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# Outer Membrane Protein Patterns Mark Clones of *Escherichia coli* O2 and O78 Strains That Cause Avian Septicemia

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**Major outer membrane proteins were isolated from 36 *Escherichia coli* strains representing six common clones of the O2 and O78 serogroups implicated in avian colisepticemia. Clonal relationships among isolates were inferred from an analysis of polymorphism at 20 enzyme-encoding loci detected by multilocus enzyme electrophoresis. For isolates of these clones, there was a high concordance (>90%) between identity in multilocus genotype and major outer membrane protein patterns. The results indicate that major outer membrane protein patterns discriminate among the genetically different clonal groups that constitute the heterogeneous O2 and O78 serogroups associated with avian disease.**

Colisepticemia is one of the most common clinical manifestations of infection by *Escherichia coli* in domesticated birds (30, 31) and is a major source of economic loss in the poultry industry (14). Surveys of outbreaks of avian colisepticemia have established that the *E. coli* isolates readily cultured from the internal organs of diseased birds are remarkably uniform in serology, with strains of the O2 and O78 serogroups accounting for the majority of cases (14, 17–21, 30). In addition, O2 and O78 strains have been recovered more than twice as often (52% versus 21%) from birds with colisepticemia than from birds with other types of infections (31). As a consequence of these findings, representative O2 and O78 strains have been used extensively in experimental studies of infection (4, 7, 13, 15, 23) and in laboratory trials for vaccine development (9, 10, 16).

Despite the serological uniformity among isolates from avian colisepticemia, recent studies of polymorphisms in outer membrane proteins (OMPs), pili, and metabolic enzymes have revealed substantial genetic variation among isolates of the O2 and O78 serogroups (1, 25, 32, 34, 35). Achtman et al. (1) described two major clonal groups among 46 O2:K1 isolates originally collected from cases of avian septicemia, human urinary tract infection, and bovine mastitis based on variation in the electrophoretic OMP pattern and multilocus enzyme genotype. Picard et al. (25) distinguished four electrophoretic types (ETs) among 24 O78 isolates recovered from humans and domesticated animals. Whittam and Wilson (35) found that the 48 (61%) of 79 isolates from diseased chickens of either the O2 or O78 serogroup belonged to 14 ETs representing three major clone clusters. Remarkably, one of these clones (ET 19) had isolates of both the O2 and O78 serotypes (35). Although the phenomenon of serotypic heterogeneity among isolates of the same ET was described previously (8, 24, 27), it was the nature of the heterogeneity that was surprising—a single ET was composed of isolates expressing two of the major serotypes associated with avian disease. These observations indicate that the O2 and O78 somatic antigens are expressed by a variety of avian strains with diverse chromosomal backgrounds and suggest that transitions between different O antigens may occur frequently in nature.

Here we have assessed the variation in the major OMPs of O2 and O78 *E. coli* strains that have been repeatedly recovered from outbreaks of avian disease for the purpose of discriminating clones among isolates of the same serogroup. The study includes a total of 36 *E. coli* isolates (Table 1), collected originally from chickens and turkeys with acute respiratory disease (airsacculitis), colibacillosis, or septicemia. The isolates were selected to represent the most frequently recovered ETs of O2 and O78 strains that we have observed among a diverse collection of more than 450 isolates of avian origin. For comparative purposes, we included three strains of the common ETs with serotypes other than O2 or O78: one isolate (820970) of serotype O5 and two O-nontypeable strains (S84 and 820954). Isolates were characterized by enzyme mobility variants detected by multilocus enzyme electrophoresis (26) and classified into distinct ETs by the variation in 20 enzyme-encoding loci (34, 35).

Among the 36 isolates, 8 of the 20 enzyme loci were polymorphic, and comparisons of the multilocus enzyme profiles resolved the strains into six ETs (Table 2), representing commonly isolated O2 and O78 clones from avian colisepticemia. Four of these clones, identified by distinct ETs, have been described previously (34, 35). The 10 isolates of ET 1 (Table 1) belong to a common clone (referred to as ET 2 on the basis of analysis of 15 enzymes in reference 35) frequently recovered from birds with airsacculitis or pericarditis from the Delmarva (Delaware–Maryland–Virginia) peninsula. The four isolates of ET 2 were originally collected from birds with colisepticemia in Spain and are members of a clone marked by ET 17 of White et al. (34). Five of the 10 isolates of ET 4 and three of the four isolates of ET 5 are O2 strains previously characterized for electrophoretic variation of 15 enzymes and represent, for the most part, ETs 19 and 35 of reference 35. The remaining two clones, designated ETs 3 and 6, are newly defined and are each represented by avian isolates from both France and the United States.

**Isolation of OMPs.** OMPs were isolated by the method described by Deneer and Potter (11) with minor modification. Bacteria were grown overnight at 37°C in 100 ml of Luria broth, and cells were recovered by centrifugation (6,000 × *g* for 10 min at 4°C), suspended in 3 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Sig-

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TABLE 1. Properties of 36 representative isolates of the major O2 and O78 clones of avian *E. coli*

| ET[a] | Isolate no. | O serogroup[b] | OMP pattern | Locality | Source[c] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Year | Host | Origin |
| 1 | 820905 | O2 | 5 | USA (Del.) | 1982 | C | Air sac |
| | 820949 | O2 | 5 | USA (Del.) | 1982 | C | Urine |
| | 820950 | O2 | 5 | USA (Del.) | 1982 | C | Heart |
| | 830127 | O2 | 6 | USA (Del.) | 1983 | C | Sinus |
| | 830137 | O2 | 5 | USA (Del.) | 1983 | C | Heart |
| | 830153[d] | O2 | 5 | USA (Del.) | 1983 | C | Liver |
| | 830467 | O2 | 5 | USA (Del.) | 1983 | C | Air sac |
| | 830495 | O2 | 5 | USA (Del.) | 1983 | C | Air sac |
| | 830497 | O2 | 5 | USA (Del.) | 1983 | C | Sinus |
| | 830507 | O2 | 5 | USA (Del.) | 1983 | C | Heart |
| 2 | S56 | O2 | 3 | Spain | 1979 | C | Heart |
| | S70 | O78 | 3 | Spain | 1979 | C | Liver |
| | S72 | O78 | 3 | Spain | 1979 | C | Heart |
| | S84 | ON | 3 | Spain | 1979 | C | Liver |
| 3 | 820928 | O78 | 1 | USA (Del.) | 1982 | C | Air sac |
| | MT 458 | O78 | 1 | France | 1984 | T | Heart blood |
| | MT 515 | O78 | 1 | France | 1972 | C | Lung |
| | 820970 | O5 | 8 | USA (Del.) | 1982 | C | Liver |
| 4 | 820887 | O2 | 1 | USA (Del.) | 1982 | C | Skin |
| | 820889[e] | O2 | 1 | USA (Del.) | 1982 | C | Joint cavity |
| | 820917 | O2 | 1 | USA (Del.) | 1982 | C | Blood |
| | 820981 | O2 | 1 | USA (Del.) | 1982 | C | Heart |
| | 820983 | O2 | 1 | USA (Del.) | 1982 | C | Bone marrow |
| | 830121 | O78 | 2 | USA (Del.) | 1983 | C | Air sac |
| | 830148 | O78 | 1 | USA (Del.) | 1983 | C | Air sac |
| | 830158 | O78 | 1 | USA (Del.) | 1983 | C | Joint cavity |
| | T25 | O78 | 1 | USA (Minn.) | N.D. | T | N.D. |
| | T26 | O78 | 1 | USA (Minn.) | N.D. | T | N.D. |
| 5 | 820891[f] | O2 | 4 | USA (Del.) | 1982 | C | Heart |
| | 820931[g] | O2 | 4 | USA (Del.) | 1982 | C | Sinus |
| | 820964 | O2 | 4 | USA (Del.) | 1982 | C | Lung |
| | MT 78 | O2 | 4 | France | 1977 | C | Trachea |
| 6 | 820954 | ON | 4 | USA (Del.) | 1982 | C | Skin |
| | MT 181 | O2 | 4 | France | 1984 | T | Liver |
| | MT 512 | O2 | 4 | France | 1972 | C | Trachea |
| | MT 513 | O2 | 4 | France | 1972 | C | Salpinx |

[a] Electrophoretic type as defined in Table 2. ETs 1, 4, and 5 include isolates representing ETs 2, 19, and 35, respectively, as described previously (35).
[b] ON, O nontypeable.
[c] Abbreviations: C, chicken; T, turkey, N.D., no data.
[d] Electromorph for mannose-6-phosphate isomerase revised from reference 35 (see Table 2).
[e] Classified as ET 20 in reference 35 due to an electromorph difference for leucine aminopeptidase, an enzyme not assayed in the present study.
[f] Electromorph for glucose-6-phosphate dehydrogenase, revised from reference 35 (see Table 2).
[g] Electromorph for aspartate aminotransferase, revised from reference 35 (see Table 2).

ma Chemical Co., St. Louis, Mo.; 10 mM, pH 7.4), and disrupted by sonication (Braunsonic sonifier, 45 s at 50% output). Cell debris was removed by centrifugation at 6,000 × *g* for 10 min at 4°C. The supernatant was added to 0.75 ml of 2% *N*-lauroylsarcosine (Sarkosyl; Sigma Chemical Co.) and incubated for 10 min at room temperature. The mixture was centrifuged at 100,000 × *g* for 1 h (Beckman 70.1 Ti, 39,000 rpm) in order to recover the detergent-solubilized OMPs. The pelleted proteins were resuspended in 3 ml of 10 mM HEPES (pH 7.4), incubated with 1 volume of Sarkosyl at room temperature for 20 min, and recovered by ultracentrifugation as described above. The final pellet was resuspended in 1 ml of 10 mM HEPES and stored at −20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5) was carried out with a 4% stacking and a 9% separating gel after the OMP preparations were solubilized at 100°C for 7 min in 0.05 M Tris-HCl buffer (2.5% SDS, 5% 2-mercaptoethanol, 25% glycerol, and 0.003% bromophenol blue). Major protein bands were visualized with Coomassie brilliant blue R250 (Sigma Chemical Co.), and trace amounts of protein were detected with silver stain (Silver stain-Daiichi kit; Integrated Separation Systems, Hyde Park, Mass.).

**Concordance between ET and OMP pattern.** We resolved eight different electrophoretic banding patterns for the major OMPs among the 36 isolates with Coomassie blue staining. Silver staining revealed several additional minor bands that were monomorphic among isolates with the same major banding pattern. The major OMP patterns were arbitrarily numbered from 1 to 8 and are listed for each isolate in Table 1. In most cases, isolates of the same ET had identical OMP patterns; only three isolates, 830127 (ET 1), 820970 (ET 3),

TABLE 2. Alleles at seven polymorphic enzyme loci that define six ETs of avian *E. coli*

| ET[a] | No. of isolates | Allele at locus for enzyme[b]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PGI | ACO | PE2 | AK | PGD | M1P | MPI | SKD |
| 1 | 10 | 5 | 5 | 2 | 2 | 6 | 4 | 8 | 6 |
| 2 | 4 | 5 | 6 | 5 | 2 | 6 | 6 | 4 | 6 |
| 3 | 4 | 4 | 6 | 7 | 2 | 6 | 8 | 5 | 2 |
| 4 | 10 | 4 | 6 | 7 | 2 | 6 | 8 | 4 | 2 |
| 5 | 4 | 6 | 6 | 4 | 4 | 10 | 2 | 4 | 6 |
| 6 | 10 | 6 | 6 | 4 | 4 | 6 | 2 | 4 | 6 |

[a] ETs are based on distinct allele combinations among comparisons of 20 enzyme loci in the sample of 36 isolates. Only allele combinations for the eight enzymes that were polymorphic are listed here. Allele designations are not cognate with those in reference 35. All 36 isolates were monomorphic for isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, aspartate aminotransferase, glucose-6-phosphate dehydrogenase, indophenol oxidase, β-galactosidase, alcohol dehydrogenase, carbamate kinase, nucleotide phosphorylase, threonine dehydrogenase, and glutamate dehydrogenase.

[b] PGI, phosphoglucose isomerase; ACO, aconitase; PE2, peptidase; AK, adenylate kinase; PGD, gluconate-6-phosphate dehydrogenase; M1P, mannitol-1-phosphate dehydrogenase; MPI, mannose-6-phosphate isomerase; SKD, shikimate dehydrogenase.

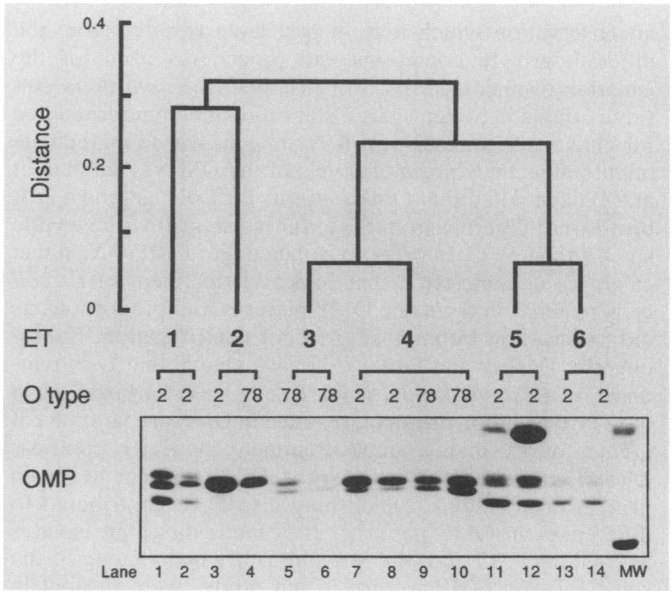


FIG. 1. Dendrogram, O serogroup, and major OMP patterns of 14 isolates representing six different ETs. Isolates are grouped by ET in lanes 1 to 14 as follows: ET 1, 830137 and 830467; ET 2, S56 and S72; ET 3, 820928 and MT 458; ET 4, 820917, 820887, 830148, and T25; ET 5, 820891 and MT 78; ET 6, MT 513 and MT 512. Lane MW contains protein size markers, with major bands at 45 kDa (top) and 29 kDa (bottom).

and 830121 (ET 4), were distinct in OMP patterns from the other isolates of the same ET. The overall association between clonal identity and OMP patterns was assessed by comparing all possible pairs of 36 isolates and cross-classifying each pair of isolates for whether the two were identical or different in ET and whether they matched or mismatched in OMP pattern (Table 3). Among the 630 pairwise comparisons, 15% of the comparisons of isolates of the same ET matched in their OMP patterns and 75% of the comparisons of isolates of different ETs had different major OMP patterns (i.e., mismatched). In contrast, only 7% of the pairwise comparisons of isolates with different ETs matched in OMP pattern, and only 3% of the comparisons of isolates with the same ET differed in OMP pattern. The overall percentage of concordant results was 94% (simple matching coefficient, $S = 0.938$) and was highly significant ($G$ test of independence, $G = 252.3$, $df = 1$, $P < 0.001$), indicating a strong correlation across these avian isolates between identity of ET and similarity in OMP pattern.

The observation that, in two cases, isolates of different ETs had the same OMP patterns (ET 3 and ET 4 had OMP-1, and ET 5 and ET 6 had OMP-4; Table 1) reflects the overall close genetic relatedness of these pairs of ETs. Figure 1 summarizes the genetic relationships among ETs and the variation in OMP patterns. Isolates of ET 3 and ET 4 (lanes 5 to 10) are closely related (distance = 0.05), differing at only a single allele out of 20 enzyme loci (Table 2), and thus the similarity in major OMPs presumably results from recent descent from a common ancestral strain. The isolates of ET 5 and ET 6 are also closely related, clustering at a distance of 0.05, and express the same major OMP pattern (OMP-4 in lanes 11 to 14 of Fig. 1); however, the ET 5 isolates expressed a high-molecular-mass protein (~45 kDa in size) that was not observed among the ET 6 isolates. Although not previously classified as a difference in the major OMPs (2), the variation in the expression of this protein reflects some divergence between the genomes of these two cell lineages. Thus, with only minor exceptions, distinct OMP patterns are highly correlated with the genetic relatedness of isolates, as indicated by similarity in ET, among the major avian clones.

TABLE 3. Concordance of ET and OMP pattern from 36 isolates of six common O2 and O78 clones

| ET | OMP pattern | |
|---|---|---|
| | Match | Mismatch |
| Same | 93 (0.15) | 21 (0.03) |
| Different | 43 (0.07) | 473 (0.75) |
| Concordance[a] | 93.8 | |

[a] Concordance equals the sum of the same ET-match and different ET-mismatch entries, expressed as a percentage of the total of 630 pairwise comparisons of isolates.

In contrast to the OMP patterns, the serotypic classification of strains does not accurately reflect the overall genetic relatedness of isolates. We were unable to identify any differences in OMP profiles between O2 and O78 strains of the same ET (Fig. 1, lanes 3, 4, and 7 to 10). We also failed to find any similarities in major OMP patterns between isolates of the same serogroup but with ETs that differed at a distance of 0.10 or greater. Because evolutionary convergence to the same multilocus enzyme genotype is highly improbable (28) and many O serotypes occur in a diversity of strains (1, 8, 24, 25, 27, 35), it seems likely that the antigenic properties of the lipopolysaccharide that determine the O2 and O78 serotypes have converged in distantly related strains, presumably through mutation and selection during the disease process. However, it is also possible that the genes specifying the serotypic properties of the O2 and O78 phenotypes have spread horizontally through the population of *E. coli*. In either case, our results suggest that selection has favored the increase in frequency and geographic spread of several genetically distinct clones that express O2 or O78 antigen and are associated with avian disease.

The OMP patterns of strains MT 515, 78, 512, 513, 181, and 458 differ from those reported by Dho-Moulin et al. (12),

an observation which may in part have resulted from the different growth conditions and procedures used for the isolation of major OMPs. For instance, these workers cultured strains in Minca agar, a starvation medium formulated for enhanced expression of K99 antigen, whose ingredients might influence expression levels of the OMPs. Achtman et al. (2) have noted that OMP patterns for isolates grown in L broth were different from those for isolates grown in tryptic soy broth at 37°C. In order to isolate major OMPs, Achtman et al. (2) demonstrated that Sarkosyl treatment of *E. coli* cells resulted in the same OMP patterns and proportions as did purification by sucrose gradient centrifugation. Subsequently, Deneer and Potter (11) have shown that two treatments of *Pasteurella multocida* lysates with Sarkosyl, as in our OMP isolation protocol, resulted in OMP preparations of greater purity than a single treatment alone. In contrast, Dho-Moulin et al. (12) used Triton X-100 in order to obtain protein preparations, which may also have contributed to differences in OMP patterns. It is interesting that isolates MT 458 and 515 and MT 512 and 513, which belong to the same ET and OMP pattern in our study, were distinct in OMP profiles in the study of Dho-Moulin et al. (12).

Although the major OMP patterns are specific for the O2 and O78 clones implicated in avian colisepticemia, the properties of the major OMPs may be irrelevant to the disease process (3). The OMPs observed here consist of a variable number of porins with molecular masses of ~40 kDa, a relatively invariable protein K between 35 and 40 kDa in size, a slightly faster-migrating protein (OmpA), and a fast-migrating plasmid-coded protein (PCP) (2). One of the major OMPs, encoded by *ompA*, has been shown to contribute to the virulence of *E. coli* with K-1 capsule in chicken embryos by a mechanism that might involve increased resistance to serum (33). The contribution of variation among the other OMPs to differences in virulence and pathogenesis of avian colisepticemia is unknown.

The correlation between OMP patterns and clonal identity of O2 and O78 strains can be exploited for the rapid identification of pathogenic clones and in the development of control measures against outbreaks of colisepticemia in the poultry industry. In conjunction with O serotyping, characterizing the OMP patterns of isolates through comparison with reference strains provides a reliable method for discriminating among the major O2 and O78 clones at a fraction of the effort of multilocus enzyme electrophoresis. The clone-specific OMP patterns may help in the selection of specific strains for developing vaccines that protect against the major O2 and O78 clones associated with avian diseases. One intriguing possibility is that any isolate from ET 4, which includes both O2 and O78 strains, may afford cross-protection against all O2 and O78 strains of this clone. Bolin and Jensen (6) have demonstrated that antibodies to iron-regulated OMPs can passively immunize against and protect turkeys from *E. coli* septicemia. In addition, the cross-protective attributes of *P. multocida* serotypes, when grown in vivo, have been ascribed to OMPs (29). And, in chickens, cell lysates of ultrasonicated O2:K1 strains have protected against infection by O78:K80 strains (22). These findings indicate that common antigens can protect chickens against infection by strains of the two major serotypes recovered from poultry. The development of successful vaccines against avian colisepticemia will be aided by the ability to identify and target the common pathogenic clones of the O2 and O78 serogroups.

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