Zbl. Bakt. Hyg. A 266, 403–411 (1987)

Isolation and Characterization of Coliphage Ω18A Specific for Escherichia coli O18ac Strains

EDITH ULMER, J. HACKER[1], E. FASSKE, and G. SCHMIDT[2]

Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, D-2061 Borstel
[1] Institut für Genetik und Mikrobiologie, D-8700 Würzburg
[2] Corresponding author

With 1 Figure · Received December 6, 1986 · Accepted March 25, 1987

Summary

The bacteriophage Ω18A, specific for *Escherichia coli* O18ac strains, was isolated from sewage. The results of host range and conjugation experiments showed that the sensitivity of bacteria to the phage is associated with the presence of O18ac antigens.

With some of the O18 strains the phage Ω18A produces clear lysis on bacterial lawns only when applied at a high multiplicity and moreover the phage does not multiply.

With the help of the phage Ω18A, *E. coli* O18ac strains could be divided into two serologically distinct subgroups called O18A and $O18A_1$. *E. coli* strains belonging to the sugroup O18A are sensitive to phage Ω18A whereas bacteria of subgroup A_1 are resistant.

Zusammenfassung

Der Bakteriophage Ω18A, der spezifisch *Escherichia coli* O18ac Bakterien lysiert, wurde aus Abwasser isoliert. Die Untersuchungen des Wirtsbereichs und Konjugationsversuche zeigten, daß die Sensitivität der Bakterien gegenüber dem Phagen mit dem Vorhandensein des O18ac Antigens assoziiert ist.

Bei einigen O18 Stämmen wird nur bei Anwendung hoher Phagenkonzentrationen eine klare Lysis auf dem Bakterienrasen erzeugt. Darüber hinaus läßt sich der Phage auf diesen Stämmen nicht vermehren.

Mit Hilfe des Phagen Ω18A konnten *E. coli* O18ac Stämme in zwei serologische Subgruppen unterteilt werden, die als O18A und $O18A_1$ bezeichnet werden. *E. coli* Bakterien der Subgruppe O18A sind gegenüber dem Phagen Ω18A sensitiv und diejenigen der Subgruppe $O18A_1$ sind resistent.

Introduction

Escherichia coli O18 strains are frequently among *E. coli* isolates identified as the etiological cause for extraintestinal diseases such as urinary tract infections and menin-

gitis (8, 15). It soon came out that the serogroup O18 comprises a number of *E. coli* strains with closely related but not identical O antigens and since 1956 the *E. coli* O18 serogroup is subdivided into O18ab and O18ac (3). By means of monoclonal antibodies (13) and chemical analysis (6) members of the serogroup O18ac could recently be differentiated further into O18A and $O18A_1$ and those of serogroup O18ab into O18B and $O18B_1$.

In our laboratory a bacteriophage was isolated from sewage which can be used to recognize *E. coli* belonging to the serologic subgroup O18A.

In the present paper the isolation of the phage and its characterization is reported.

Material and Methods

Bacteria and media

E. coli 2980K$^-$, a capsular-negative (K$^-$) mutant of *E. coli* 2980 (O18ac:K5:H5) was used as the host strain for isolation and propagation of the O18ac-specific bacteriophage. The serological typing of *E. coli* 2980 was kindly performed by *I.* and *F. Ørskov,* Copenhagen.

Other *E. coli* strains used in this study are listed in Table 1. The *E. coli* O18 strains with the Prefix C or D as well as the majority of the other strains were obtained from *I.* and *F. Ørskov,* Copenhagen. The remainder O18 strains were supplied by *M. Kist* (18), Freiburg (2980, 2954), *J. F. van den Bosch* (16), Amsterdam (AD 123) and *W. Nimmich,* Rostock, GDR (N 160, S 170, S 466). The strains B 2351, 2126 and 20629 are from the Borstel collection. For bacterial growth and phage propagation we used a medium containing Difco tryptose (10 g); Difco yeast extract (5 g); NaCl (8 g); and glucose (1 g) ad 1,000 ml distilled water. The medium was supplemented with calcium chloride to 5×10^{-3}M. For solid medium, 15 g agar and for soft agar (used for the agar layer method) 7 g per 1,000 ml were added.

Phages

For characterization of *E. coli* strains, the rough specific phages Br10 and 6SR (9, 10) and the phage U3, which is specific for *E. coli* K-12 (17) were used. For screening the phage sensitivity, drops of the phage suspension wer placed onto bacterial lawns with standardized loops (10 µl loops, Nunc, Denmark). Bacteriophages specific for capsular (K) antigens were used for the isolation of K-negative mutants from the K$^+$ parent strains. The phages K1 and K5 specific for *E. coli* exhibiting the K1 or K5 antigen, respectively have been described by *Gross* et al. (4) and *Gupta* et al. (5). The phages K7, K12 and K13 specific for the respective K antigens were recently isolated in our laboratory (*Ulmer* et al., to be published).

K-negative mutants were isolated by picking up single clones grown within the lysis zone of phage sensitive strains. The mutants were purified by repeated single colony isolation. The ability of phage resistant mutants to agglutinate in the respective O-antiserum and their resistance to rough-specific phages was taken as evidence for the presence of a smooth K-negative phenotype.

Isolation and purification of O18-specific phages

Phages were isolated from pooled samples of sewage. A mixture of 900 ml sewage, 100 ml of 10-fold concentrated broth and 10 ml of a freshly grown culture of the host bacteria were incubated overnight at 37 °C. Aliquots of the crude lysate (sterilized with chloroform) were centrifuged in a Sorvall RC-5B centrifuge for 10 min at 8000 g. 0.1 ml of serial dilutions were plated with 4 ml soft agar and 0.1 ml of the host. After 20 h incubation at 37 °C, single plaques were isolated and propagated in liquid culture on *E. coli* 2980K$^-$. Propagation and titration of phages were performed as described previously (5). In order to

obtain a pure line of bacteriophages the single plaque isolations were repeated three times. High titer stocks of purified phages were obtained by isopycnic centrifugation in a cesium chloride gradient as described earlier (5, 7). The phages were kept over chloroform at 4 °C.

Electron microscopy

A drop of suspension of purified bacteriophages was placed on formvar coated grids and stained with 1% phosphotungstic acid and inspected in an electronmicroscope (model EM 300, Philips).

Characterization of nucleic acid

For characterization of phage nucleic acid the staining method of *Bradley* (2) was used as described earlier (7). Briefly, 0.01 ml of a phage suspension containing 2×10^{11} plaque forming units (p.f.u.) per ml were placed on a microscopie slide and air dried. The spots were fixed with a mixture of glacial acetic acid, ethanol and chloroform (1 : 6 : 3) followed by staining with 0.01% acridine orange for 5 min. After washing with citrate-phosphate buffer (pH 3.8) the sample was viewed under an u.v. lamp directly and after treatment with tartaric acid. The fluorescent colour produced under various conditions and its stability after RNAse or DNase treatment allows conclusions about the type of nucleic acid.

Production of antisera and agglutination tests

The production of O-antisera in rabbits by intravenous injection of heat-killed K^- organisms and exhaustive absorption of sera were performed as described previously (10).

Since there are indications that heat-stable components of fimbrial antigens may interfere with O-agglutination (own unpublished observation), fimbriae-negative mutants were applied for immunization. These mutants were obtained by mutagenization of the K^- strains *E. coli* 2980K^- and C311K^- (s. Table 1) with N-methyl-N-nitro-N-nitroso guanidine (NTG) C1, 18).

For slide agglutinations tests cells of the K^- strains were used.

Conjugation experiments

As donor *E. coli* 20915 was used, which was obtained by introduction of the F'lac: :Tn10 plasmid from the auxotrophic *E. coli* K-12 strain NK 5549 into the prototrophic *E. coli* 2980 by selection of tetracyclin-resistant exconjugants on minimal agar containing 15 µg tetracyclin per ml.

As recipient in crosses with *E. coli* 20915 the auxotrophic *E. coli* K-12 strain AB 1133 (*his*$^-$, *thr*$^-$, *leu*$^-$, *argE*$^-$, *proA*$^-$, *rpsL*$^-$) was used.

Selection of hybrids was achieved on appropriately supplemented minimal agar as described earlier (14). The streptomycin sensitive donor was counterselected by addition of streptomycin (100 µg per ml).

Results

Plaque morphology and host range of phage Ω18A

The *E. coli* bacteriophage Ω18A which proved to be specific for *E. coli* O18ac was isolated from pooled samples of sewage. On the host *E. coli* 2980K^- the phage produces plaques of clear lysis with a diameter of around 2 mm, surrounded by an opaque lysis zone.

The host range of this phage was examined in the drop test with supensions containing approximately 5×10^9 plaque forming units (p.f.u.) per ml on a large number of *E.*

coli strains belonging to different serotypes (see Table 1). With the exception of strains D-M3219, C311 and their K⁻ derivatives all *E. coli* O18ac strains tested were sensitive to Ω18A.

When various concentrations of the phage were used the strains 2954, 2980, C96 and the respective K⁻ mutants were completely lysed even when the suspension applied was diluted down to 10^3 p.f.u. per ml (see Table 1). The remainder *E. coli* O18ac strains were sensitive only at high bacteriophage concentrations (ca. 10^9 p.f.u. per ml). Moreover in broth cultures the phage did not multiply on these latter strains.

Table 1. Host range of bacteriophage Ω18A

Strain Nr.	Serotype	lytic activity of Ω18A p.f.u./ml[1] 5×10^9	1×10^3
2980	O18ac:K5:H5	+	+
2980K⁻	O18ac:K⁻:H5	+	+
2954	O18ac:K5:H⁻	+	+
2954K⁻	O18ac:K⁻	+	+
C96	O18ac:K5:H⁻	+	+
C96K⁻	O18ac:K⁻:H⁻	+	+
AD123	O18ac:K5:H⁻	(+)	–
AD123K⁻	O18ac:K⁻:H⁻	+	–
N160	O18ac:K5:H⁻	(+)	–
N160K⁻	O18ac:K⁻:H⁻	+	–
S170	O18ac:K5:H1	(+)	–
S170K⁻	O18ac:K⁻	+	–
S466	O18ac:K5:H1	(+)	–
S466K⁻	O18ac:K⁻:H1	+	–
D–M3219	O18ac:K5:H7	–	
D–M3219K⁻	O18ac:K⁻:H7	–	
C311	O18ac:K5:H⁻	–	
C311K⁻	O18ac:K⁻:H⁻	–	
C5	O18ac:K5:H⁻	(+)	–
C5K⁻	O18ac:K⁻:H⁻	+	–
C12	O18ac:K1:H7	(+)	–
C12K⁻	O18ac:K⁻:H7	+	–
C1976–79K⁻	O1:K1⁻:H7	–	–
Su65–42K⁻	O4:K12⁻:H⁻	–	
Su4344–41K⁻	O6:K13⁻:H1	–	
Pus3432–41K⁻	O7:K7⁻:H4	–	
E56bK⁻	O8:K27⁻:H⁻	–	
E69K⁻	O9:K30⁻:H12	–	
Bi8337–41K⁻	O10:K5⁻:H4	–	
C949–78	O18ab:H⁻	–	
B2351	O19ab:H?	–	
E3bk⁻	O75:K5⁻:H5	–	
20629	O78:H⁻	–	
2126	O111:H⁻	–	

+, complete lysis; (+) opaque lysis; –, no lysis

[1] Number of plaque forming units in the phage suspension.

The inability to propagate the phage and the presence of the capsule, which obviously impedes with the accessibility of the phage receptor may be the reason for the weak reactivity (opaque lysis) of Ω18A on some of the O18ac:K^+ strains.

E. coli strains belonging to serotypes other than O18ac and even *E. coli* O4, O18ab and O19 which are serologically related to O18ac (12) proved to be resistant to phage Ω18A. This result suggested that the phage Ω18A is reactive only on *E. coli* O18ac strains (for details see Table 1).

Electron microscopy and characterization of bacteriophage nucleic acid

Electron microscopic inspection showed that the phage Ω18A has a head diameter of 27–30 nm and a tail length of 50–53 nm. The tail, 5–6 nm in diameter, ends in a plate with pins which in total has a size of 7–9 nm (see Fig. 1). The structure of this phage resembles that of the T-even phages of *E. coli* (e.g. T2) although it is smaller than T2.

The fluorescent staining with acridine orange (2) provides a simple method for differentiation between various types of nucleic acid (2DNA, 1DNA, 2RNA, 1RNA).

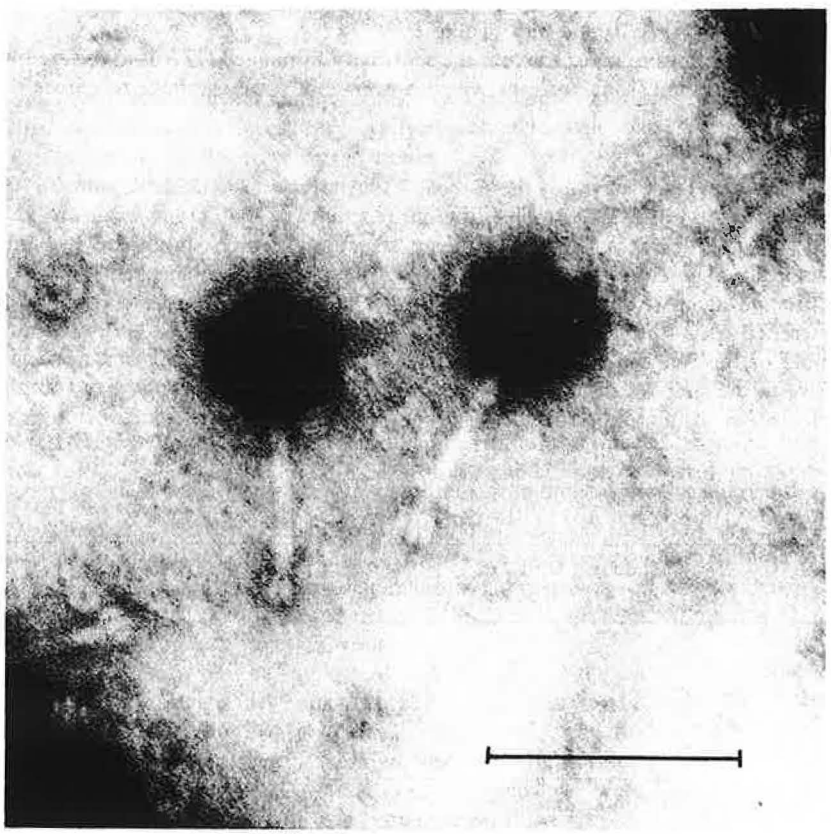

Fig. 1. Coliphage Ω18A as revealed after preparation by negative staining with phosphotoungstic acid. Bar indicates 50 nm.

Yeast RNA, and the double stranded DNA of the T4 phage served as control. The fluorescent colours of Ω18A and T4 but not of the RNA were yellow green in phosphate buffer, orange in tartaric acid, were unchanged after treatment with RNase and vanished after treatment with DNase. These results suggest that the nucleic acid of Ω18a is double stranded DNA like that of phage T4.

Characterization of the phage receptor sites

Conjugation experiments were performed to find out whether the phage sensitivity is associated with the presence of the O18ac antigens. For this purpose the *E. coli* donor 20915 (O18ac:K5) and the *E. coli* K-12 recipient AB1133 *(his)* were crossed with the aim to transfer the donor *his*-linked *rfb* region controlling the synthesis of O-specific polysaccharides (11) into *E. coli* K-12 and to test the resulting hybrids for sensitivity to the phage Ω18A.

His$^+$hybrids were selected from such a cross on appropriately supplemented minimal agar. Most of the resulting hybrids (76 of 92) were agglutinated in slide tests with an anti-O18ac serum indicating that O18ac-specific polysaccharides were expressed in consequence of the introduction of the donor *rfb* genes (see Table 2). These O18-positive hybrids were also lysed by the phage Ω18A and were resistant to the rough-specific phages U3, Br10 and 6SR (Table 2).

This result allows to conclude that the sensitivity to phage Ω18A is associated with the presence of the O18ac antigens which presumably serve as phage receptors.

Serological tests

As shown in Table 1 two of the *E. coli* O18ac strains (D-M3219K$^-$ and C311K$^-$) were resistant to phage Ω18A. The different reactivity of the O18ac strains to Ω18A might reflect differences in the O antigen structures, which should be recognized serologically. Therefore antisera were produced against the phage-sensitive *E. coli* 2980K$^-$ and against the phage-resistant *E. coli* D-M3219K$^-$. In order to avoid any interference with fimbrial antigens, antisera were produced with fimbriae-negative mutants of the two *E. coli* strains. By cross adsorption factor antisera, specifically reactive in the slide tests with bacteria of the homologous strain only, were obtained (Table 3). According to their reaction in the slide tests with the two factor antisera the

Table 2. Serological analysis and phage sensitivity of his$^+$ hybrids from a cross between E. coli 20915 (O18:K5) and AB1133(K–12)

No. of hybrids	slide test with anti O18ac serum[1]	reaction with phage[2]				
		K5	Ω18A	Br10	6SR	U3
76	+	–	+	–	–	–
16	–	–	–	+	–	+
parents						
20915(O18:K5)	+[3]	+	+	–	–	–
AB1133(K–12)	–	–	–	+	–	+

[1] +, agglutination; –, no agglutination
[2] +, lysis; –, no lysis
[3] bacteria of 20915 (K5$^+$) are agglutinable only after heating for 1 hr at 100 °C
[4] U3 phage is specific for the unsubstituted LPS core of *E. coli* K–12.

Table 3. Results of slide agglutination tests with different E. coli O18:K⁻ strains

Strain Nr.	reactivity[1] with Ω18A	reaction[2] in serum			
		anti-2980K⁻		anti-C311K⁻	
		unab-sorbed	abs. with C311K⁻	unab-sorbed	abs. with 2980K⁻
2980K⁻	+	+	+	+	–
2954K⁻	+	+	+	+	–
C96K⁻	+	+	+	+	–
AD123K⁻	+	+	+	+	–
N160K⁻	+	+	+	+	–
S170K⁻	+	+	+	+	–
S466K⁻	+	+	+	+	–
C5K⁻	+	+	+	+	–
C12K⁻	+	+	+	+	–
D–M3219K⁻	–	+	–	+	+
C311K⁻	–	+	–	+	+

[1] +, lysis; –, no lysis
[2] +, agglutination; –, no agglutination

O18ac strains could clearly be divided into two serologic subgroups (see Table 3). The strains sensitive to Ω18A were specifically agglutinated with factor serum 2980K⁻ and those resistant to Ω18A were reactive only in factor serum C311K⁻. Thus the phage Ω18A can be used to recognize serologic subgroups of *E. coli* O18ac.

Discussion

The bacteriophage Ω18A isolated from sewage is specific for *Escherichia coli* O18ac strains. The results presented here infer that the sensitivity to the phage is associated with the expression of the O18ac antigens. Further studies are required to elucidate the role of the O antigen in the phage adsorption process.

Two of the O18ac strains tested proved to be resistant to phage Ω18A and moreover were serologically distinct from Ω18A-sensitive strains. Thus, two serologic subgroups of serotype O18ac were established with the help of this phage. The result obtained by *Pluschke* et al. (13) with monoclonal antibodies also led to subdivision of O18ac bacteria into two subgroups called O18A and $O18A_1$, respectively. According to their reaction pattern with a set of monoclonal antibodies, strains sensitive to phage Ω18A have been shown to belong to subgroup O18A and those resistant against Ω18A to subgroup $O18A_1$ (*Pluschke*, pers. commun.).

The rational basis for the serological differentiation is the chemical composition of the respective 0 antigens (6). Thus the O18A repeating units of *E. coli* O18A consist of pentasaccharides having N-acetylglucosamine as side branch. The $O18A_1$ repeating units have an additional glucose residue, also in a side position.

A number of the *E. coli* O18ac:K⁻ strains tested here were lysed only with high concentrations of phage Ω18A and moreover no phage progeny was produced with them. The phage obviously acts on these strains like a colicin. Possibly these strains are missing a factor which may play the role of a secondary receptor necessary to trigger

the injection of Ω18A DNA into the bacterial cells. On the other hand it is conceivable that the inability of the phage to propagate in certain O18ac strains may be due to a DNA restriction system active in cells of these strains. Experiments are set out to elucidate this point.

Acknowledgement. The expert help of *Ulrike Venema, Sabine Löbau,* and *Heike Kühl* is gratefully acknowledged. – We are grateful to many of our colleagues for generous help and advice. *E. coli* strains were obtained from *J. F. van den Bosch,* Amsterdam, *M. Kist,* Freiburg, *I.* and *F. Ørskov,* Copenhagen and *W. Nimmich,* Rostock, GDR. *E. M. Lederberg,* Plasmid Reference Center, Stanford, supplied the F′ strain NK5549 of *E. coli* K-12. *G. Pluschke,* Basel, supplied us with data of unpublished results and tested some of our strains with monoclonal antibodies. *K. Jann,* Freiburg, kindly informed us of unpublished results about the chemical structure of O18 antigens.

References

1. *Adelberg, E. A., M. Mandel,* and *G. C. C. Chen:* Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli.* Biochem. Biophys. Res. Commun. 18 (1965) 788–795
2. *Bradley, D. E.:* The fluorescent staining of bacteriophage nucleic acids, J. Gen. Microbiol. 44 (1966) 383–391
3. *Ewing, W. H., K. E. Tanner,* and *H. W. Tatum:* Investigation of *Escherichia coli* O group 18 serotypes isolated from cases of infantile diarrhea. Publ. Hlth Lab. 14 (1956) 106–115
4. *Gross, R. J., T. Cheasty,* and *B. Rowe:* Isolation of bacteriophages specific for the K1 polysaccharide antigen of *Escherichia coli.* J. Clin. Microbiol. 6 (1977) 548–550
5. *Gupta, D. S., B. Jann, G. Schmidt, J. R. Golecki, I. Ørskov, F. Ørskov,* and *K. Jann:* Coliphage K5, specific for *E. coli* exhibiting the capsular K5 antigen. FEMS Microbiol. Lett. 14 (1982) 75–78
6. *Gupta, D. S., B. Jann,* and *K. Jann: Escherichia coli* O18ac antigen: structure of the O-specific polysaccharide moiety. Infect. Immun. 45 (1984) 203–209
7. *Jann, K., G. Schmidt, B. Wallenfels,* and *E. Freund-Mölbert:* Isolation and characterization of bacteriophage Ω8 specific for *E. coli* strains belonging to sero-group O8. J. Gen. Microbiol. 67 (1971) 289–297
8. *Korhonen, T. K., M. V. Valtonen, J. Parkkinen, V. Väisänen-Rhen, J. Finne, F. Ørskov, I. Ørskov, S. B. Svenson,* and *P. H. Mäkelä:* Serotype, hemolysin production, and receptor recognition of *Escherichia coli* strains associated with neonatal sepsis and meningitis. Infect. Immun. 48 (1985) 486–491
9. *Lindberg, A. A.:* Bacteriophage receptors. Ann. Rev. Microbiol. 27 (1973) 205–241
10. *Mayer, H.* and *G. Schmidt:* The occurrence of three different lipopolysaccharide cores in Shigella and their relationship to known enterobacterial core types. Zbl. Bakt. Hyg., I. Abt. Orig. A 224 (1973) 345–354
11. *Mäkelä, P. H.* and *B. A. D.:* Genetics of lipopolysaccharide. Handbook of Endotoxins (*R. Proctor,* ed.), Volume 1: Chemistry of Endotoxins (*E. Th. Rietschel,* ed.), pp. 59–137. Elsevier, Biomedical Press, Amsterdam (1984)
12. *Ørskov, F.* and *I. Ørskov:* Serotyping of *Escherichia coli.* Meth. Microbiol. 14 (1984) 43–112
13. *Pluschke, G., A. Moll, B. Kusecek,* and *M. Achtmann:* SDS-PAGE and monoclonal antibodies as tools for the subgrouping of *Escherichia coli* lipopolysaccharide O18 and O23 antigens. Infect. Immun. 51 (1986) 286–293
14. *Schmidt, G.:* Genetical studies on the lipopolysaccharide structure of *Escherichia coli* K-12. J. Gen. Microbiol. 77 (1973) 151–160

15. *Väisänen-Rhen, V., J. Elo, E. Väisänen, A. Siitonen, I. Ørskov, F. Ørskov, S. B. Svenson, P.H. Mäkelä,* and *T. H. Korhonen:* P-fimbriated clones among uropathogenic *Escherichia coli* strains. Infect. Immun. 43 (1984) 149–155
16. *van der Bosch, J. F., U. Verboom-Sohmer, P. Postma, J. de Graaff,* and *D. M. MacLaren:* Mannose-sensitive and mannose-resistant adherence to human uroepithelial cells and urinary virulence of *Escherichia coli.* Infect. Immun. 29 (1980) 226–233
17. *Watson, G.* and *K. Paigen:* Isolation and characterization of an *Escherichia coli* bacteriophage requiring cell wall galactose. J. Virol. 8 (1971) 669–674
18. *Wevers, P., R. Picken, G. Schmidt, B. Jann, K. Jann, J. R. Golecki,* and *M. Kist:* Characterization of pili associated with *Escherichia coli* O18ac. Infect. Immun. 29 (1980) 685–691

Dr. *G. Schmidt,* Forschungsinstitut Borstel, Inst. f. Experimentelle Biologie und Medizin, D-2061 Borstel