

## TRANSPOSON MUTAGENESIS AND MEMBRANE PROTEIN STUDIES IN AN AVIAN COLISEPTICAEMIC *Escherichia coli* STRAIN

Wanderley Dias da Silveira, Fabiana Fantinatti and Antonio F.P. de Castro

### ABSTRACT

The pathogenicity, antibiotic resistance, plasmid DNA and membrane protein profiles of a colisepticaemic *Escherichia coli* strain (362) isolated from chickens was studied. It was verified that this strain harboured at least five plasmids. One 88.0 MD plasmid is non conjugative and is responsible for the production of colicin V and serum resistance. The 43.0 MD plasmid is responsible for resistance to bactericidal activity of serum and ampicilin. Curing of these plasmids did not decrease the pathogenicity of the strain. Transposon mutagenesis (Tnpho A) of strain 362 yielded a non-pathogenic derivative strain which had lost a 40.7 kD membrane protein, which is not correlated to the aerobactin and enterochelin systems. We suggest that this protein subunit is involved in the pathogenicity process of avian septicaemic *E. coli* strains.

### INTRODUCTION

*Escherichia coli* strains have been implicated as causative agents of septicaemia in chickens (Sojka and Carnaghan, 1961). It has been proposed that this disease starts as a secondary infection triggered by an initial mycoplasmal or viral infection (Gross, 1961; Aycardi and Lafont, 1969; Dho and Lafont, 1982) which would be followed by an invasive phase by pathogenic *E. coli* strains, leading to organ lesions (Gross, 1961). Therefore, colonization of the trachea would be essential for the first steps in the development of the illness (Dho and Lafont, 1982, 1984) and the presence of fimbriae on the surface of invasive strains would help this process (Nagaraja *et al.*, 1983; Naveh *et al.*, 1984; Suwanickul and Panigrahy, 1986, 1988; Suwanickul *et al.*, 1987). After the septicaemic *E. coli* strain has invaded the blood stream it is able to overcome the iron-limiting conditions in the body fluids with an iron-acquisition system that is mediated by either chromosomal or plasmid genes (Rosemberg and Young, 1974; Hancock *et al.*, 1976; Williams, 1979; Williams and

Warner, 1980; Warner *et al.*, 1981; Pierce *et al.*, 1983; Williams and Carbonetti, 1986). Recently Góes *et al.* (1993) found that recombinant strains harboring and expressing the aerobactin system or virulent *E. coli* were not pathogenic.

We investigated the relationship between serum resistance and the electrophoretic profile of membrane proteins with pathogenicity in a septicaemic *E. coli* strain (362) isolated from chickens as well as some non-pathogenic derivatives, using bacterial genetic assays and transposon mutagenesis.

### MATERIAL AND METHODS

#### *Bacterial strains*

*E. coli* 362 was a wild type col V producer strain isolated from a chicken with colisepticaemia. Strain SM10  $\lambda$  *pir* (pRT733 - TnphoA) was a gift from Dr. James B. Kaper from the Center for Vaccine Development, Maryland, USA. *E. coli* strain MS101 was a *lac*<sup>-</sup>, *Sm*<sup>r</sup> non-pathogenic for poultry. *E. coli* strain 362/3 was derived from strain 362, which had lost a 88.0 MD plasmid through treatment with SDS. *E. coli* T16 was a non-pathogenic transconjugant isolated after conjugation between 362 and SM1  $\lambda$  *pir* strains.

### Pathogenicity tests

Strains to be tested for pathogenicity were grown at 37°C overnight in LB medium (Sambrook *et al.*, 1989), diluted (1:100) into the same medium and incubated for three hours with agitation (150 rpm). Aliquots of 0.4 ml of 10-fold dilutions of these cultures were injected subcutaneously into a group of six one-day-old chicks. They were observed for seven days, and mortality rates were recorded. The LD<sub>50</sub> was calculated by the method of Reed and Muench (1938).

### Antibiotic resistance

Antibiotic resistance was determined by growth on LB medium containing increasing concentrations of the antibiotics ampicillin (Ap), kanamycin (Km), streptomycin (Sm), tetracycline (Tc), chloramphenicol (Cm) and nalidixic acid (Nal). The level of resistance of each strain was considered the highest concentration in which growth occurred.

### Transposon mutagenesis

Mutagenesis with the transposon TnphoA (Boquet *et al.*, 1987) using plasmid pRT733 was accomplished as described by Taylor *et al.* (1989).

### Preparation of outer membrane proteins

Outer membrane proteins were prepared as described by Schaitman (1971), with some modifications as follows. The bacteria were grown in LB medium or LB medium containing 200 µM alpha-alpha-dipyridil (an iron chelator, Sigma Chemical Co.) and incubated overnight. The cells were collected by centrifugation (8000xg for 5 min. at 4°C) and resuspended in cold 3.3 mM Tris-HCl, pH 7.4. After this procedure, the bacterial cells were mixed using a OmniMixer (Sorval) for 1 min. on ice and centrifuged at 8000xg for 5 min. at 4°C. Cells were resuspended and centrifuged once in cold 10 mM EDTA; 10 mM Tris-HCl pH 7.2 and once in 10 mM MgCl<sub>2</sub>; Tris-HCl pH 7.2. Cells were resuspended in cold 75 mM sucrose; 10 mM glycine pH 9.2 containing 0.1 mg ml<sup>-1</sup> lysozyme (Sigma Chemical Co.) and incubated on ice for 30 min. The suspension was sonicated for four 30 sec. cycles, at 20 Khz, 70 - 90 W. Cell debris was removed by centrifugation at 8000xg for 5 min. at 4°C and the supernatant was centrifuged at 37000 g for 20 min. at 4°C. The pellet was resuspended in cold 3.3 mM Tris-HCl pH 7.4. and was centrifuged under the same conditions. The proteins were resuspended in 85 mM Tris-HCl pH 6.8; 4% Triton X-100 and shaken by inversion (45 rpm) at room temperature for 20 min. This suspension was centrifuged

at 12000xg for 15 min. The pellet containign the Triton X-100 insoluble membrane fraction was resuspended in 100 mM Tris-HCl pH 6.8; 20% glycerol; 4% SDS; 0.2% bromophenol blue; 10% beta mercaptoethanol, boiled for 5 min. and stored at -20°C until ready for use. The concentration of protein was determined as described for Bradford (1976).

### Analysis by SDS - PAGE

Proteins were separated by SDS - PAGE as described by Laemmli (1970). A 3% stacking gel and a 10% separating gel was used. 2.0 µg of each sample was applied to each well. Protein bands were visualized by silver staining as described by Blum *et al.* (1987). Molecular weights were estimated by comparison with the relative molecular mobilities of standard protein markers (Sigma MW-S-70).

### SDS cure of plasmid DNA

The plasmid DNA was cured as described by Tomoeda *et al.* (1968), using SDS (sodium dodecyl sulfate) at a final concentration of 10%.

### Plasmid DNA extraction and agarose gel electrophoresis

Plasmid DNA for electrophoresis on agarose gels was extracted as described by Sambrook *et al.* (1989). Twenty µl samples of plasmid DNA were loaded into wells of a 0.7% agarose gel (LKB-Pharmacia) and run at 5 Volts/cm until the blue dye reached the end of the gel. Gels were stained with ethidium bromide and visualized on an ultra-violet 2011 Macrovue transilluminator (LKB-Pharmacia). Photographs were taken using a 100 TMAX Kodak film.

### Serum resistance determination

The resistance of the different strains to the bactericidal activity of the serum was determined by a quantitative method. For this purpose, the strains were grown overnight and then diluted 1:100 in 1% Peptone - 1% glucose medium and grown under the same conditions. When the cultures were in log-phase (A<sub>600</sub> = 0.5), 250 µl was inoculated into 1.0 ml of a pool of chicken sera and again incubated at 37°C. At intervals of 1 hour (for three hours), 10-fold dilutions of the cultures were made in sterile 0.85% NaCl solution and then plated on agar. After 18-20 hours incubation, colonies were counted. The rate of growth and hence the bactericidal activity of the serum was measured using 0 hour as the control.

## RESULTS

Strain 362 was resistant to ampicillin and tetracycline. Agarose gel electrophoresis of plasmid DNA of pathogenic *E. coli* strain 362 showed that it harbours 88.0 MD, 54.0 MD, 43.0 MD, 4.0 MD, 2.0 MD, 1.9 MD and 1.0 MD plasmids (Figure 1).

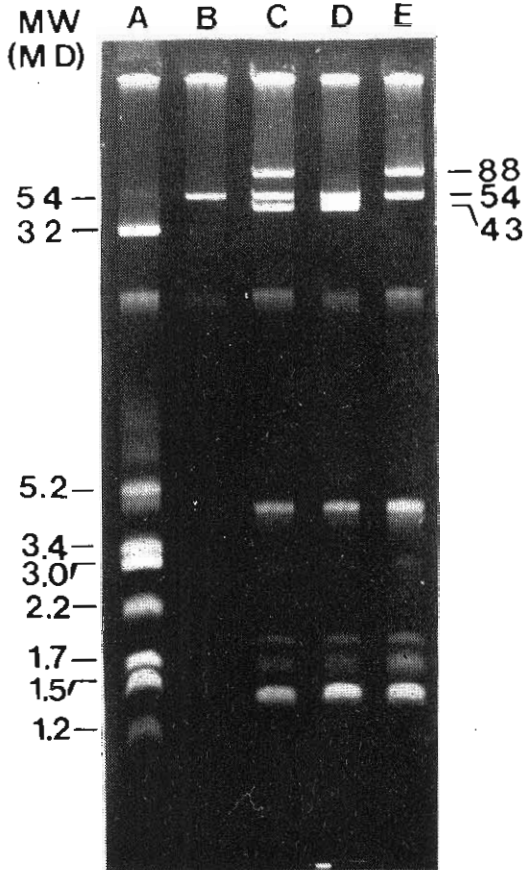


Figure 1 - Agarose gel electrophoresis of plasmid DNA of the colisepticaemic *E. coli* strain 362 and its derivatives. Lane A, 32.0 MD, 5.19 MD, 3.48 MD, 3.03 MD, 1.69 MD, 1.51 MD reference plasmids. Lane B, reference plasmid p307, 54.0 MD. Lane C, strain 362. Lane D, strain 362/3. Lane E, strain 366.

SDS treatment of strain 362 eliminated two plasmids (Figure 1). Elimination of the 43.0 MD plasmid produced strain 366 which was rendered ampicillin sensitive and curing of the plasmid of 88.0 MD gave rise to strain 362/3 (Figure 1). Serum resistance determination of these strains compared with strain 362 (Figure 2) demonstrated that either the elimination of plasmid 43.0 MD (strain 366) or plasmid 88.0 MD (strain 362/3) decreased the capacity of the respective strains to survive the bactericidal action of the serum. Transfer, by conjugation, of plasmid 43.0 MD to a serum sensitive, non-pathogenic *E. coli* strain (MS101) rendered it

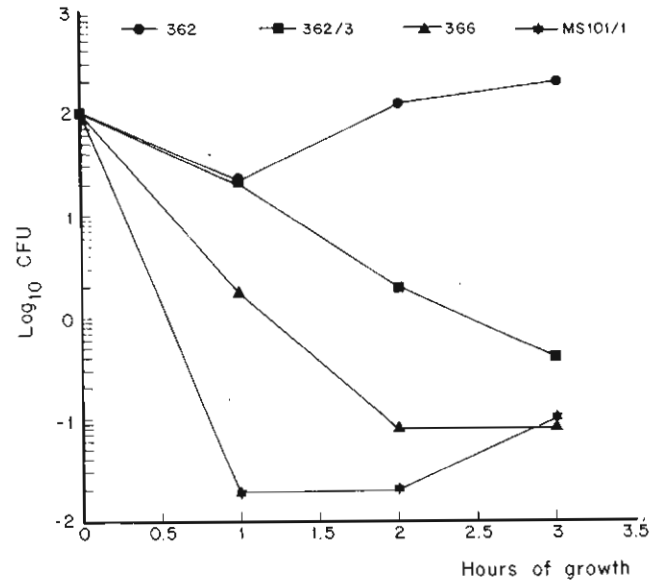


Figure 2 - Serum resistance determination of colisepticaemic strain 362, plasmidless derivatives (362/3 and 366) and transconjugant strain MS101 that received the 43.0 MD plasmid (MS101/1).

somewhat resistant to serum. However the transconjugant strain was not pathogenic in the one-day-old chick assay. Both strains 366 and 362/3 were still pathogenic by this assay. With the elimination of plasmid 88.0 MD strain 362/3 did not produce colicin V and its serum resistance decreased, while strain 366 remained a colicin V producer. Plasmid 88.0 MD was not a F-like plasmid since we were unable to transfer it to strain MS101 after it had been marked with kanamycin resistance from transposon TnphoA. We found 19 blue-kanamycin resistant colonies using transposon TnphoA. One of these (T16) was not pathogenic in the one-day-old chick assay (Table I).

Table I - LD<sub>50</sub> in number of bacteria obtained for strains 362, 362/3, 366 and T16 in the one-day-old chick assay.

Strain	362	362/3	366	T16
	4.3 10 <sup>6</sup>	2.0 10 <sup>7</sup>	8.0 10 <sup>9</sup>	1.2 10 <sup>12</sup>

The SDS-PAGE of membrane proteins showed that strain 366 was identical to strain 362 (results not shown); strain 362/3 expressed three extra major protein subunits (38.1 KD, 27.6 KD and 17 KD) in a derepressed mode; and strain T16 did not express the 40.7 KD protein subunit that was expressed by strains 362, 362/3 and 366, and it also synthesized protein of 17 KD in a derepressed manner (Figure 3).

SDS-PAGE of membrane proteins of strains grown in the presence of alpha-alpha-dipyridil (iron

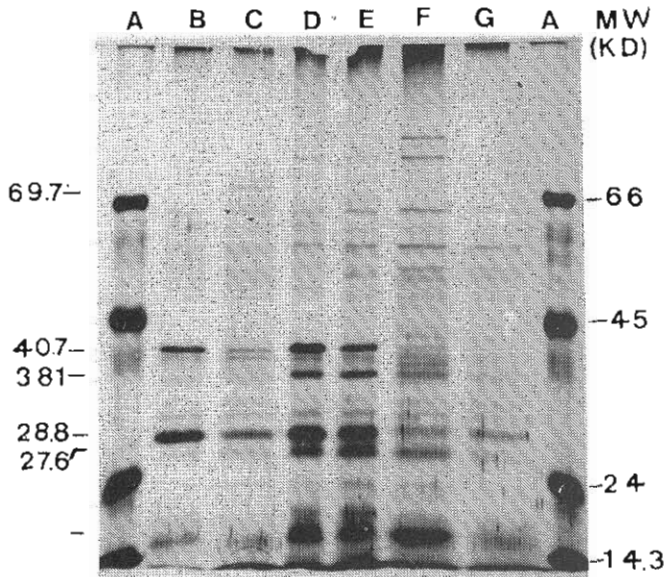


Figure 3 - Membrane protein profile of colicepticaemic strain 362 and its derivatives grown in the presence and absence of iron separated by SDS-PAGE. Lane A molecular weight markers of 66 KD, 45 KD, 18 KD and 14.3 KD. Lanes B and C, strain 362. Lanes D and E, strain 362/3. Lanes F and G, strain T16. Lanes B, D, F, presence of iron. Lanes C, E, G, absence of iron.

chelator) when compared with the same strains grown without this drug (Figure 3) showed that in strain 362 a protein subunit of 69.7 KD was induced by the absence of iron. On the other hand, in strain 362/3 under the same iron-deprived conditions this band was absent. Protein subunits of 40.7 KD, 38.1 KD, 28.8 KD and 27.6 KD were expressed by strain 362/3 in the presence or absence of iron. Strain T16 did not express the 69.7 KD protein subunit independently of the presence or absence of iron.

## DISCUSSION

Strain 362 was able to survive the bactericidal action of the serum, and derivative strain 366 which had lost a 43.0 MD plasmid had a lower capacity to survive the serum, but was still pathogenic in one-day-old chicks. As plasmid 43.0 MD is a conjugative plasmid, the serum resistance showed by this strain could be due to the outer membrane proteins *tra* T, as found for other *E. coli* strains (Moll *et al.*, 1980). Derivative strain 362/3, which had lost the 88.0 MD plasmid showed a slight decrease in serum survival, maintaining, however, its pathogenicity. Plasmid 88.0 MD is a non-conjugative ColV plasmid (Bindereif *et al.*, 1982) which probably specifies outer membrane proteins related to the iron-acquisition system of

aerobactin. This was confirmed by SDS-PAGE membrane protein analysis of this strain grown in the presence and absence of alpha-alpha-dipyridyl, which is a iron chelator. When strain 362/3 was grown in the presence of alpha-alpha-dipyridyl it was not able to express the 69.7 KD protein subunit when compared with strain 362 grown in the absence of iron. In addition, strain 362/3 expressed three other new protein subunits of 38.1 KD, 27.6 KD and 20.4 KD, either in the presence or in the absence of iron. These protein subunits are, probably, repressed by the presence of the 88.0 MD plasmid.

Transposon mutagenesis of wild type strain 362 produced strain T16, which is not pathogenic and SDS-PAGE analysis of the membrane proteins of this strain showed that the 69.7 KD and 40.7 KD protein subunits were missing. The 69.7 KD protein subunit corresponds to the protein described for the aerobactin system and was not responsible for the pathogenicity since strain 363/3, despite the loss of the 88.0 MD plasmid, was still pathogenic and had this protein subunit. The 40.7 KD protein subunit does not correspond either to the proteins associated with the plasmidial DNA (Bindereif *et al.*, 1982) or chromosomal DNA aerobactin systems (Carbonetti *et al.*, 1986) or to the enterochein system (Hancock *et al.*, 1976).

Agarose gel electrophoresis of plasmid DNA and SDS-treatment for plasmid DNA elimination (results not shown) of non pathogenic derivatives obtained by transposon mutagenesis demonstrated that transposon *TnphoA* was inserted into the 88.0 MD plasmid and in the chromosome. Since strain 362/3 still expressed the 40.7 KD protein subunit and did not have the 88.0 MD plasmid and all the other plasmids did not have transposon insertions, we concluded that the genes responsible for the expression of this subunit were located on the chromosome. Recently, Góes *et al.* (1993) demonstrated that recombinant *E. coli* strains harboring and expressing the aerobactin system were not pathogenic either for chicken or mice and suggested that this system is not responsible for the virulence shown by the wild type strain. The 40.7 KD protein subunit could participate in the process of pathogenicity of avian septicaemic *E. coli* strains and the 28.8 KD protein subunit does not participate in this process.

## ACKNOWLEDGMENTS

This work was supported by grants no. 89/3918-8 and 91/5217-7 from the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP and grants no. 300121/90-3 and 300374/88-7 from CNPq. The authors thank Dr. David H. Moon for reviewing the manuscript.

Publication supported by FAPESP.

## RESUMO

Uma amostra septicêmica de *Escherichia coli* (362) foi isolada em galinha e estudada quanto a sua patogenicidade, resistência a drogas antimicrobianas, perfil plasmidial e de proteínas de membrana. Tal amostra possui cinco plasmídios. O plasmídio de 88.0 MD é o responsável pela produção de colicina V e resistência sérica, entretanto este plasmídio não é conjugativo. O plasmídio de 43.0 MD é responsável pela resistência sérica e carrega os genes para resistência à ampicilina. A cura do plasmídio de 43.0 MD não altera a patogenicidade. A mutagênese da amostra 362 com o transposon Tnpho A, originou a amostra T16 que se mostrou não patogênica para pintos de um dia de idade e perdeu a expressão de uma proteína de membrana de 40.7 KD. Esta proteína não está relacionada com os sistemas de aerobactina e enteroquelina. Nós sugerimos que a proteína de 40.7 KD pode participar no processo de patogenicidade de amostras de *E. coli* septicêmicas para aves.

## REFERENCES

- Aycardi, J. and Lafont, J.P. (1969). Données récentes sur l'étiologie et l'épizootologie de la mycoplasmosse du poulet. Conséquences prophylactiques. *Bull. Off. Int. Epizoot.* 72: 351-380.
- Bindereif, A., Braun, V. and Hantke, K. (1982). The cloacin receptor of ColV-bearing *Escherichia coli* is part of the Fe<sup>3</sup>-aerobactin transport system. *J. Bacteriol.* 150: 1472-1475.
- Blum, H., Beier, H. and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8: 93-99.
- Boquet, P.L., Manoil, C. and Beckwith, J. (1987). Use of TnphoA to detect genes for exported proteins in *Escherichia coli*: identification of the plasmid-encoded gene for a periplasmic acid phosphatase. *J. Bacteriol.* 169: 1663-1669.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein - dye binding. *Anal. Biochem.* 72: 248-254.
- Carbonetti, N.H., Boonchai, S., Parry, S.H., Vaisanen-Rhen, V., Korhonen, T.K. and Williams, P.H. (1986). Aerobactin-mediated iron uptake by *Escherichia coli* isolated from human extraintestinal infections. *Infect. Immun.* 51: 966-968.
- Dho, M. and Lafont, J.P. (1982). *Escherichia coli* colonization of the trachea in poultry: Comparison of virulent and avirulent strains in gnotoxigenic chickens. *Avian Dis.* 26: 787-797.
- Dho, M. and Lafont, J.P. (1984). Adhesive properties and iron uptake ability in *Escherichia coli* lethal and nonlethal for chickens. *Avian Dis.* 28: 1016-1025.
- Góes, C.R., Gaziri, L.C.J. and Vidotto, M.C. (1993). Cloned genes of the aerobactin system of virulent avian *Escherichia coli* do not confer virulence to recombinant strains. *Brazilian J. Med. Biol. Res.* 26: 261-275.
- Gross, W.B. (1961). The development of "air sac disease". *Avian Dis.* 5: 431-439.
- Hancock, R.E.W., Kantke, K. and Braun, V. (1976). Iron transport in *Escherichia coli* K-12: involvement of the colicin B receptor and of a citrate-inducible protein. *J. Bacteriol.* 127: 1370-1375.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Moll, A., Manning, P.A. and Timis, K.N. (1980). Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the *traT* gene product, is responsible for plasmid-specified serum resistance in *Escherichia coli*. *Infect. Immun.* 28: 359-367.
- Nagaraja, K.V., Every, A., Newman, J.A. and Poveroy, P.S. (1983). Identification and isolation of somatic pili from pathogenic *Escherichia coli* of turkeys. *Am. J. Vet. Res.* 44: 282-287.
- Naveh, M.W., Zusman, T., Skutelsky, E. and Ron, E.Z. (1984). Adherence pili in avian strains of *Escherichia coli*: effect on pathogenicity. *Avian Dis.* 28: 651-661.
- Pierce, J.R., Pickett, C.L. and Earhart, C.F. (1983). Two *fep* genes are required for ferriterochelin uptake in *Escherichia coli* K-12. *J. Bacteriol.* 153: 330-336.
- Reed, L.J. and Muench, H. (1938). A simple method for estimating fifty per cent end points. *Am. J. Hyg.* 27: 493-497.
- Rosenberg, H. and Young, I.G. (1974). Iron transport in the enteric bacteria. In: *Microbial Iron Metabolism: a Comprehensive Treatise* (Neiland, J.B., ed.). Academic Press, New York, pp. 67-82.
- Sambrook, J., Fritsh, E.F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. 2nd Edition. Cold Spring Harbor Laboratory Press, New York, pp. 1584.
- Schaitman, C.A. (1971). Solubilization of the cytoplasmic membrane of *Escherichia coli* by triton X-100. *J. Bacteriol.* 108: 545-552.
- Sojka, W.J. and Carnaghan, R.B.A. (1961). *Escherichia coli* infection in poultry. *Res. Vet. Sch.* 2: 340-351.
- Suwanichkul, A. and Panigrahy, B. (1986). Biological and immunological characterization of pili of *Escherichia coli* serotypes 01, 02, 078 pathogenic to poultry. *Avian Dis.* 30: 781-787.
- Suwanichkul, A. and Panigrahy, B. (1988). Diversity of pilus subunits of *Escherichia coli* isolated from avian species. *Avian Dis.* 32: 822-825.
- Suwanichkul, A., Panigrahy, B. and Wagner, R.M. (1987). Antigenic relatedness and partial amino acid sequence of pili of *Escherichia coli* serotypes 01, 02, 078 pathogenic to poultry. *Avian Dis.* 31: 809-813.
- Taylor, R.K., Manoil, C. and Mekalanos, J.J. (1989). Broad-host-range vectors for delivery of TnphoA: Use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. *J. Bacteriol.* 171: 1870-1878.
- Tomoda, M., Inuzuka, M., Kubo, N. and Nakamura, S. (1968). Effective elimination of drug resistance and sex factors in *Escherichia coli* by sodium dodecyl sulfate. *J. Bacteriol.* 95: 1978-1989.
- Warner, P.J., Williams, P.H., Bindereif, A. and Neilands, J.B. (1981). ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. *Infect. Immun.* 33: 540-545.

- Williams, P.H.** (1979). Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. *Infect. Immun.* 26: 925-932.
- Williams, P.H. and Carbonetti, N.H.** (1986). Iron, siderophores, and pursuit of virulence. Independence of the aerobactin and enterochelin iron uptake systems of invasive strains of *Escherichia coli*. *Infect. Immun.* 51: 942-947.
- Williams, P.H. and Warner, P.J.** (1980). ColV plasmid-mediated, colicin V-independent uptake system of invasive strains of *Escherichia coli*. *Infect. Immun.* 29: 411-416.

Received June 15, 1993)