Salmonellae of serotypes Gallinarum and Pullorum grouped by biotyping and fimbrial-gene probing

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Summary. When salmonellae of serotypes Gallinarum (50 isolates) and Pullorum (36 isolates), that produce non-adhesive (type-2) fimbriae, were tested for their reactions in biochemical tests, 81 (94%) were found to belong to three distinct biochemical groups, I–III. Interaction of *Hinf*I-digested DNA of both Gallinarum and Pullorum with a probe of accessory genes of type-1 fimbriation in serotype Typhimurium gave one type of Southern hybridisation pattern that was readily distinguished from that of Typhimurium strains. With a probe of the Typhimurium fimbrial subunit gene, Pullorum isolates were separated into strongly and weakly probe-reactive groups which showed restriction fragment-length polymorphism; these latter groups corresponded to biochemical groups II and III, respectively.

Introduction

Salmonellae of most serotypes have a wide host range. However, a few serotypes show high specificity for particular animal hosts in which they usually cause systemic or invasive infections. Thus, the host-adapted salmonella serotypes Abortusovis, Typhi and Typhisuis are confined, respectively, to sheep, man and swine. Another two host-adapted serotypes, Pullorum and Gallinarum, are highly specific for avian hosts in which they cause quite distinct diseases.

The first of these, pullorum disease, occurs in chicks and poults in the first 7–10 days after hatching. The incidence of the disease and the resultant mortality are high, with serotype Pullorum organisms being isolated from the blood and internal organs of diseased young birds. Only sporadic deaths occur in adult birds in which active infection is uncommon. Female survivors act as reservoirs of infection and become carriers of Pullorum so that disease is commonly spread by transovarian transmission of infection to eggs and resultant chicks.^{1,2}

The second important avian salmonellosis is fowl typhoid, an acute septicaemic disease caused by salmonellae of serotype Gallinarum. Unlike pullorum disease, fowl typhoid is seldom present in the first days after hatching; it occurs after the first week and persists throughout life so that both young

and adult birds may succumb to this infection, mortality from which may be as high as 100% but is breed dependent.^{1,3,4}

Both Gallinarum and Pullorum are described by the same antigenic formula (1,9,12:—:—); hence, they cannot be distinguished serologically. Most strains, however, are readily differentiated by biochemical tests such as ornithine decarboxylase.^{5,6} Current practice denies status to both these serotype names; accordingly, in the latest edition of the Kauffmann-White scheme, serotype Gallinarum, having priority, is maintained whereas Pullorum, because it is considered to be a biotype variant of an established serotype, is suppressed.⁷ Nevertheless, both serotype names will be used in this paper to circumvent that nomenclatural difficulty.

Strains of Gallinarum and Pullorum are unusual among salmonellae in being non-motile and non-flagellate. Their permanent non-flagellate status is thought to result from deletion and multiple mutations in the structural and other flagellar genes.⁸ That there are no reports of spontaneously motile or flagellate strains of these serotypes is in keeping with that hypothesis.

Gallinarum and Pullorum strains are also unusual with regard to the production of fimbriae. Thus, whereas the majority of naturally occurring strains of most serotypes of *Salmonella* form type-1 fimbriae which bestow on the bacteria diverse adhesive properties,^{9–11} strains of Gallinarum and Pullorum form type-2 fimbriae which, whilst resembling type-

1 fimbriae morphologically, lack their adhesiveness.⁹ Recent experiments showed that the DNA of type-2 fimbriate strains of Gallinarum and Pullorum hybridised strongly with Typhimurium gene probes of type-1 fimbriation,¹² suggesting that type-2 fimbriae are non-adhesive forms of type-1 fimbriae. In this paper we report our findings from studies in which restriction-endonuclease digests of chromosomal DNA of strains of Gallinarum and Pullorum were examined by Southern hybridisation with additional gene probes and discuss our observations with regard to the evolution of these two fowl-adapted *Salmonella* serotypes.

Materials and methods

Bacteria

The 86 type-2 fimbriate strains of *Salmonella* of serotypes Gallinarum and Pullorum examined had been isolated between 1921 and 1987 and included isolates from Africa, Australia, Europe and the USA; they have been described elsewhere.^{9,11,12} We confirmed that all strains belonged to serogroup D and were non-flagellate as judged by their reactions in slide-agglutination tests with somatic O9 and flagellar polyvalent H (phases 1 and 2) and non-specific H (factors 1, 2, 5, 6, 7) antisera from Wellcome Diagnostics (Dartford). The serotype designations used in this paper are those supplied by the donors. Thus, there were 50 strains of Gallinarum and 36 strains of Pullorum; the latter strains had also been phase-typed.

Control strains used in DNA-hybridisation experiments included the type-1 fimbriate strains of serotype Typhimurium (naturally occurring strain S615 and the laboratory-derived strain LT2) and *Escherichia coli* strain HB101¹³ which does not form type-1 fimbriae.

Strains were stored on Dorset's egg slopes at ambient temperature (c. 18°C) until subcultured for testing on plates of Columbia Blood Agar Base (Oxoid) containing defibrinated horse blood (Difco) 5% w/v.

Media

L broth without glucose¹⁴ was used for the culture of salmonellae for preparation of DNA (*vide infra*).

Sugar-peptone water medium was Peptone Water (Oxoid), prepared according to the manufacturer's instructions, containing carbohydrate (dulcitol, D-glucose, maltose, L-rhamnose or D-xylose) 0.5% w/v and bromocresol purple indicator 0.002% w/v; it was dispensed in 4-ml amounts in screw-capped bottles (6 ml) and steamed at 100°C for 30 min.¹⁵ Production of gas from glucose fermentation was detected in an inverted Durham tube.

Decarboxylase basal medium and L-ornithine decarboxylase medium were prepared and dispensed as described before.¹⁵

Biochemical tests

A single colony from a pure culture of the *Salmonella*

strain grown on blood agar was suspended in physiological saline (NaCl, 8.5 g/L) to a density of c. 5×10^8 cfu/ml. After inoculation of sugar-peptone water and decarboxylase media with c. 0.2 ml of bacterial suspension, bottle caps were screwed firmly. Tests were incubated at 37°C and examined daily for up to 7 days.

Extraction of bacterial and plasmid DNA

For the preparation of chromosomal DNA, strains of serotypes Gallinarum, Pullorum and Typhimurium were grown in L broth (5 ml) in 25-ml bottles shaken overnight at 37°C at 100 rpm. Thereafter, extraction of whole-cell DNA from these cultures was by previously published methods.¹²

Plasmid DNA from *E. coli* strain HB101 transformed with pISF141,¹⁶ a subclone of pISF101 (fig. 1)¹³ containing the type-1 fimbrial subunit gene of Typhimurium strain LT2, was purified from cells amplified with chloramphenicol 200 µg/ml, as described by Crichton *et al.*¹²

Preparation of gene probes

For preparation of the Typhimurium type-1 fimbrial subunit gene probe (P4), which differs in specificity from probe P1 used in an earlier study¹² in that only the sequence encoding the structural protein is included, purified pISF141 DNA was digested to completion with the restriction enzymes *Bst*EII and *Hind*III according to the instructions of the manufacturer (Gibco BRL). After separation of the digestion products by electrophoresis through Ultrapure Low Melting Point Agarose (Gibco BRL) 0.5% w/v in Tris-borate-EDTA (TBE) buffer (pH 8.0) at 4°C, the appropriate 900-base fragment was cut out along with a minimal amount of gel and the DNA was stored in 25-ng amounts at -20°C. This subunit probe (designated P4) and the *Hpa*I-*Eco*RI accessory-gene probe P3, available from a previous study,¹² were labelled directly with deoxycytidine 5'-[α-³²P] triphosphate by the Multiprime system (Amersham International).

Southern hybridisations

Digestion of 2-µg amounts of cellular DNA from representative isolates of Gallinarum and Pullorum was performed in 20-µl volumes with restriction endonuclease *Hinf*I; digestion fragments of different sizes were separated by electrophoresis at ambient temperature in Agarose (type 1 low EEO, Sigma) 0.7% w/v gels containing ethidium bromide 0.5 µg/ml in TBE buffer. After electrophoresis, gels were photographed over a mid-range (302 nm) UV transilluminator; DNA was denatured in 1.5 M NaCl containing 0.5 M NaOH for 30 min and transferred by capillary blotting to Hybond-N membranes (Amersham) with alkali buffer comprising 0.2 M NaOH and 1.5 M NaCl.

The interaction of these separated chromosomal fragments with the two gene probes P4 and P3, used in

turn, was assessed by hybridisations at 65–67°C following previously published techniques,¹⁷ described in detail elsewhere.¹²

After high-stringency washes at 65–67°C,¹² the remaining bound probe was visualised by exposing the membrane to X-ray film for 2–4 days at –70°C, followed by its development in a Kodak X-OMAT Processor.

Probe 4 was then removed from the membrane by incubation at 45°C, first in 0.4 M NaOH for 30 min and then in a solution of 0.1 × SSC (20 × SSC contains 3 M NaCl and 0.3 M sodium citrate), sodium dodecyl sulphate 0.1% w/v and 0.2 M Tris (pH 7.5) for 15 min. Blots were autoradiographed to ensure complete removal of probe 4 before hybridising with probe 3.

Results

Biochemical tests

When isolates of *Salmonella* of serotypes Gallinarum and Pullorum were examined for their ability to metabolise the substrates detailed above, they gave the results shown in the table. Most, but not all, of them were readily distinguished as strains of Gallinarum or Pullorum on the basis of their reactions in tests with dulcitol, maltose and ornithine (table), substrates generally considered helpful for their differentiation.^{5,6} Indeed, 81 (94%) of the 86 isolates examined were readily assigned to one of three distinct biochemical groups (I–III, table).

Thus, among the 50 cultures received as Gallinarum, 49 gave identical results in the five biochemical tests used for their characterisation, that is, they were anaerogenic, fermented dulcitol and maltose, but not rhamnose, and did not decarboxy-

late ornithine (group I, table). Despite their behaviour as a biochemically homogeneous group, these 49 isolates originated from such geographically diverse countries as Greece, Saudi Arabia, Tanzania, the UK and the USA. Unlike these isolates, one Gallinarum culture (strain 1109) gave results which might be considered aberrant for this serotype^{5,6} in two of the five biochemical tests—it did not ferment dulcitol and decarboxylated ornithine (table). However, that strain (formerly NCTC 1109) was discarded from the National Collection of Type Cultures in 1958 (Dr L. R. Hill, personal communication).

The 36 Pullorum isolates, most of which came from different locations within the UK, were biochemically more diverse than the Gallinarum strains. Nevertheless, amongst them a further two biochemically homogeneous groups (II and III, table) were recognised on the basis of gas production, and fermentation of rhamnose and xylose. Group II comprised 15 Pullorum isolates that were aerogenic, decarboxylated ornithine and fermented rhamnose and xylose, but not dulcitol or maltose (table). Group III contained 17 Pullorum isolates which were anaerogenic and decarboxylated ornithine but did not ferment any of the four carbohydrates tested (table).

Another four strains, received as Pullorum, possessed properties that did not strictly conform to the biochemical profiles of either of the Pullorum groups II or III. Strain Sal663 was anaerogenic and strain NCTC 5776 was ornithine decarboxylase-negative—results obtained, respectively, with 10% and 5% of Pullorum strains;⁵ thus, each differed

Table. Biochemical properties of strains of Gallinarum and Pullorum and their reactions with type-1 fimbrial-gene probes

Serotype	Bio-chemical group	Number of strains (and, if 1, strain no.)	Gas production	Fermentation of				Decarboxylation of ornithine	Reaction with gene probe	
				dulcitol	maltose	rhamnose	xylose		P3	P4
Gallinarum	I	49	—	+	+	—	...	—	+	+
		1 (1109)	—	—	+	—	...	+	+	+
Pullorum	II	15	+	—	—	+	+	+	+	+
		1 (Sal663)	—	—	—	+	+	+	+	+
		1 (NCTC 5776)	+	—	—	+	+	—	+	+
	III	17	—	—	—	—	—	+	+	W
		1 (SL303)	—	+	—	+	+	—	+	+
		1 (8044)	+	+	+	—	—	+	+	+

W, weak reaction (see text), ..., not tested.

P3, probe of accessory genes of type-1 fimbriation in Typhimurium LT2.¹²

P4, probe of type-1 fimbrial subunit gene of Typhimurium LT2.

from Pullorum strains of group II in only one of the six biochemical properties tested (table).

The biochemical properties of the remaining two Pullorum strains were aberrant in several characters. Strain SL303, which came originally from the series examined by Blaxland *et al.*,¹⁸ was more like a Gallinarum strain because it was anaerogenic, fermented dulcitol and did not decarboxylate ornithine; its maltose and rhamnose characters, however, were not those expected of a Gallinarum strain (table). Strain 8044 fermented dulcitol and maltose, like Gallinarum strains, but, like Pullorum strains of group II, it was aerogenic and decarboxylated ornithine (table). This latter strain (formerly NCTC 8044) was discarded from the National Collection of Type Cultures in 1958 (Dr L. R. Hill, personal communication).

Southern hybridisations

Under stringent conditions, a probe (P3) of accessory genes of type-1 fimbriation in *Salmonella* of serotype Typhimurium (fig. 1) hybridised with *Hinf*I-digested total DNA of each of 26 type-2 fimbriated isolates—14 chosen as representative of the 49 isolates of Gallinarum group I, and six each of the 15 Pullorum group-II and the 17 Pullorum group-III isolates. Each of these isolates, as well as five biochemically atypical strains (table), gave an identical pattern of hybridised fragments that was different from the patterns obtained with the two

(control) type-1 fimbriated strains of Typhimurium examined (fig. 2) and with strains of other serotypes (data not shown). This restriction fragment-length polymorphism indicates that there is a difference between these adhesive and non-adhesive salmonellae in the nucleotide-base sequence in that region of the gene cluster coding for accessory proteins. Probe P3 did not hybridise with the DNA prepared from *E. coli* strain HB101 (fig. 2).

The type-1 fimbrial-subunit probe (P4) also hybridised with the DNA from all of the 26 biochemically typical cultures of Gallinarum and Pullorum examined. On the basis of results obtained, isolates were assigned to two types, each with a characteristic, readily recognisable band pattern: (i) a pattern characteristic of Typhimurium strains and of isolates of Gallinarum and Pullorum of groups I and II; (ii) a pattern characteristic of the anaerogenic Pullorum isolates of group III (fig. 3). It was not surprising to find that strains Sal663 and NCTC 5776, which differed from Pullorum group-II isolates in only one biochemical character (table), also showed the strong hybridisation pattern seen with the six isolates typical of that group.

The hybridisation pattern typical of group-I and -II isolates was found unexpectedly in strain 1109 (table), indicating that this strain was unlikely to belong among isolates of group III which it resembled in many biochemical characteristics. Furthermore, the biochemically aberrant strains SL303 and 8044 (table) also gave a strong reaction

Fig. 1. Physical map of the recombinant plasmid pISF101 encoding type-1 fimbriation in Typhimurium LT2 and its deletion derivative pISF141. The approximate locations of the fimbria-associated genes¹⁶ are indicated by hatched boxes. The sizes of the probes P3 and P4 of accessory and subunit genes, respectively, are shown by the solid lines.

Figs 2 and 3. Autoradiographs of digested chromosomal DNA from Gallinarum and Pullorum isolates and (control) strains of Typhimurium and *E. coli* after hybridisation with the ^{32}P -labelled probes, P3, of Typhimurium type-1 fimbrial accessory genes, and P4, of the Typhimurium type-1 fimbrial subunit gene. Track 1, representative Gallinarum strain of biochemical group I; tracks 2 and 13, Typhimurium strains LT2 and S615; tracks 3-7, strains 1109, Sa1663, NCTC 5776, SL303 and 8044; track 8, standard mol.-wt markers; track 9, representative Pullorum strain of group II; tracks 10 and 12, representative Pullorum strains of group III; track 11, *E. coli* strain HB101. Arrows (fig. 3) indicate the positions of the very weak hybridisation bands of *E. coli* strain HB101.

with probe 4 and, therefore, were likely to belong to groups I or II rather than to group III. It should be noted, however, that these three problematic strains of *Salmonella* of serogroup D formed non-adhesive fimbriae, were non-motile and gave hybridisation patterns suggesting that they were strains of serotypes Gallinarum or Pullorum.

Probe P4 hybridised very weakly with the DNA of *E. coli* strain HB101 to give a barely visible pattern that was quite different from that of Typhimurium, Gallinarum or Pullorum strains (fig. 3). This result, perhaps not surprising in view of the low immunological cross-reactivity between fimbriae of *E. coli* and Typhimurium strain LT2,¹⁹ may reflect the presence of DNA sequences encoding the homologous amino-acid sequences of the type-1 fimbrial proteins previously described in these two organisms.²⁰

Discussion

The apparent O-antigenic identity of Gallinarum and Pullorum strains has proved a major difficulty for those investigators who, by other criteria, might have wished to regard them as distinct salmonellae.

However, in addition to the distinct pathology that they cause in avian hosts, Gallinarum and Pullorum strains have also been differentiated by growth characteristics,^{2,3,18} biochemical reactions, particularly with dulcitol, maltose and ornithine,^{5,6} and the virtual and significant absence of O-antigen form variation amongst Gallinarum strains.¹⁸ In contrast with the antigenic homogeneity of Gallinarum strains isolated worldwide, Pullorum strains have been subdivided not only on the basis of their variable production of O antigen-12 subfactors¹⁸ but also on biochemical activity.^{18,21} In this study, we have confirmed the existence of different biochemical types of Pullorum and have shown that anaerogenic Pullorum strains of group III failed to ferment rhamnose or xylose after incubation for 7 days. They were also commonly of phage types (such as 2 and 3) not found amongst aerogenic Pullorum strains (data not shown). The restricted distribution of phage types amongst anaerogenic Pullorum strains is in agreement with earlier observations²¹ which indicated that Lilleengen phage types 3–6 contained only aerogenic Pullorum strains; anaerogenic Pullorum strains belonged, along with most of the Gallinarum strains, to Lilleengen phage type 2.²¹ Again, although all 17 of our anaerogenic Pullorum strains from group III originated in the UK, that particular Pullorum subgroup is probably more widely distributed. Although the majority of anaerogenic Pullorum

strains from the international collection studied by Lilleengen²¹ also came from the UK, others were reported from South Africa, Denmark and Finland but not, surprisingly, from Sweden.

Early work showed that type-1 fimbriation is a common feature of Enterobacteriaceae, including *Salmonella*.⁹ However, although electronmicroscopy revealed the presence of fimbriae on all of 14 isolates of Gallinarum, they were detected on only 11 of 30 Pullorum strains; whether or not they were fimbriate, Gallinarum and Pullorum strains were always non-haemagglutinating and non-adhesive.⁹ Our recent immuno-electronmicroscopy and haem-agglutination studies have confirmed that all Gallinarum isolates have non-adhesive (type-2) fimbriae antigenically similar to type-1 fimbriae; furthermore, immune-gold labelling of bacteria from representative cultures showed that the non-adhesive fimbriae lack the adhesin present in type-1 fimbriae.¹² Southern hybridisations with a probe of type-1 fimbrial accessory genes show that *HinfI*-digested DNA of both Gallinarum and Pullorum give a hybridisation pattern that is quite distinct from that of Typhimurium (fig. 2) and other *Salmonella* serotypes tested. Genetic studies are in hand to define more accurately the site of the fimbrial lesion(s) in Gallinarum and Pullorum.

By the use of improved techniques for the preparation of specimens for electronmicroscopy, we also detected fimbriae, albeit in small numbers, in cultures of all 36 Pullorum isolates¹² including some previously thought, from tube-agglutination and the then available electronmicroscopy methods, to lack fimbriae.^{9,22} It was noteworthy that all of the sparsely fimbriate Pullorum strains belonged to anaerogenic group III.

Previous dot-blot hybridisations of cellular DNA with a probe encompassing the type-1 fimbrial subunit gene of Typhimurium also suggested that Pullorum strains might be divisible into two distinct groups.¹² In the present study, Southern hybridisations of *HinfI*-digested DNA with a specific subunit probe, together with the results of biochemical tests, have confirmed that idea: thus, there are strongly and weakly probe-reactive groups (II and III, respectively) which show restriction fragment-length polymorphism (fig. 3). Although comparative sequence studies have not been performed, the sizes of hybridised *EcoRI* fragments in the two groups (data not shown) indicate that a major deletion may have occurred in the subunit gene of group-III isolates. As was seen in the P fimbrial subunit gene,^{23,24} such a deletion may destroy the correct spacing between regions of the fimbrial protein involved in biogenesis and thereby influence

the number of fimbriae without altering their morphology; that explanation would go some way towards understanding the difficulties of earlier workers in detecting fimbriae by electronmicroscopy in some Pullorum isolates of our group III.

The detailed virulence mechanisms contributing to the pathogenicity of salmonellae remain unknown, although many factors including motility and adhesiveness have been proposed.²⁵⁻²⁷ It is of interest, therefore, that Gallinarum and Pullorum strains are not only non-motile but, like the bird-associated Typhimurium strains of biotypes 29-32 of the FIRN clone,²⁸⁻³¹ lack adhesive fimbriae and yet have maintained an important role as pathogens of avian hosts.

Because Gallinarum and Pullorum are both described by the same antigenic formula, it is surprising to find so much evidence supporting their delineation as different types. The phenotypic traits suggesting that Gallinarum and Pullorum strains be considered separately are supported by data from multilocus enzyme-electrophoresis analysis, a technique that measures evolutionary distance between pairs of strains.³² Mathematical interpretation of the data on 74 isolates of 29 different serotypes of *Salmonella* confirmed their genetic relatedness³³ although, as observed with

DNA hybridisation,³⁴ subspecies V strains showed least relatedness to other subspecies. The single isolates of Gallinarum and Pullorum examined belonged to readily distinguishable electrophoretic types; furthermore, Gallinarum apparently clustered more closely with Hadar, another avian-associated serotype, than with Pullorum.³³

It is possible that ancestral strains of distinct genetic origin have evolved by host adaptation and convergent evolution in antigenic structure into the strains that we currently recognise as Gallinarum and Pullorum. Information obtained from multilocus enzyme electrophoresis should help greatly in elucidating the evolutionary history of Gallinarum and Pullorum strains which, despite their serological similarity, can be differentiated by so many other characteristics. It should also help to establish the true relationships amongst the three biochemical groups reported in this study and clarify whether the biochemically aberrant strains are indeed members of the Gallinarum or Pullorum serotypes.

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