natural subsurface environment, changes in the mobility of these pathogens mediated by intrinsic outer membrane properties may lead to contamination of potable water supplies due to increased migration (25). In this work, electrophoretic mobility (EM) measurements were employed to determine the overall cell surface charge, and titration of bacterial cells along with the modeling of the experimental data was utilized to determine the pK_a values of the major proton-active functional groups at the cell surface. ATR-FTIR spectroscopy was then used to characterize the effects of the growth and acclimation conditions on the overall biochemical composition of the cells, and the pH dependence of the spectra was examined to obtain information on proton-active functional groups and macromolecular structures that might be involved in the development of the cell surface charge of the pathogens studied.

Materials and Methods
Bacteria Selection, Incubation, and Acclimation. The pathogenic bacteria Escherichia coli O157:H7 ATCC 700927 and Yersinia enterocolitica ATCC 23715 were grown and acclimated as described in the Supporting Information. Briefly, the bacteria were grown either anaerobically (ANA) or aerobically (AER), then suspended in 10 mM KCl (pH 6.7) of either low dissolved oxygen (lowDO) or saturated DO (satDO) concentration. Following a 24 h acclimation period in lowDO or satDO electrolyte solution, bacterial surface properties were examined by potentiometric titration and electrophoretic mobility measurements, and the biochemical composition of the cells was studied by ATR-FTIR spectroscopy.

Bacterial Cell Characterization. (a) ATR-FTIR Spectroscopy. ATR-FTIR spectra were recorded on a Varian Excalibur 3100 FTIR spectrometer (Varian, Melbourne, Australia) equipped with a DTGS detector and a SensIR single-bounce diamond ATR accessory (Smith Scientific, CT). The instrument was purged with dry air to minimize spectral contributions from CO_2 and water vapor. For each of the four treatments examined, cell suspensions (4 × 10^9 cells/mL) were washed after acclimation (21 h at 9°C) and were then immediately concentrated by centrifugation to ∼5 × 10^9 cells/mL. Subsequently, cell suspensions with pH values of 4.0, 6.7, and 9.5 were prepared separately by adding appropriate quantities of 0.1 M HCl or 0.1 M NaOH. After allowing the cells to equilibrate with the electrolyte solution for 1 h, a 1.5-mL sample of each bacterial suspension was spun down into a wet pellet paste (9000 × g for 4 min, microCL 17R, Thermo). For FTIR spectral acquisition, a portion of the paste was collected with a 1-mm loop and spread on the diamond ATR element. The ATR-FTIR spectrum of the wet pellet paste was collected by coaddition of 100 scans at a resolution of 1 cm^-1 and ratioed against a background spectrum previously collected from the clean ATR crystal. For all four experimental treatments, a reference spectrum of preconditioned 10 mM KCl water solution was subtracted from the spectrum of each bacterial sample, with the subtraction scaling factor set such as to obtain a constant ratio of the maximum absorbance in the amide I region (1700–1600 cm^-1) to that in the amide II region (1600–1500 cm^-1) of 1.4 ± 0.02. The resulting spectrum was then normalized to unit height of the amide I band. ATR-FTIR measurements were conducted at room temperature (22°C).

(b) Electrophoretic Mobility Measurements. EM measurements were previously reported in ref 25. Briefly, EPM was measured at 11°C using acclimated cell suspensions (∼4 × 10^9 cells/mL, pH 6.7, 10 mM KCl) (ZetaSizer Nano ZS, Malvern). Laser Doppler electrophoretic measurements were repeated using at least three different bacterial suspensions, measured at least three times each. Bacteria ζ-potentials were calculated from the EPM measurements using the Smoluchowski equation (26).

(c) Titrations of Bacteria. Potentiometric titrations were conducted (11°C) to determine the dissociation constants (pK_a) and proton binding site concentrations (S) of acid/base functional groups located in the macromolecules forming the bacterial outer cell wall. Details regarding the titration procedure and data analysis are provided in the Supporting Information.

Results and Discussion
Electrokinetic Characterization of Bacteria. Several studies on the transport and fate of colloids and microorganisms have demonstrated the relevance of ζ-potential as a predictor of particle stability and interaction with abiotic surfaces (9, 15, 25, 27–29). Bacteria ζ-potentials were calculated from EPM measurements (taken from Castro and Tufenkji (25)). Both E. coli and Y. enterocolitica exhibit a net negative charge at pH 6.7 (Figure 1). However, there is a considerable difference in the charge of the two organisms over the range of treatments considered. Y. enterocolitica has a substantially more negative ζ-potential than E. coli (ranging from 5 to 15 times larger, depending on the treatment). The data in Figure 1 shows that E. coli exhibits a more negative overall charge when grown in the absence of oxygen versus growth in the presence of oxygen, with a larger negative charge for the ANA/low condition than the ANAtsat condition. This latter result suggests that the DO concentration during acclimation influences the overall cell surface charge. Interestingly, Y. enterocolitica exhibits an opposite trend whereby cells grown aerobically are slightly (p < 0.01) more negatively charged than those grown anaerobically (Figure 1). Roberts et al. (30) reported a similar trend with the organism Shewanella putrefaciens whereby aerobically grown cells were slightly more negatively charged than anaerobically grown cells between pH 2–4.

Potentiometric Titration. To better understand the nature of the functional groups present in the cell wall of the two organisms when grown and acclimated at different DO
concentrations, the data obtained from potentiometric titrations were analyzed using chemical speciation software (FITTEQL 4.0) (31). The pK\textsubscript{a} values and concentrations of the proton binding sites determined from this analysis are summarized in Table S1 (Supporting Information). In general, the dissociation constants and site densities determined in this study are comparable to those determined in previous studies using other organisms (5, 17). The most acidic proton binding site determined for *E. coli* has pK\textsubscript{a} values in the range of 3.4–4.1, falling within the pK\textsubscript{a} range for carboxylic groups (pK\textsubscript{a} = 2–6) as well as overlapping with the upper end of the pK\textsubscript{a} range reported for phosphodiester (pK\textsubscript{a} = 3.2–3.5) (32, 33). The second proton binding site for *E. coli*, with pK\textsubscript{a} between 5.2 and 5.4, can be attributed to the carboxylic groups of organic (diprotic) acids, whereas the third pK\textsubscript{a} (7.4–7.6) can be associated with phosphoryl groups and the most basic pK\textsubscript{a} (~10) likely corresponds to either amine or hydroxyl (phenolic) groups (19, 20). The fitted pK\textsubscript{a}s determined for *E. coli* O157:H7 are generally comparable to those reported for the same organism grown under different conditions by Kim et al. (22). For *Y. enterocolitica*, FITTEQL predicts three major proton binding sites and the fitted pK\textsubscript{a}s can be tentatively assigned to carboxyl (pK\textsubscript{a}), phosphoryl (pK\textsubscript{a}), and amine/hydroxyl (pK\textsubscript{a}) groups (Table S1 of the Supporting Information). Predicted pK\textsubscript{a} values for the two organisms in the four treatments are not very different (Table S1 of the Supporting Information), suggesting that similar types of functional groups are present on both cell surfaces.

To allow comparison of the titration data with the ζ-potential measurements, the concentration of deprotonated functional groups at the pH of interest (6.7) was determined. Specifically, the pK\textsubscript{a} values determined by FITTEQL were used to quantify the number of deprotonated groups at pH 6.7 for the four treatments. The contribution of each site to the total deprotonated charge at pH 6.7 is presented in Figure 1 for both bacteria. At pH 6.7, *E. coli* has a significantly larger amount (at a 95% confidence level) of deprotonated sites (groups bearing a negative charge) per cell when grown anaerobically than when grown in the presence of oxygen. At this pH, the number of charged groups in the AER\textsubscript{low} condition is greater than in the AER\textsubscript{sat} condition. In contrast, it remains comparable in the ANAlow and ANAsat treatments, suggesting little influence of the DO acclimation treatment when *E. coli* is grown anaerobically. However, closer examination of Figure 1 shows that there is a difference in the relative contributions of the individual site types to the total charge, with the site S\textsubscript{1} (tentatively assigned to phosphodiester groups) corresponding to nearly 50% of S\textsubscript{i} in the ANAlow condition, in comparison with 38% in the ANAsat condition.

Titration data presented in Figure 1 also provides insight into the surface charge of *Y. enterocolitica* at pH 6.7. In contrast to *E. coli*, *Y. enterocolitica* exhibits more charged groups when grown aerobically than anaerobically, as was also observed for *Shewanella putrefaciens* by Haas (3), and the number of charged groups for both growth conditions is not strongly dependent on the acclimation protocol. Interestingly, the ANAlow condition, which is one of the treatments exhibiting the lowest amount of proton binding sites (S\textsubscript{i}), in turn has the highest percentage of deprotonated groups per cell (48%) at pH 6.7, with S\textsubscript{i} being the dominant charge-contributing group (Figure 1). When grown aerobically, the average contribution of deprotonated groups to the total titrated charge per cell at pH 6.7 is much lower (32%).

Figure 1 allows for a direct comparison of ζ-potential data with the number of charged groups at pH 6.7. For both organisms, ζ-potential is generally well associated with the total concentration of charged functional groups at pH 6.7. Specifically, the treatments with the greatest amount of charged groups determined by titration analysis (and associated model predictions) are generally associated with the highest absolute ζ-potentials. When the data for all four treatments are combined, a simple linear regression of the total concentration of charged functional groups at pH 6.7 against ζ-potential for *E. coli* O157:H7 reveals a rather weak correlation (R\textsuperscript{2} = 0.66) between the two sets of measurements. However, correlating only S\textsubscript{2} (concentration of most acidic functional group determined by analysis of the titration data) and ζ-potential, the regression yields an R\textsuperscript{2} of 0.87. When ζ-potential is regressed against S\textsubscript{3} for all treatments except AER\textsupersat (which has a relatively low S\textsubscript{3}), the R\textsuperscript{2} value is 0.999. In the case of *Y. enterocolitica* (Figure 1b), the trend in ζ-potential is in better agreement with the observed changes in total charged functional groups at pH 6.7. A similar linear regression analysis of the total concentration of charged functional groups at pH 6.7 against ζ-potential for *Y. enterocolitica* (for all four treatments) reveals a correlation with an R\textsuperscript{2} of 0.89, whereas correlating only S\textsubscript{1} and ζ-potential yields an R\textsuperscript{2} of 0.94.

**ATR-FTIR Spectra of Bacteria Samples.** We employed ATR-FTIR spectroscopy as a rapid means of examining the bacterial biochemical composition as a function of changes in DO during cell growth and acclimation. To detect variations in the occurrence of certain functional groups when cells were grown or acclimated at different DO levels, the spectrum measured for one treatment was subtracted from the spectrum measured for another treatment. Figure 2 shows the difference spectra for both organisms generated in this manner from their respective spectra (measured at pH 6.7) to examine the influence of the growth protocol. A similar analysis was completed to examine the influence of the acclimation protocol on the spectra (Supporting Information, Figure S1). It should be noted that all the spectra recorded in this study were normalized to unit height of the protein amide I band to compensate for the inherent variability in the effective path length of the ATR measurements. As such, the difference spectra in Figures 2 and S1 of the Supporting Information are intended to highlight the changes in the spectra that most likely correspond to changes in biological composition.

![FIGURE 1. Concentration of titrated charged sites at pH 6.7 following the four treatments for (a) *E. coli* O157:H7 and (b) *Y. enterocolitica* (bar graph). Scatter plots represent the ζ-potential, where the error bars represent the 95% confidence interval. S\textsubscript{1}, S\textsubscript{2}, and S\textsubscript{3} represent the site concentrations associated with each proton binding site.](image-url)
Information effectively represent relative changes with respect to protein content.

The predominant features in the spectra presented in Figure 2 are a broad band in the “carbohydrate” absorption region of the spectrum (1200–950 cm$^{-1}$), in which C–OH, C–C, and C–O–C vibrations give rise to extensively overlapped bands, and a somewhat weaker, broad band at 1250–1200 cm$^{-1}$. Absorptions in the latter region are often assigned to the antisymmetric stretching vibration of phosphate groups (denoted $\nu_s$(PO$_2^-$)), but carbohydrates also exhibit bands in this region, whereas $\nu_s$(PO$_4^{3-}$), which is observed at $\sim$1074 cm$^{-1}$, as well as C–O–P and P–O–P vibrations contribute to absorptions in the “carbohydrate” region (note: a summary of band assignments is provided in Supporting Information, Table S2). Thus, the difference spectra in Figure 2 indicate that significant increases in phosphate and carbohydrate absorptions are associated with switching from aerobic to anaerobic growth. It is interesting to note that both organisms show the same trend, in contrast to the opposing trends seen in Figure 1 for both $S_{sat}$ and $\xi$-potential with the change from aerobic to anaerobic growth. For both organisms, the integrated intensity between 1200 and 950 cm$^{-1}$ in the spectra recorded for the four treatments was found to be strongly correlated with the integrated intensity between 1250 and 1200 cm$^{-1}$ ($R^2$ values of 0.999 and 0.989 for E. coli and Y. enterocolitica, respectively), suggesting that the absorptions observed in Figure 2 in these two regions may originate from one molecular species. Given the presence of both phosphate groups and carbohydrates in LPS, these observations are consistent with increased production of LPS when cells are grown under oxygen-depleted conditions, as Landini and Zehnder (23) observed with E. coli K12 grown in the absence of oxygen. Indeed, the difference spectra in Figure 2 are qualitatively similar to the spectra of LPS isolated from a different organism (Pseudomonas aeruginosa) over the spectral range presented in the literature (1500–900 cm$^{-1}$) (34). The spectra in Figure 2 also exhibit a weak $\nu(C=O)$ band at $\sim$1705 cm$^{-1}$, which could originate from the lipid A portion of LPS.

The integrated intensity between 1200 and 950 cm$^{-1}$ is plotted for each of the four treatments in Figure 3. As noted above, because these spectra have been normalized to unit height of the protein amide I band, this integrated intensity is a measure of the phosphate+CHO:protein ratio. The data for E. coli clearly illustrate a larger integrated area for the ANA growth treatment in comparison to the AER growth conditions, which may be linked to increased production of LPS under anaerobic growth conditions (see above). Figure 3 also shows that the acclimation treatment (either lowDO or satDO) does not strongly influence the phosphate+CHO:protein ratio measured in the E. coli spectra, whereas the data obtained from potentiometric titrations and $\xi$-potential (Figure 1) indicate that the DO level during cell acclimation does influence cell surface charge when cells are grown aerobically.

The phosphate+CHO:protein ratios determined for Y. enterocolitica suggest that the ANA low condition presents a unique physicochemical profile in comparison to the three other treatments examined. In addition, the spectrum for the ANA low condition was found to exhibit a unique absorption band at $\sim$1032 cm$^{-1}$, which is discernible in the ANA low–AER low difference spectrum in Figure 2, but assignment of this band to a specific molecular species was not possible. It is interesting to note that the anomalous spectral behavior observed for the ANA low condition is consistent with the unusual adhesion behavior of this organism reported previously (25) whereby the transport behavior of this organism in quartz sand for the ANA low treatment could not be explained by DLVO theory. The data presented in Figure 3 may suggest increased production of LPS for Yersinia cells grown anaerobically and acclimated in lowDO electrolyte. Such an increase in LPS content may explain the enhanced attachment observed in laboratory sand column experiments under this condition (25). It would be of interest to undertake further studies aimed at elucidation of the nature of the unique spectral profile observed in the case of Y. enterocolitica for the ANA low treatment.

Finally, the influence of pH on the ATR-FTIR spectra was investigated to obtain information that may assist in definitive identification of the functional groups associated with the $pK_a$ values determined by titration of the bacterial cells. For this purpose, spectra were recorded at three pH values (4.8, 6.7, and 9.5), and difference spectra were generated for each condition by subtracting the spectrum recorded at pH 4.8 from that at pH 6.7 as well as that recorded at pH 6.7 from that recorded at pH 9.5. In addition, the variance of all the spectra for each organism was calculated to ascertain which peaks were sensitive to pH (Figure S2 of the Supporting Information). The first set of difference spectra (pH 6.7–4.8) obtained for both organisms is presented in Figure 4. Comparison of the absorbance scales in this figure with those in Figure 2 clearly shows that the spectral changes resulting from varying the pH are relatively minor. The second set of
suremeasurements conducted at pH 6.7 demonstrate a correlation between the deprotonated cell surface functional groups and bacteria ζ-potential for both *E. coli* O157:H7 and *Yersinia enterocolitica*. For each organism, the amount of deprotonated groups at pH 6.7 generally agrees well with the trends observed in the cell ζ-potentials. However, when comparing the data for the two organisms, a considerable difference in the magnitude of the ζ-potential between the two species is observed, which is not reflected in the calculated amount of deprotonated groups (i.e., the amount of deprotonated groups is quite similar for the two organisms). Hence, our results show that it is not always appropriate to directly compare the amount of titratable charge (evaluated using a nonelectrostatic chemical speciation model) to the measured electrophoretic mobility of cells. In a previous study, Hong and Brown (36) showed that accurate modeling of bacterial cell surfaces requires use of an electrostatic model that accounts for the charge-regulated nature of the cell wall surface. Using such an electrostatic model, these researchers were able to show good agreement between potentiometric titration data and ζ-potential for both a Gram-negative and a Gram-positive organism. Application of an electrostatic model using measurements obtained over a range of electrolyte compositions may thus provide better agreement between titration and electrophoretic mobility data.

FTIR spectra of bacterial samples can provide insight into the biochemical composition of the cells and, when combined with *pK*_a values from analysis of titration data, may allow for tentative identification of the functional groups responsible for the cell surface charge, including carboxylic, phosphate, phosphodiester, hydroxyl, and amine groups. The ATR-FTIR spectra measured in this work provided clear evidence of variations in cell biochemical composition as a function of growth conditions (aerobic vs anaerobic) and, in the case of *Y. enterocolitica* also showed a definitive effect of oxygen tension during acclimation. ATR-FTIR spectroscopy proved to be less informative with respect to the functional groups responsible for the cell surface charge owing to the limited sensitivity of the spectra to pH changes over the range examined. Overall, the FTIR results indicate that this system warrants closer examination via extraction of specific cell components (e.g., LPS fraction, protein fraction, etc.) to definitively assign measured changes in IR spectra to
biochemical changes in the bacterial cell. Moreover, a genome-wide transcrip
tional analysis using microarray technology could be useful in determining how gene expres-
sion of the organisms examined is affected by the variations in oxygen tension during cell growth and accli-
mmation.

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Supporting Information Available
Details regarding preparation of bacterial suspensions, experi-
mental techniques, and supporting data in the form of
tables and figures. This material is available free of charge
via the Internet at http://pubs.acs.org.

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