Cells and Materials

Volume 6
Number 1 *Numbers 1-3*

Article 18

1996

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Recommended Citation

McLean, Robert J. C.; Stickler, David J.; and Nickel, J. Curtis (1996) "Biofilm Mediated Calculus Formation in the Urinary Tract," *Cells and Materials*: Vol. 6 : No. 1 , Article 18.
Available at: https://digitalcommons.usu.edu/cellsandmaterials/vol6/iss1/18

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Cells and Materials, Vol. 6, No. 1-3, 1996 (pages 165-174) 1051-6794/96$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

BIOFILM MEDIATED CALCULUS FORMATION IN THE URINARY TRACT

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(Received for publication February 7, 1996 and in revised form June 16, 1996)

Abstract

Mineralization and subsequent calculus formation is a common complication of biofilm infections. In the urinary tract, these infected calculi often arise from infections by urease-producing bacteria. Ammonia, liberated by bacterial urease activity, increases urine pH, resulting in the precipitation of Ca and Mg as carbonate-apatite $\{Ca_{10}(PO_4,CO_3)_6(OH,CO_3)_2\}$ and struvite ($NH_4MgPO_4 \cdot 6H_2O$). These minerals become entrapped in the organic matrix which surrounds the infecting organisms and ultimately grow into mature calculi. When the causative organisms grow on urinary catheters and stents, the resulting mineralization can partially or completely obstruct urine flow. Mineralization may also exacerbate tissue damage, lead to a loss of kidney function, and aid in the dissemination of microorganisms into deeper tissues. Several factors influence mineral formation and growth during struvite urolithiasis. These include host factors such as urine chemistry and anatomy of the urinary tract, the presence and characteristics of any foreign objects such as catheters, and bacterial factors such as the type of organisms present and their virulence factors. This review will address these and other factors which influence biofilm mineralization and calculus formation in the urinary tract.

Key Words: Biofilm, mineral, struvite, carbonate-apatite, urinary calculus, urolithiasis.

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Introduction

Proteus (P.) mirabilis and other urease-producing bacteria are a major cause of urinary tract infections (UTI) reviewed in (Gleeson and Griffith, 1993; McLean *et al.*, 1988). The major risk in these UTI is the development of urinary calculi which typically contain struvite ($NH_4MgPO_4 \cdot 6H_20$) and carbonate-apatite $\{Ca_{10}(PO_4,CO_3)_6(OH,CO_3)_2\}$ as the predominate mineral components (Gleeson and Griffith, 1993; Hesse *et al.*, 1992; McLean *et al.*, 1988; Stickler *et al.*, 1993a, b). Infection stones account for only 5-20% of all urinary calculi, however, they represent a much more significant health problem and danger to the organs of the urinary tract than do conventional metabolic stones (McLean *et al.*, 1988; Schmitz *et al.*, 1993; Stickler *et al.*, 1993b). The association of infection with urinary stones has been known since the time of Hippocrates. The association of urease-producing bacteria with struvite calculi was documented long ago (McLean *et al.*, 1988). Griffith *et al.* (1976) illustrated the fundamental role of urease in the pathogenesis of this infection. Urea hydrolysis by bacterial urease activity elevates urine pH and leads to Mg and Ca precipitation as struvite and other minerals. Jones *et al.* (1990) showed that isogenic urease-minus mutants of *P. mirabilis* induced less tissue damage when experimentally infected into an ascending mouse model of UTI. This is due to a lack of stone production as well as an elimination of ammonia-induced tissue damage (Johnson *et al.*, 1993). Ultrastructural examination of infection stones reveals the growth of microorganisms throughout these calculi (McLean *et al.*, 1989; Takeuchi *et al.*, 1984; Winters *et al.*, 1995). Microorganisms within mineralized material would be shielded from the effects of antimicrobial agents (Rocha and Santos, 1969). Of equal or greater importance, any dislodged calculus fragments would contain viable organisms and could therefore act as seeds for the rapid development of new calculi. These two features explain the high recurrence of these calculi ($\sim$ 50%) in spite of conventional surgical techniques (McLean *et al.*, 1988).

Clinical therapy

Current surgical approaches for urinary calculi include: extracorporeal shockwave lithotripsy (ESWL), percutaneous lithotripsy, and anatrophic nephrolithotomy (Segura, 1990). In the case of lithotripsy, the rationale is to disintegrate the stone *in situ* after which the sand-like fragments are passed by the patient (ESWL) or are aspirated with a catheter in the case of percutaneous lithotripsy. More conventional surgical approaches (i.e., anatrophic nephrolithotomy) are based on surgically removing the stone from the kidney and other regions of the urinary tract. In either case, the patient is generally given antibiotic therapy infrequently coupled with urinary acidifiers (Seftel and Resnick, 1990). At least two studies have shown that ESWL itself has little adverse effect on the infecting microorganisms (Reid *et al.*, 1990; Stoller and Workman, 1990). Although ESWL is non-invasive, some tissue damage does occur (Smith *et al.*, 1991). The chief reason for calculus recurrence is failure of the patient to pass all residual stone fragments (Seftel and Resnick, 1990; Segura, 1990). Conventional surgery exhibits the highest success rate (i.e., lowest recurrence), however, reinfection does occur in many patients. There are the standard risks associated with any invasive surgery. Yet, left untreated, this condition can result in the loss of the kidney and mortality within 5-10 years (Gleeson and Griffith, 1993).

Contributing Host Factors

Urinary tract defence mechanisms

All infectious diseases including UTI, represent an ecological interaction between host defenses and microbial virulence. We now address several defence mechanisms in the urinary tract and their influence on UTI. The major defence mechanisms in the urinary tract are mechanical in nature. These include periodic emptying of the bladder (voiding) to remove unattached bacteria, prevention of bacterial adhesion to tissue surfaces (sIgA production, glycosaminoglycan mucous layer, and uroepithelial cell turnover), and the length of the male urethra which increases the length that uropathogens must ascend (Anderson, 1986; Aronson *et al.*, 1988; Fukushi and Orikasa, 1981; Orikasa and Hinman, 1977). Growth of organisms in urine is inhibited by its high osmolality due to urea and salts, Zn-containing antibacterial secretions by the prostate, and by the low concentration of iron in urine (McLean *et al.*, 1988). Dissemination of organisms to deeper tissues is prevented by the integrity of the uroepithelial tissue (a physical barrier) and by cellular mediated immunity (McLean *et al.*, 1988). The humoral immune system generally combats infections in the upper urinary tract. It does not work well in the presence of large quantities of urine such as exist in the bladder. When one or more of these non-specific defence mechanisms is circumvented, the risk and incidence of UTI is greatly increased. The most common problems involve the complete or partial loss of voiding function due to congenital problems, pathology related to another disease (e.g., cancer) or neuropathy induced by trauma or neurological disease. The other major risk factor is the presence of a foreign object such as a catheter, stent, or calculus. The loss of voiding will increase the residence time of potential pathogens and the likelihood that they will be able to initiate infection. Foreign objects present potential pathogens with an immunologically inert (i.e., lacking sIgA), non-shedding surface to which they can attach and form biofilms.

Although many urologists consider the bladder and upper urinary tract to be sterile (i.e., $< 10^3$ colony forming units per ml urine), the bladder is intermittently exposed to low concentrations of bacteria. Sterile foreign Zn disks, implanted into male rat bladders become rapidly colonized by biofilm-forming bacteria within 24 hours (Nickel, Olson and McLean, unpublished). The risk factor of urinary infection associated with foreign objects is therefore understandable (Stickler and Zimakoff, 1994).

Urine composition

Aside from its influence on microbial growth, urine chemistry also has a significant impact on mineralization processes. The basic premise behind mineral formation in the urinary tract is that the solubility of one or more urine solutes (e.g., Mg^{2+}, Ca^{2+}, PO_4^{3-}, etc.) is reduced. The pH of urine is a crucial factor influencing not only ion solubility, but also the effectiveness of several antibiotics such as trimethoprim and tetracycline (A. Hesse, personal communication). Depending upon the urine chemistry and the presence of microorganisms, one or more minerals then form. Typically, calcium oxalate and calcium phosphate calculi are most frequently encountered. When urea hydrolysing microorganisms are present, struvite is commonly encountered. Urologists may advise their patients to increase fluid intake and to reduce intake of foods and liquids containing high levels of Ca and Mg. The rationale here is to reduce the concentration of ions in urine. Hedelin *et al.* (1986) showed that mineralization processes which could occur in artificial urine were inhibited in the presence of increasing concentrations of human urine. More recent studies on struvite mineralization *in vitro* have shown that its formation could be inhibited by citrate (McLean *et al.*, 1990; Wang *et al.*, 1993), pyrophosphate (McLean *et al.*, 1991a), and albumen (Hugosson *et al.*, 1990). *In vitro* tests using cat urine showed that, whereas Tamm-Horsfall glycoprotein enhanced struvite formation, albumen had no effect (Buffington *et al.*, 1994).

In contrast to their effects on calcium oxalate mineralization, heparin sulfate and chondroitin sulfate did not interfere with struvite mineral growth *in vitro* (McLean *et al.*, 1990). Crystallisation inhibitors, when present, greatly reduce the tendency of dissolved Mg^{2+} and Ca^{2+} to precipitate and form minerals. Edin-Liljegren *et al.* (1992) showed that urine from several human volunteers differed in buffering capacity and urease-inhibiting capability.

Bacterial Factors

Isolates

The predominant feature of most organisms associated with struvite urolithiasis is their urease activity {reviewed in McLean *et al.* (1988)}. The few isolates which fail to show urease activity generally represent a minor component of the calculus or urine microflora. The predominate isolates include *P. mirabilis* and related organisms, particularly *P. vulgaris, Providencia* sp., and *Morganella morganii*. Gram positive cocci (*Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp.) are also commonly observed. *Corynebacterium* sp. D2, a gram positive rod has been associated with struvite urolithiasis (Soriano *et al.*, 1986). There have been several cases of struvite calculi associated with the *Ureaplasma urealyticum*, a mycoplasma (Grenabo *et al.*, 1988; Hedelin *et al.*, 1984).

Virulence factors

In vitro experiments (Griffith *et al.*, 1976) and genetic deletion of urease coupled with animal model tests (Jones *et al.*, 1990) have shown urease to be an essential virulence factor in the development of these calculi. Ammonia, generated by urea hydrolysis, elevates urine pH and induces production of ammonium (NH_4^+), carbonate (CO_3^{2-}), phosphate (PO_4^{3-}), and hydroxide (OH^-) ions. Mg^{2+} and Ca^{2+} are relatively insoluble at alkaline pH and precipitate as struvite and carbonate-apatite due to the aforementioned other ions. Also, *P. mirabilis* mutants, lacking urease, are much less able to colonize and cause tissue damage in a mouse model of ascending UTI (Jones *et al.*, 1990). Other virulence factors also play a role. To date, hemagglutinin, pili, IgA protease, siderophore production, capsule polysaccharide (CPS) production, and toxins {hemolysin and lipid A (endotoxin)} have been identified (McLean *et al.*, 1988; Mobley and Chippendale, 1990). These factors are important in that they enable *P. mirabilis* to colonize and survive in the urinary tract, evade host defense mechanisms, and induce tissue damage. The distinguishing features of struvite urolithiasis are its urease-induced mineral formation, rapidity of growth, and high rate of recurrence.

Bacteria can also influence calculus formation indirectly by reducing or removing crystal growth inhibitors from urine. *In vitro* experiments by Edin-Liljegren *et al.* (1995) showed that *Escherichia coli* could reduce citrate concentrations in human urine. Similar removal of crystal growth inhibitors or production of crystal growth promoters by bacteria would certainly enhance calculus formation.

Ultrastructural studies of struvite urolithiasis have shown the bacteria, responsible for this disease, to be present throughout these calculi (McLean *et al.*, 1988), and to be intimately associated with both the crystalline and matrix components. The majority of these bacteria possess urease activity and produce CPS in the immediate vicinity of struvite crystals. This has been observed in both clinical samples (McLean *et al.*, 1989) and in stones produced in an animal model (Nickel *et al.*, 1987). It has been proposed that the bacterial CPS is largely responsible for initiating matrix deposition and crystal binding, and may even be crucial in the process of crystal nucleation through creating an alkaline, metal (Ca and Mg) saturated microenvironment (Clapham *et al.*, 1990; Dumanski *et al.*, 1994; McLean *et al.*, 1991b). Although considerable morphological evidence has been obtained as to the role of *P. mirabilis* CPS in this infection, definitive evidence of its importance has not yet been shown using acapsular strains of *P. mirabilis* in an animal model experiment.

Biofilm formation

Biofilms are an integral aspect of many types of chronic UTI such as device associated infections, chronic prostatitis, and struvite calculi (McLean *et al.*, 1995; Stickler and McLean, 1995; Stickler and Zimakoff, 1994). In device-associated infections, bacteria colonize the surfaces of catheters and stents forming encapsulated biofilm communities. When growing as biofilms, bacteria are notoriously resistant to host defenses and antimicrobial therapy (Stickler *et al.*, 1989, 1991). From this protected environment, they can disseminate to urinary tissues such as the kidney. In general terms, biofilm formation occurs when organisms encounter a surface, attach to the surface, then spread over the surface, thereby growing into an adherent biofilm community. The surfaces of implanted devices rapidly become coated with conditioning films of protein from body fluids. These coatings can provide receptor sites for bacterial attachment (Stickler and McLean, 1995). In the case of urinary catheters, Ohkawa *et al.* (1990) found that catheters removed from patients after just 1-3 days were coated with a fibrous material which specifically reacted with antihuman fibrinogen fluorescent conjugate. Such organic conditioning films have been well-documented in a variety of environments (Busscher *et al.*, 1995;

Hawthorn and Reid, 1990; Reid *et al.*, 1992; Schneider *et al.*, 1994). Conditioning films can alter the chemistry of a surface in several ways. They can mask an otherwise harmful surface coating, directly or indirectly provide a source of nutrition, or provide receptors for bacterial adhesion. The initial encounter with the surface can be facilitated by cell motility due to flagella. Alternatively, the flow of liquids such as urine over a surface will ensure exposure of microorganisms. Bacterial attachment to a substratum is facilitated by adhesions, i.e., structures on the bacterial cell surface, notably fimbriae and CPS, whose primary function is adhesion (Marshall *et al.*, 1971; McLean *et al.*, 1994). Several cellular functions are triggered by surface adhesion. One notable characteristic is CPS production (Davies and Geesey, 1995), a major component of biofilms. As adherent cells grow, they form small encapsulated microcolonies which are small clumps of morphologically identical cells (often 2-10 cells) immediately adjacent to each other. Growth of adjacent microcolonies towards each other will lead to the development of a mature biofilm. Details of this process and other aspects of biofilm biology have been published by Korber *et al.* (1995).

Mineralization of Biofilms

The presence of any solid in the lumen of a pipe will adversely affect the flow of liquids. When biofilms are present, they can reduce the effective diameter of the lumen and increase friction for liquid flow (Costerton *et al.*, 1987; Stickler *et al.*, 1993b). The same can be said for catheters and stents since they represent medically important pipes. Mineralization of biofilms exacerbates this problem. In general terms, encrusted biofilms are thicker and possess a rougher surface than non-mineralized biofilms (Cox and Hukins, 1989; Cox *et al.*, 1989; Hukins *et al.*, 1983; Liedberg *et al.*, 1991; Ohkawa *et al.*, 1990; Stickler *et al.*, 1993a). The thicker coating further reduces the lumen diameter and may also inhibit the activity of antimicrobial agents against the organisms (Stickler and Hewett, 1991). The rougher surface of mineralized biofilms can exacerbate tissue damage by the catheter (Hukins *et al.*, 1983; Norlén *et al.*, 1988), thus providing a portal of entry for urinary pathogens to deeper host tissues (Stickler and Zimakoff, 1994).

Biological and physical factors will influence biofilm mineralization. Biological influences include the types of organisms present, their metabolism (e.g., urease activity), and the influence of cell surface materials such as CPS. Physical factors controlling biofilm mineralization include the influence of the underlying substratum (e.g., catheter and any conditioning film) to which the biofilm is attached (Lopez-Lopez *et al.*, 1991; Schmitz *et al.*, 1993; Stickler *et al.*, 1989, 1991), and the overall urine chemistry, addressed earlier. The majority of microorganisms associated with biofilm mineralization in the urinary tract share a common attribute of urease activity. As in stone formation, urease represents the major driving force behind mineralization of catheter biofilms, creating the alkaline conditions that induce crystallisation of the calcium and magnesium salts (Cox *et al.*, 1989; Stickler *et al.*, 1993a).

Biomineralization is not unique to catheter encrustation. Indeed, it is widely documented in the geological and microbiological literature (for recent reviews, see Beveridge, 1989; McLean and Beveridge, 1990). Uronic acids, pyruvate residues and/or phosphate groups present in cell walls and CPS give bacteria an overall negative charge. This enables bacteria to sequester cations such as Mg^{2+}, Ca^{2+}, and Fe^{3+} onto their cell surface. Under appropriate chemical conditions, these bound cations can precipitate and form minerals (Beveridge, 1989; McLean and Beveridge, 1990). Clapham *et al.* (1990) first investigated this phenomenon in relation to struvite crystal growth. They found that struvite crystals grown adjacent to *P. mirabilis* and its CPS polymers adopted dendritic crystal habits characteristic of rapid growth. In contrast, struvite crystals grown at a distance from *P. mirabilis* adopted a more tabular crystal habit, reflecting slower growth. Further experiments by Dumanski *et al.* (1994), showed that CPS enhancement of struvite growth *in vitro* was a unique feature of *P. mirabilis* CPS.

Mineralization processes within biofilms are influenced by several factors. Certainly, anionic CPS and bacterial cell envelope polymers will bind and concentrate metal ions as described above. Since the majority of cells within biofilms are buried within CPS, diffusion of substances through the biofilm is slowed. Diffusion gradients, coupled with metabolic activities of biofilm microorganisms, result in the establishment of heterogeneous chemical microenvironments within biofilms (Korber *et al.*, 1995), which are often quite different from the chemical environment in the surrounding fluids. McLean *et al.* (1991b) showed that biofilms of *P. mirabilis* created an alkaline microenvironment and were able to protect acid-labile struvite crystals from dissolution when exposed to artificial urine pH 5.8.

Examination of struvite crystals by transmission electron microscopy (TEM) shows that mineralization within biofilms occurs at or near the surface of *P. mirabilis* cells (Fig. 1) (McLean *et al.*, 1985; Winters *et al.*, 1995). In the mouth, another type of calculus forms due to plaque mineralization (Mishima *et al.*, 1992). In contrast to struvite urinary calculi, plaque (biofilm) mineralization by $Ca_3(PO_4)_2$ occurs throughout the cells

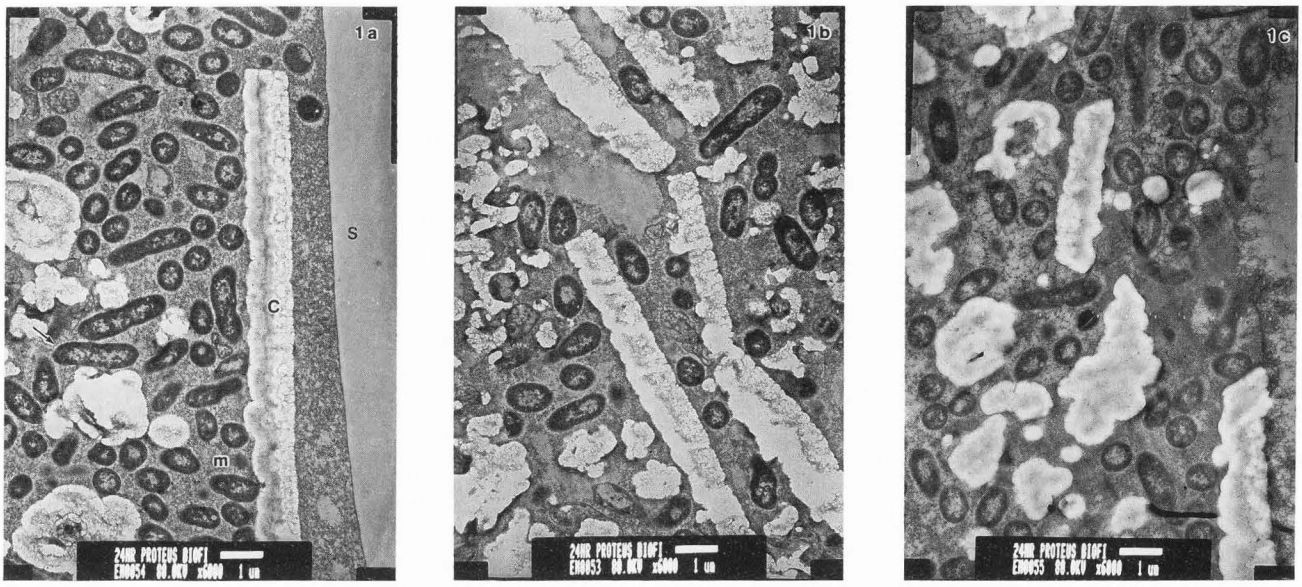

Figure 1. Transmission electron micrograph of sections of an encrusting biofilm that had grown over 24 hours on a silicone catheter due to inoculation with *Proteus mirabilis* and *Escherichia coli* (Winters *et al.*, 1995). Micrographs represent regions taken immediately adjacent to the catheter surface (S) (**Fig. 1a**), in the central region of the biofilm (**Fig. 1b**) and at the luminal surface (**Fig. 1c**). Gram negative bacterial cells (arrow) are quite evident throughout the anionic, ruthenium-red-staining polysaccharide matrix (m) of the biofilms. The unstained areas (C) are remnants of struvite and apatite crystals which have dissolved during sample preparation. Bars = 1 μm.

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and organic matrix along a broad front. This would suggest that biofilm-mediated calculus formation can occur by several different mechanisms.

Physiology of biofilm organisms is also heterogeneous. In contrast to planktonic bacteria, growth rates of biofilm organisms are slowed because of diffusion gradients of nutrients and metabolic waste products within biofilms, as well as competition for nutrients from cells in close proximity to each other. Notwithstanding plasmid- and chromosome-encoded antibiotic resistance mechanisms, biofilm organisms exhibit markedly lowered susceptibility to antibiotics because of low growth rates and low diffusion of antibiotics into biofilms (Gilbert and Brown, 1995).

Conclusions and Prospects for Future

The identification of factors that promote biofilm formation and mineralization, suggests a variety of alternative strategies to those currently used to deal with the problem. The key features of the process: (**a**) the colonization of the urinary tract by *P. mirabilis*, (**b**) the development of bacterial communities embedded in a common polysaccharide matrix, (**c**) the attraction of the calcium and magnesium ions into the gel of the polysaccharide, and (**d**) the generation of alkaline conditions in the biofilm matrix by the activity of urease, can all be considered as potential targets.

Inhibition of extracellular polysaccharide production by *P. mirabilis* or dispersion of the polysaccharide matrix would undermine biofilm formation and prevent the concentration of calcium and magnesium ions in a gel which promoted crystallisation. The absence of an extensive biofilm matrix could also enhance the effectiveness of crystallisation inhibitors such as citrate.

The instillation of acid solutions directly into the bladder or the oral administration of urinary acidifying agents such as ascorbic acid are commonly used to try to control catheter encrustation. Clinical experience of these methods is poor, however, and the work of Bibby and Hukins (1993) has shown that the addition of hydrogen ions to urine containing urease simply causes more urea to be converted into ammonia, and alkaline conditions are quickly restored. Inhibitors of urease such as acetohydroxamic acid and flurofamide have been shown to be much more effective in lowering pH in *P. mirabilis* infected urine (Rosenstein and Hamilton-Miller, 1984). A controlled clinical trial with acetohydroxamic acid also demonstrated that it significantly inhibited the growth of struvite stones (Williams *et al.*, 1984). Half of the patients receiving the drug, however, experienced adverse reactions (tremulousness and phlebothrombosis).

A less toxic molecule would, of course, be required for the long-term inhibition of the urease activity that would be necessary to control mineralization.

The biomaterials currently used in the manufacture of catheters provide highly attractive surfaces for bacterial colonization and biofilm formation. Alteration of the physicochemical nature of catheter surfaces by coating with hydrophilic hydrogels or the incorporation of antibacterial agents such as silver have been used to try to control encrustation (Liedberg *et al.*, 1991). While these surfaces resist encrustation in *in vitro* tests with artificial urine to which urease has been added, there is no evidence that they are effective in patients infected with *P. mirabilis* where conditioning films will rapidly coat the catheter surface, where struvite crystals formed in the urine will attach to the catheter and where debris from pioneering cells that might be killed by the antibacterial, all provide foundations for subsequent colonization shielded from the antimicrobial in the catheter. A biomaterial capable of releasing the active agent at a steady rate over prolonged periods, producing bactericidal concentrations in the urine bathing the catheter surfaces, might be more successful in preventing biofilm development. The constant sloughing away of epithelial cells from the bladder surface is an important defence mechanism against bacterial colonization. A catheter surface that slowly dissolved away as urine flowed over it, washing away any bacteria or crystals that had managed to attach themselves, could also be effective.

The root of the problem is, of course, the infection of the urinary tract with *P. mirabilis*. The radical solution is therefore aggressive antibiotic therapy targeted specifically at this organism. In the presence of stones or catheters, however, these infections are almost impossible to eradicate by current antibiotic regimes (Slack, 1992). The development of appropriate drug regimes might be feasible if more was known about the response of this species to antibiotics in the various stages of biofilm formation and mineralization.

Acknowledgements

Research in the authors' laboratories has been supported by grants from the Welsh Scheme for the Development of Health and Social Research (DJS), the Kidney Foundation of Canada (RJCM, JCN), the Natural Sciences and Engineering Research Council of Canada (RJCM) and a Research Enhancement Grant from Southwest Texas State University (RJCM). We express our appreciation to Carol Winters for providing the transmission electron micrographs.

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Discussion with Reviewers

G. Wolfaardt: Can the authors provide some more information on the natural microflora in urine?

Authors: Natural microflora in urine are generally considered to be present only in the distal urethra. Any which ascend to the bladder or kidneys are considered to be potential pathogens. Two detailed studies on the urethral flora of human females were carried out by Marrie *et al.* (1978, 1980). They found the aerobic flora to be dominated by *Lactobacillus sp* and *Staphylococcus epidermidis*, and the anaerobic flora to be dominated by *Bacteroides melaninogenicus*. The predominant species varied among premenarchal, reproductive age, and post-menopausal women. Of note, aerobic gram negative rods such as *Escherichia coli*, *Proteus mirabilis*

were only present in any significant numbers during urinary tract infection.

G. Wolfaardt: Please define CPS (capsular polysaccharides). Is CPS really largely responsible for initial matrix deposition?
Authors: The literature is a little ambiguous as to the use of capsule, slime, glycocalyx, extracellular polysaccharides, etc. As extracellular polymers in some organisms, e.g., *Bacillus anthracis* are polymers of gamma-D-glutamic acid, this precludes the use of glycocalyx. Similarly, using extracellular polymers might lead to confusion with lipopolysaccharide, or teichoic acids, hence our rationale for using the term capsule polysaccharides (CPS). The origin of the organic matrix of urinary calculi has still not been firmly established. While the mineral component of calculi varies widely, the organic matrix component is relatively constant and is comprised largely of host-derived material (Boyce, 1968; Morse and Resnick, 1988). Vermeulen and Lyon (1968) postulated that the stone matrix formed as a consequence of mineral crystallization. The presence of matrix calculi (Allen and Spence, 1966) containing little or no mineral component would refute this claim. Our suggestion that matrix formation is induced by bacterial CPS is based on our observations that bacteria growing in the urinary tract always produce CPS (McLean *et al.*, 1989) and that the urinary tract has low levels of bacteria, even in many healthy individuals. These bacteria are available to interact with particulates, including calcium oxalate crystals, and so may induce matrix formation. Definitive testing of this hypothesis awaits the development of isogenic acapsular mutants of *Proteus mirabilis* and testing in an animal model.

G. Wolfaardt: **"Mineralization of biofilms"** is misleading. What the authors really mean is accumulation or deposition of inorganic materials (crystals or mineralization products) in (organic) biofilms. The organic components of biofilms (cells and polymers) are not mineralized. Note that the term "mineralization" is also frequently used to describe the process in which organic contaminants (such as chlorinated hydrocarbons) are completely degraded by microbes to produce H_2O and CO_2.
Authors: While mineralization has a connotation in the decomposition of organic carbon to inorganic carbon, we have used it in a geological context in which mineralization means the formation and accumulation of minerals.

G. Wolfaardt: In the fifth paragraph of **Mineralization of biofilms**, the authors state: "Examination of struvite crystals by TEM shows that mineralization within biofilms occurs in discrete sites at or near the surface of *P. mirabilis* cells (Fig. 1)." I am afraid that I cannot see these discrete sites. The cells seem to be evenly distributed, and with the crystals larger than the cells, I cannot agree that such a conclusion can be drawn from Figure 1, especially considering that a major portion of the crystals could have dissolved during sample preparation.
Authors: By discrete sites, we mean that the crystals occur sporadically throughout the biofilm as seen in Figure 1 and McLean *et al.* (1985). In contrast, dental calculi exhibit a broad mineralization front in which all components of the dental plaque (biofilm) become simultaneously mineralized (Mishima *et al.*, 1992; McLean and Beveridge, unpublished results). This includes the cells and surrounding matrix.

G. Wolfaardt: What proportion of the calculi consist of struvite and apatite crystals? In Figure 1 legend, it is stated that the crystals have dissolved during sample preparation. Is it possible that these calculi consist mainly of organic material (microbial cells and polymers) in which the crystals became trapped and grown? Is there enough evidence to state that microbes grow throughout these calculi, or is this an illusion created by streaming of the dissolved (crystal) material which resulted in mixing of bacteria and the remaining smaller crystals? How much protection can this "mineralized material" really provide against antimicrobial agents?
Authors: Current thinking on the percentage of struvite and apatite in mineralized calculi is approximately 10-50%. There is considerable evidence that bacteria grow throughout these calculi and that this mode of growth protects them from antimicrobial agents. The original references for this work are Rocha and Santos (1969) and Takeuchi *et al.* (1984).

J.R. Lawrence: Can the authors suggest a method to inhibit CPS production?
Authors: Several attempts have been made to control CPS production in bacteria. While developing a isogenic CPS minus mutant is the best way, this is not practical in a clinical setting. Recently, Domeneco *et al.* (1993) have shown that bismuth salicylate may be effective in blocking CPS production in some bacteria. This is worthy of further investigation.

J.R. Lawrence: What type of materials could be used to create a dissolving surface for catheters? Are there models in other areas of biofouling that could be applied?
Authors: Our comments regarding developing catheters with "shedding surfaces" were made in the context of trying to mimic the natural defenses that occur in the urethra and bladder and in the intestinal tract. To date, these are merely ideas and not yet working hypotheses

for study.

Reviewer IV: Are patients diagnosed with conventional metabolic stones predisposed to bacterial stones, or is there no correlation?
Authors: Patients diagnosed with conventional metabolic stones or possessing any other foreign body in the urinary tract, such as a stent or catheter, are predisposed to bacterial stones. See reviews by Griffith (1978) and others.

Reviewer IV: Do patients exhibit kidney infection prior to stone formation, or are they often asymptomatic?
Authors: Some patients do exhibit acute urinary infection before stone formation. Whether or not stones actually form is due in some part to natural inhibitors found in the urine as described by H. Hedelin and his colleagues (Hugosson *et al.*, 1989; Hedelin *et al.*, 1986, 1989, 1991; Edin-Liljegren *et al.*, 1994, 1995).

Reviewer IV: Do the authors think that the alkaline conditions of the bladder and the calculi may also be a primary factor in antibiotic resistance. And has the pH been studied within the mineralized biofilms?
Authors: The alkaline conditions of the bladder may influence antibiotic effectiveness given the various pH optima of antibiotics; pH studies within mineralized biofilms have been done by J.R. Lawrence, D. Korber, D.E. Caldwell, and others with the advent of scanning confocal laser microscopy and the development of fluorescent pH probes (reviewed in Korber *et al.*, 1995).

Reviewer IV: Are these calculi usually composed of single species, or are they mixed bacterial communities as found in dental plaque?
Authors: Urinary calculi are generally comprised of several species. Often one species will predominate, but this is not always the case.

Reviewer IV: Have the authors found that the bacteria grow and divide within the calculi after mineralization or do they become dormant?
Authors: We have no indication that bacteria within a mineralized biofilm grow or become dormant. They do not die, however, as they can be cultured from calculus fragments.

A. Hesse: Are there any indications that in the case of UTI there is any increase in the excretion of Tamm-Horsfall protein (THP) or glycosaminoglycans (GAGs) in the urine?
Authors: I am not aware of any studies in which urine THP or GAGs have been measured as a consequence of infection.

A. Hesse: Are the high-molecular weight glycoproteins merely an absorption medium, or are they also a source of sustenance for the bacteria?
Authors: The high molecular weight glycoproteins could easily be a source of nutrition to bacteria as well as an absorption medium. I must emphasize that these substances do incorporate into the matrix of calculi and indeed form the major matrix component.

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