INFECTION AND IMMUNITY, Apr. 1985, p. 35–39 Vol. 48, No. 1
0019-9567/85/040035-05$02.00/0
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In Vivo Evidence That Bacteria in Urinary Tract Infection Grow Under Iron-Restricted Conditions

GEOFFREY H. SHAND,[1] HOSMIN ANWAR,[1] JAGATH KADURUGAMUWA,[1] MICHAEL R. W. BROWN,[1*] STANLEY H. SILVERMAN,[2] AND JACK MELLING[3]

Microbiology Research Group, Department of Pharmacy, University of Aston in Birmingham, Birmingham B4 7ET,[1] General Hospital, Birmingham B4 6NH,[2] and Vaccine Research and Production Laboratory, Centre for Applied Microbiology and Research, Public Health Laboratory Service, Porton Down, Salisbury, Wiltshire SP4 0JG,[3] United Kingdom

Received 21 September 1984/Accepted 19 December 1984

The outer membrane protein composition of bacteria isolated directly and without subculturing from the urine of two patients with urinary tract infections was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results indicated that the bacteria grew under iron-restricted conditions, as revealed by the expression of several high-molecular-weight outer membrane proteins which could also be observed when the same isolates were grown under iron-depleted conditions in laboratory media. The antigenicity of outer membrane components of the bacteria isolated was studied by immunoblotting with serum samples from the patients. The results indicated that the sera from the patients contained antibodies against major outer membrane components of the bacteria present in the urine, including the iron-regulated membrane proteins.

Urinary tract infections (UTIs) are a major cause of human morbidity and mortality. The prevalence of bacteriuria in adult women is 5 to 6% and is higher in hospital patients than in ambulant patients at home (17). Twenty percent of cases of end-stage renal failure in patients needing hemodialysis or transplantation are caused by chronic pyelonephritis (7). Predisposing factors for UTIs include female sex, anatomical or physiological conditions causing urinary stasis, and instrumentation or catheterization. This last factor is important in hospitals, where many patients are catheterized because of neurological diseases (stroke or paraplegia) or during the course of major surgery.

The restriction of iron availability to microorganisms growing in the body is thought to play an important role in host defense (8, 10, 13, 24, 25). Relatively large amounts of iron are known to be excreted in normal urine (10). This iron may be withheld from infecting bacteria by the presence of chelating agents such as transferrin and lactoferrin. Gram-negative bacteria are known to respond to iron restriction by synthesizing iron-chelating compounds called siderophores and inducing high-molecular-weight outer membrane proteins (OMPs), which act as receptors for the iron-siderophore complex (18). Brown et al. (6) recently found that mucoid *Pseudomonas aeruginosa* isolated directly from the lungs of a cystic fibrosis patient expressed iron-regulated membrane proteins (IRMPs). Similar observations have been made for experimentally induced *Escherichia coli* infections in the peritoneal cavities of guinea pigs (14) and *Vibrio cholera* infections in the intestinal tracts of infant rabbits (20). Much work, including dilution models, has been done to mimic UTIs in vitro (12). Nevertheless, little is known about the physiological and antigenic states of the bacteria in vivo. In this study, we present our preliminary observations of the OMP compositions of organisms isolated directly and without subculturing from the urine of two patients who had UTIs and were catheterized, one because of a stroke and the other because of an enterovesical fistula. We have also investigated the antigenicity of the outer membrane (OM) components in the sera from the patients.

* Corresponding author.

MATERIALS AND METHODS

Collection and identification of bacteria. Urine was collected from two catheterized patients (females, 69 and 86 years old) with UTIs every day for 5 days during hospitalization and was stored at 4°C. In separate control experiments, we found that the OMP profiles of bacteria stored at 4°C for 5 days in urine were not altered as compared with those of bacteria harvested immediately from the urine (data not shown). After 5 days, patient 2 was treated by surgical closure of an enterovesical fistula and received one dose of ceftriaxone (Rocephin; Hoffmann-La Roche Inc., Basel, Switzerland). The urine subsequently collected was sterile. Patient 1 had an asymptomatic UTI and was not treated with antibiotics. Microbiological analysis of the urine yielded *Proteus mirabilis*, *Proteus morganii*, and *Klebsiella pneumoniae* from patient 2 and *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus faecalis* from patient 1. The urine from each patient was coarsely filtered to remove cell debris. Material retained by the filter was washed with 0.85% saline, and the filtered washings were added to the bulk of the filtered urine. The filtered urine from each patient was pooled, and bacteria were harvested by centrifugation at 5,000 × *g* for 10 min. One sample of urine from patient 2 was found to contain a pure culture of *P. mirabilis* and was harvested separately. *E. coli* and *P. aeruginosa* used in control experiments were isolated from infected urine.

Cultivation of bacteria. The gram-negative bacteria isolated from the urine were cultivated in a variety of media under iron-sufficient and iron-restricted conditions. Iron-deficient chemically defined medium (Fe–CDM), prepared by the method described by Klemperer et al. (16), consisted of the following: 35 mM glucose, 25 mM NH_4Cl, 1.5 mM KCl, 0.4 mM $MgSO_4$, 0.045 mM NaCl, and 66.6 mM Na_2HPO_4-NaH_2PO_4. The phosphate buffer had previously been passed twice through a column of Chelex 100 ion-exchange resin (Bio-Rad Laboratories, Watford, United Kingdom) to remove iron. For iron-sufficient chemically defined medium,

0.02 mM $FeSO_4$ was added to Fe−CDM (Fe+CDM). A micronutrient solution of essential metal ions was added to both Fe+ and Fe− CDM to yield the following final concentrations: 5×10^{-7} M $CaCl_2$, 5×10^{-7} M HBO_3, 5×10^{-8} M $CoCl_2$, 10^{-8} M $CuSO_4$, 10^{-8} M $ZnSO_4$, 10^{-7} M $MnSO_4$, and 5×10^{-9} M $(NH_4)_6Mo_7O_{24}$. We removed unbound iron from tryptone soy broth (London Analytical and Bacteriology Media Ltd., Salford, United Kingdom) by passing it five times through a column of Chelex 100 ion-exchange resin. This decreased its iron content by ca. 80% to a value of less than 10^{-7} M (Fe−TSB). We prepared the Chelex 100 column by washing it with 1 M HCl (2 volumes), distilled water (5 volumes), 1 M NaOH (2 volumes), distilled water (5 volumes), and 0.7 M sodium phosphate buffer (pH 7.4) until the pH of the eluant was constant. After the iron was removed, other essential metal ions were replaced by the addition of 0.4 mM $MgSO_4$ and the micronutrient solution described above. For iron-sufficient tryptone soy broth (Fe+ TSB), $FeSO_4$ at 0.02 mM was added to Fe− TSB.

Bacteria were cultivated at 37°C for 24 h in an orbital shaking incubator, harvested by centrifugation at 5,000 × *g* for 10 min, and washed once with saline.

OM preparation and SDS-PAGE. The bacterial pellet was suspended in 20 ml of distilled water and broken by two 60-s pulses of sonication in an ice bath, with one 30-s interval for cooling. Unbroken cells, which included the gram-positive organisms isolated from patient 2, were removed by centrifugation at 5,000 × *g* for 10 min. Sarkosyl (*N*-lauroylsarcosine, sodium salt; Sigma Chemical Co., Poole, England) was added to the supernatant to a final concentration of 2%. The mixture was incubated for 30 min and then centrifuged at 38,000 × *g* for 1 h. The membrane pellets were washed twice with distilled water, resuspended in a small volume of distilled water, and stored at −20°C. Membrane preparations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the system described by Anwar et al. (3) with 14% acrylamide gels and purified sodium dodecyl sulfate (specially purified; BDH, Poole, United Kingdom).

Serum. Serum was separated from blood obtained by venipuncture from the patients during the course of clinical investigations. A small portion of the blood was allowed to clot at 37°C for 2 h and then centrifuged at 2,000 × *g* for 10 min, and the supernatant serum was collected and stored at −20°C.

Immunoblotting. OMPs separated on polyacrylamide gels were transferred to nitrocellulose (NC) paper, and antigenic sites were visualized by a modification of the method of Towbin et al. (21). The NC paper was incubated first with 10 mM Tris-hydrochloride–0.85% saline (pH 7.4) (TBS) containing 0.3% Tween 20 (4, 22) for 1 h to saturate nonspecific protein-binding sites and then with patient serum diluted 1/50 in TBS–0.3% Tween 20 for 4 h at 37°C. The paper was then washed thoroughly with TBS and incubated for a further 2 h at 37°C with horseradish peroxidase goat anti-human immunoglobulin G conjugate (Miles-Yeda Ltd., Rehovot, Israel) diluted 1/2,000 in TBS–0.3% Tween 20. After incubation, the NC paper was again washed thoroughly, and antigenic sites were visualized with a 25-μg/ml solution of 4-chloro-1-napthol in TBS containing 0.01% H_2O_2. To show complete qualitative transfer of protein bands from polyacrylamide gels to the NC paper, we directly visualized blotted protein bands on duplicate NC paper by staining the paper with 1% amido black (data not shown).

Determination of iron concentrations. Iron concentrations in urine and media were determined by atomic absorption

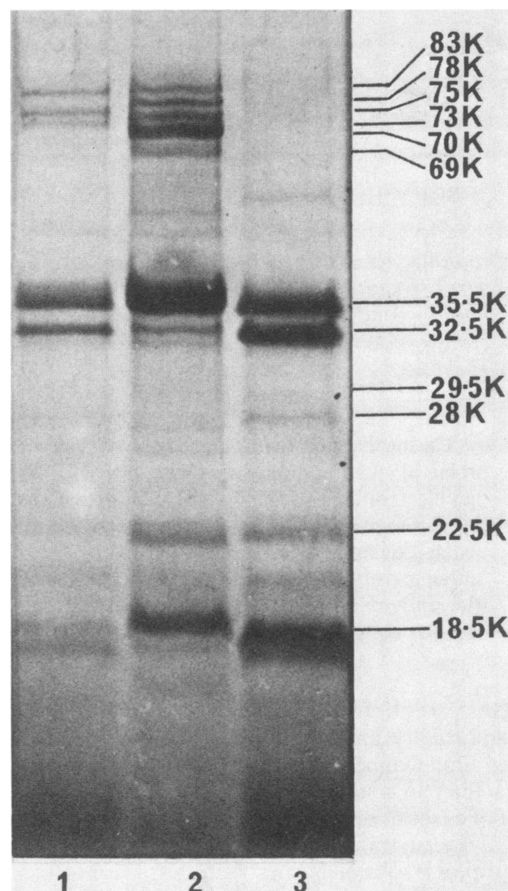

FIG. 1. SDS-PAGE of OMPs of *K. pneumoniae* isolated directly from urine (lane 1) and of the same isolate grown in Fe−TSB (lane 2) and Fe+TSB (lane 3) laboratory media.

with a Perkin-Elmer model 360 SG apparatus fitted with a deuterium background corrector and an HGA-74 graphite furnace.

RESULTS AND DISCUSSION

Figure 1 shows the OMP profiles of the pure *K. pneumoniae* isolated directly from the urine of patient 1 (lane 1) and of the same isolate grown in vitro in Fe−TSB (lane 2) and Fe+TSB (lane 3). Three high-molecular-weight proteins were present in the OM of the cells grown in vivo (73K, 75K, and 83K); these corresponded to proteins that were induced in the iron-depleted medium (lane 2) and that disappeared when the organism was grown in the iron-rich medium (lane 3). The number of high-molecular-weight bands observed in lane 2 was greater than that seen in lane 1, and it is possible that other environmental conditions and factors, such as pH or growth on the epithelial surface, affected the expression of these high-molecular-weight proteins. It is not known which of these IRMPs are involved in specific iron-uptake mechanisms, but the facts that they were induced when the organism was grown in the iron-depleted medium (lane 2) and repressed when it was grown in the iron-rich medium (lane 3) make it clear that the organisms growing in the urine were iron restricted. Figure 2 shows the immunoblots of the OMP shown in Fig. 1 probed with the patient's own serum. The porins (32.5K and 35.5K) of the organism under all three growth conditions were highly immunogenic. IRMPs were also seen to be immunogenic but were fainter on the blot of

the isolate from the patient's urine (lane 1) than on that of the cells grown in Fe−TSB (lane 2). In addition, an antigen with a molecular weight of 48,000 seemed to be highly immunogenic, although it was not evident on the Coomassie blue-stained polyacrylamide gel (Fig. 1). Proteins with molecular weights below 32,500 in the sample isolated directly from the urine were not recognized by the patient's serum (Fig. 2, lane 1). The immunogenic profiles of cells isolated directly from the urine were not identical to those of cells grown in Fe−TSB. Further work is necessary to mimic more closely the growth environment in urine.

Figure 3 shows the OMP profiles of *P. mirabilis* isolated from the urine from patient 2 and cultured in Fe+TSB (lane 1) and Fe−TSB (lane 2), *P. morganii* cultured in Fe+TSB (lane 4) and Fe−TSB (lane 5), and *K. pneumoniae* cultured in Fe−TSB (lane 7), Fe+TSB (lane 8), Fe−CDM (lane 9), and Fe+CDM (lane 10). The pure culture isolated directly from the urine is shown in lane 3, and the mixed culture is shown in lane 6. The OMP profile of the pure culture was similar to that of the iron-depleted *P. mirabilis* culture shown in lane 2. The mixed culture in lane 6 had a mixture of *Proteus* and *Klebsiella* protein profiles. Thus, in this patient, the organisms isolated directly from the urine had OMPs which could be closely mimicked when the same isolates were grown in laboratory media under iron-depleted conditions. Figure 4 shows the antigenic profiles after the proteins were blotted onto NC paper and probed with the patient's own serum. The antigenic profile of *P. mirabilis* isolated directly from the urine (lane 3) was very similar to that of *P. mirabilis* grown in Fe−TSB (lane 2). It is worth noting that the patient's serum contained antibodies which reacted with porin proteins and IRMPs from all three organisms present in the urine.

Control experiments were carried out to determine the cross-reactivity of antibodies present in each patient's serum with OM antigens of members of the family *Enterobacteriaceae* and of a representative of an unrelated family—*P. aeruginosa*. Figure 5a shows the OMP profiles of *K. pneumoniae* (lane 1), *P. mirabilis* (lane 2), *E. coli* (lane 3), and *P. aeruginosa* (lane 4) isolated from human UTIs and grown in Fe−TSB. Figures 5b and c show immunoblots obtained when the antigens shown in Fig. 5a were probed with sera from each of the two patients. The sera from the patients contained antibodies which reacted with antigens in the OMs

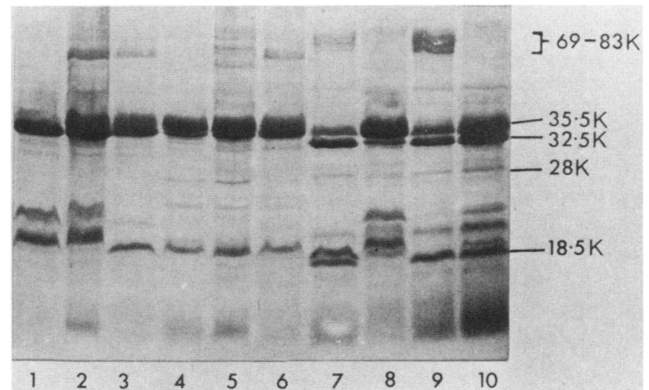

FIG. 3. SDS-PAGE of OMPs of gram-negative bacteria isolated directly from urine and of the same isolates grown in laboratory media. Lanes: 1 and 2, *P. mirabilis* grown in Fe+TSB and Fe−TSB media, respectively; 3, *P. mirabilis* isolated directly from urine; 4 and 5, *P. morganii* grown in Fe+TSB and Fe−TSB media, respectively; 6, mixed culture isolated directly from urine; and 7, 8, 9, and 10, *K. pneumoniae* grown in Fe−TSB, Fe+TSB, Fe−CDM, and Fe+CDM, respectively.

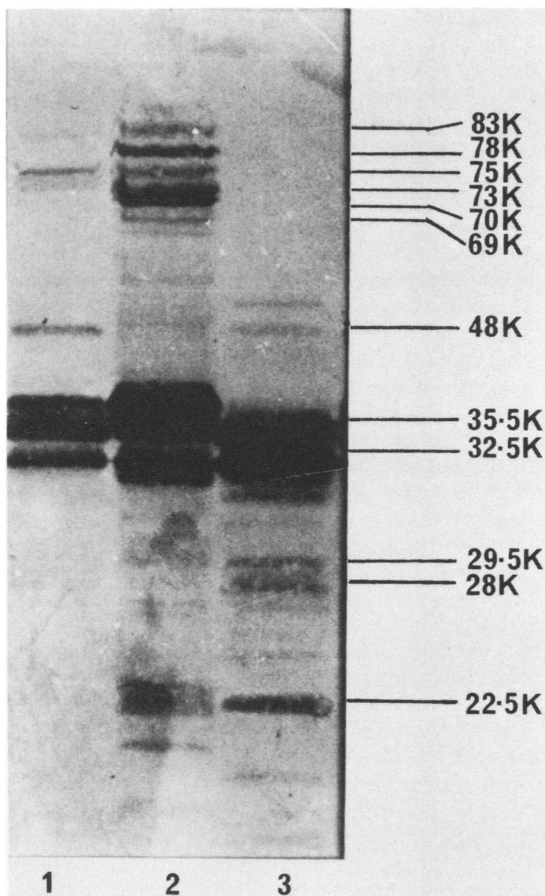

FIG. 2. Immunoblots of the OMPs shown in Fig. 1 electrophoretically transferred to NC paper and reacted with the patient's own serum. Lanes are the same as in Fig. 1.

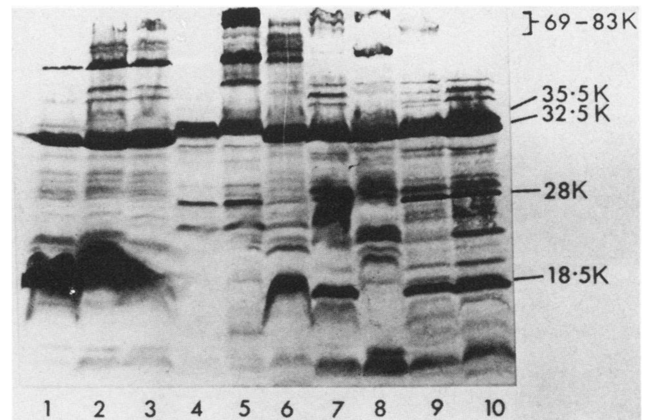

FIG. 4. Immunoblots of the OMPs shown in Fig. 3 electrophoretically transferred to NC paper and reacted with the patient's own serum. Lanes are the same as in Fig. 3.

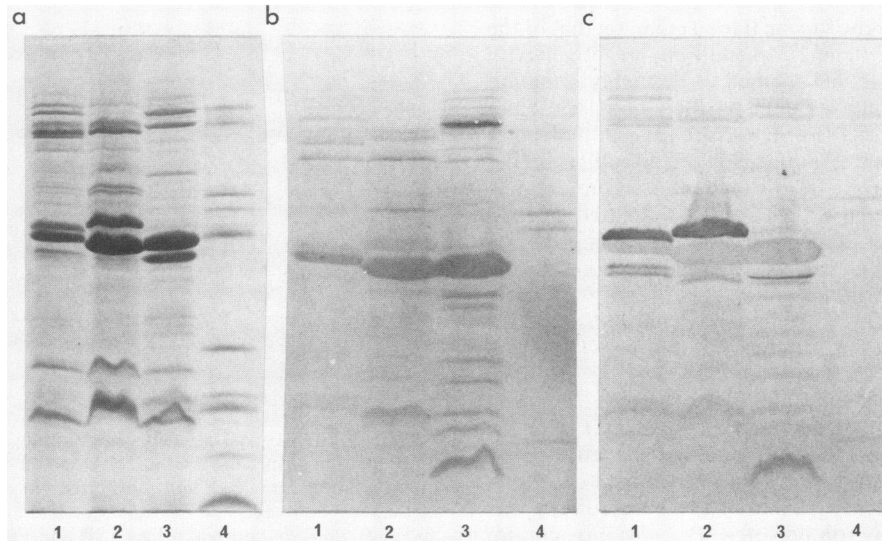

FIG. 5. (a) SDS-PAGE of OMPs of *K. pneumoniae* (lane 1), *P. mirabilis* (lane 2), *E. coli* (lane 3), and *P. aeruginosa* (lane 4). All strains were grown in Fe−TSB. (b and c) Immunoblots of the OMPs shown in panel a reacted with serum from patient 2 (b) and with serum from patient 1 (c). Lanes are as in panel a.

of *K. pneumoniae*, *P. mirabilis*, and *E. coli* (lanes 1 through 3) but which demonstrated a very weak response to antigens in the OM of *P. aeruginosa* (lane 4). To further demonstrate the lack of cross-reactivity between *Enterobacteriaceae* and *Pseudomonadaceae* OM antigens, we probed the OM samples shown in Fig. 5a with sera from patients with UTIs caused by *E. coli* and *P. aeruginosa*. Figure 6a shows that serum from a patient with an *E. coli* infection recognized OM antigens of *K. pneumoniae*, *P. mirabilis*, and *E. coli* (lanes 1 through 3) but only faintly reacted with the OM antigen of *P. aeruginosa* (lane 4). Serum from a patient with an infection caused by *P. aeruginosa*, however, reacted with antigens from all the strains used (Fig. 6b). This was expected, as members of the *Enterobacteriaceae* are part of the normal gut flora. When sera from six female volunteers (ages, 19 to 47 years) who had no history of UTIs were used to probe the samples shown in Fig. 5a, there was a faint reaction to the porins and IRMPs of all three *Enterobacteriaceae* spp. but not to the antigens of *P. aeruginosa* (data not shown). These control experiments taken as a whole indicated that there was a possibility of a cross-reaction between the sera from the patients and the three species of *Enterobacteriaceae*. It is probable that all normal individuals have a low antibody titer against some OM antigens of the normal gut flora; when an individual contracts a UTI caused by one of these organisms, the amounts of antigens increase and trigger the synthesis of antibodies against these antigens. Reactions between these antigens and antibodies are strongly detected by our system. The exact nature of the cross-reactions between the strains remains to be investigated with a more specific system, possibly with monoclonal antibodies against these antigens.

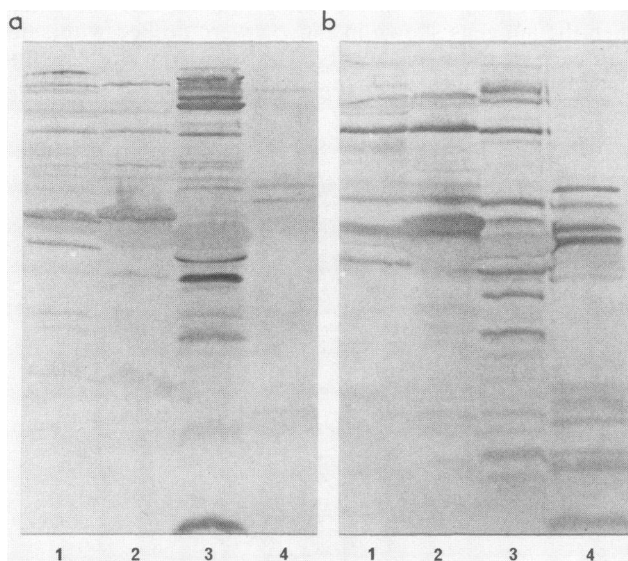

FIG. 6. Immunoblots of the OMPs shown in Fig. 5a reacted with serum from a patient with a UTI caused by *E. coli* and with serum from a patient with a UTI caused by *P. aeruginosa* (b). Lanes are as in Fig. 5a.

Impaired immunological status has been suggested as a possible contributory reason for the high incidence of UTIs in elderly hospitalized patients (11, 15). This study appears to show that the immune systems of the two patients studied effectively recognized the OM antigens of the infecting organisms.

The data presented in this paper indicate that *P. mirabilis* and *K. pneumoniae* grew in the urine of the patients under conditions of iron restriction. In both patients studied, the iron concentration in the urine was found to be less than 10^{-6} M. This concentration of iron in the urine, which was lower than normal (10), may have been a physiological response to the infection or to the underlying illness. We are currently investigating the concentrations of host chelating agents, the titer and nature of immunoglobulins in both serum and urine, and the interaction of these agents with surface components of bacteria in the urine of UTI patients. The limitation of nutrients, including iron, has been shown to have profound effects on the envelope composition (1), including penicillin-binding proteins (23) of bacteria, with consequent changes in sensitivity to antibiotics (5) and host defense mechanisms (2, 9) as well as virulence factor production (19). These findings support the use of iron-restricted growth conditions for the

in vitro study of UTIs. Further work is needed to establish whether all UTIs are iron restricted.

ACKNOWLEDGMENTS

G.H.S. was supported by a grant from The Pharmaceutical Society of Great Britain. This work was also supported in part by grants from the Cystic Fibrosis Research Trust of the United Kingdom (grant 175) and Ciba-Geigy, Basel, Switzerland).

We thank G. Domingue for iron determinations in urine and P. Williams for the defined medium for *Klebsiella* sp.

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