Serum Resistance and Aerobactin Iron Uptake in Avian *Escherichia coli* Mediated by Conjugative 100-Megadalton Plasmid

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**ABSTRACT.** A total of 115 strains of *Escherichia coli* isolated from chickens with colisepticemia in Japan were examined for chicken lethality and virulence factors. It was found that serum resistance and aerobactin-mediated iron uptake are the most prevalent characteristics in these strains. Among them, S-20, a representative virulent strain of serotype O2, was further studied. S-20 harbored a conjugative 100-megadalton (Mdal) plasmid, designated pKI100. Curing and reintroduction experiments showed that pKI100 encodes both serum resistance and aerobactin-mediated iron uptake, and the diminished virulence of the pKI100-cured strain was fully restored by the reintroduction of the plasmid. These results demonstrated that pKI100 is the virulence plasmid of the S-20 strain, and that serum resistance and aerobactin-mediated iron uptake are the virulence factors in *E. coli* strains which cause avian colibacillosis.—**KEY WORDS:** aerobactin, avian *E. coli*, iron uptake, serum resistance, virulence plasmid.


Colibacillosis, *Escherichia coli* infection, in chicken is manifested in several forms of septicemia, granuloma, hemorrhagic enteritis, panophthalmitis, pericarditis, and salpingitis, and this disease is responsible for the major economic losses incurred by the poultry industry [7, 15]. Frequently isolated O serotypes of avian *E. coli* are O1, O2, and O78, which are different from O serotypes of *E. coli* isolated from other animals [7, 15]. We have previously reported that serotypes of *E. coli* strains isolated from septicemic chickens in Japan are mostly O2 and O78, and they express type 1 and/or Fmsha pili [19]. It has been reported that serum resistance [13], iron-sequestering [11, 22], chick lethal toxin [36, 37], congo red binding activity [3, 14], adhesion to epithelial cells by type 1 (-like) pili [2, 16, 17, 30] or mannose resistance adhesin [11, 17], and autoagglutination [33] can be the virulence factors in *E. coli* causing chicken colibacillosis. These facts indicate that the virulence factor in avian *E. coli* is multifactorial.

The purposes of the present study were to investigate firstly what kinds of virulence factors are expressed in *E. coli* recently isolated in Japan from septicemic chickens, and secondly which virulence factors are in fact involved in chicken lethality. The present study demonstrated that serum resistance and aerobactin-mediated iron uptake, which were frequently found in the strains, were encoded by the conjugative plasmid.

**MATERIALS AND METHODS**

**Bacterial strains:** One hundred and fifteen strains of *E. coli* isolated from septicemic chickens [19] were used in this study. *E. coli* LG1315 harboring plasmid ColV-K30 has two components, the hydroxamate siderophore aerobactin and an inducible outer membrane protein which is the receptor for ferric-aerobactin ([iuc", iut"] [42, 43], and *E. coli* LG1522 is defective in the synthesis and secretion of aerobactin ([iuc, iut"] [6]). LG1315 and LG1522 were provided by Dr. H. W. Williams. *E. coli* N3406 harboring plasmid pJN73 produces cloacin DF13 bacteriocin [38], and was provided by Dr. F. K. de Graaff. *E. coli* K1-2 derivative, Row, is a colicin susceptible strain [23]. Nalidixic acid resistant (*naf") *E. coli* CR34 strain carrying pTH10 [18] was used as a donor strain for insertion mutagenesis using an ampicillin resistant (*amp") transposon Tn1. *E. coli* C600 and HB101 *naf" are our laboratory strains.

**Sera, media, and chemicals:** Chicken serum was obtained from at least 10 healthy 4-week-old SPF chickens (Line M, Nippon Institute for Biological Science, Japan) and allowed to clot at 37°C for 1 hr and then at 4°C for 1 hr. Serum was separated by centrifugation, pooled, and frozen at −80°C until used. Anti-O serotypes, and anti-type 1 and -Fmsha pili sera were prepared as reported previously [19].
Trypticase soy broth and Trypticase soy agar (BBL Microbiol., systems, U.S.A.), Heart infusion agar, Nutrient agar, and Pennassay broth (Difco Laboratories, U.S.A.), and M9 minimal agar [31] were used for the cultivation of bacteria. Dulbecco’s PBS + containing Ca2+ and Mg2+ was used for phosphate-buffered saline (PBS). 2,2’-dipyridyl and congo red dyc (Wako Pure Chemicals Industries, Japan) were purchased commercially.

Virulence test: Virulence of the wild-type E. coli strains was classified into three classes according to the criteria of Dho and Lafont [11] by injecting ca. 1 × 10⁸ CFU cells from the overnight culture in Trypticase soy broth intraperitoneally into 1-day-old chicks. Five chicks were used for each strain, and observed for 7 days postinoculation. To estimate the LD₅₀ value, the exponentially growing cultures were suspended in saline and introduced intratracheally into 4-week-old Line M chickens.

Aerobactin-mediated iron uptake: Synthesis and secretion of aerobactin was examined by the crossfeeding test of Williams and Warner[43]. Approximately 10⁶ cells of LG1522 strain were seeded in M9 minimal agar containing 200 μM 2,2’-dipyridyl, and cross-feed with wild-type E. coli. The strains that produce and secrete aerobactin (iuc+) are able to support the growth of LG1522, making a halo around the inoculum after incubation overnight at 37°C. Ferric aerobactin transport was assayed as follows. Approximately 10⁵ cells of wild-type E. coli were seeded in M9 minimal agar containing 200 μM 2,2’-dipyridyl. The agar plate was spotted with 20 μl of a solution of colacin DF13 prepared from N3406 strain [38], and incubated overnight at 37°C. Strains that are active in ferric aerobactin transport across the cell membrane (iut+) are susceptible to colacin DF13 bactericidal action. LG1315 (iuc+, iut+) and C600 were used as strains susceptible and resistant to colacin DF13 bactericidal action, respectively.

Serum resistance: Serum resistance of 115 strains of wild-type E. coli was assayed by the method of Taylor [35]. The bacterial cultures grown at 37°C for 24 hr were added to the original volume of pre-warmed Trypticase soy broth, and further incubated for 2 hr. The cells (approximately 2.5 × 10⁹ CFU) were resuspended in 25 μl gelatin-veronal buffer containing 0.15 mM CaCl₂ and 0.5 mM MgCl₂ (pH 7.4), and mixed with 225 μl of chicken serum (final concentration; 90%). After incubation for 3 hr, the number of viable bacteria was determined on Trypticase soy agar.

Serum resistance for serotype O2 and laboratory E. coli strains with or without 100-megadalton (Mdal) plasmid was determined by the method of Moll et al. [27]. Overnight bacterial cultures were diluted 100 times in Pennassay broth, and incubated at 37°C for 3 hr. Cells harvested by centrifugation were washed and resuspended in PBS at a final concentration of 2×10⁹ CFU/ml. The 0.5 ml bacterial suspension was mixed with 2.0 ml PBS containing appropriately diluted chicken serum. After the mixture was incubated for 90 min, the number of viable bacteria was determined on Pennassay broth agar.

Other virulence factors: Hemolysis was determined by streaking of wild-type E. coli onto Heart infusion agar containing 5% (v/v) defibrinated sheep and horse blood. Congo red binding was measured according to the method of Ishiguro et al. [20]. For the colicin production assay, bacteria grown on nutrient agar were killed with chloroform, overlaid with nutrient soft agar containing E. coli K-12 Row strain [23], and incubated overnight at 37°C. A clear zone was observed for colicinogenic strains. Production of enterotoxin was assayed by the sucking mouse test [9] for heat-stable enterotoxin, and by passive reversed latex agglutination test for heat-labile enterotoxin with a commercial kit (VET-RPLA, Denka Seiken, Japan).

Analysis of plasmids: Plasmids were isolated by the method of Kado and Liu [21], and analyzed with using 0.8% agarose gel (Seakem GTG, FMC Bioproducts, U.S.A.). Electrophoresis was carried out with TBE buffer containing 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0) with a constant voltage of 100 V.

Transfer experiment of plasmid: The donor and the recipient cells in the exponential phase were mixed, centrifuged, and placed on Pennassay agar plate and kept at 30°C for 4 hr. The cells were picked up, suspended with Pennassay broth, and spread on selective agar plate containing suitable antibiotics. The colony on these plates was used as the transconjugant.

Tagging of plasmid with Tn1: The method for tagging plasmid with Tn1 was the same as reported previously [8]. The pTH10 plasmid in E. coli CR34 nal+ was transferred to a rifampicin resistant (rif+) strain of avian E. coli by conjugation at 30°C. After transconjugants were kept in Pennassay broth at 30°C for 7 days, they were spread on Pennassay broth agar containing amp, and incubated at 42°C to
select the amp<sup>+</sup> E. coli strains.

RESULTS

Chicken lethality of wild-type E. coli strains: We have previously reported O and pilus types of E. coli strains isolated from chickens with colisepticaemia [19]. In this study, the randomly selected 97 strains with serotypes O1 (1), O2 (26), O5 (1), O8 (3), O9 (1), O11 (2), O14 (2), O15 (7), O18 (4), O20 (1), O24 (7), O25 (3), O78 (20), O101 (1), O111 (1), O119 (2), O140 (1), O141 (1), O152 (13), and 18 untypable strains were used (figures in parenthesis indicate the number of strains tested). According to the criteria of Dho and Lafont [11], the virulence of the action of these strains on 1-day-old chicks was divided into three classes. Forty one strains were shown to kill all five chicks (lethal class, LC), 50 strains to kill 4 to 2 chicks (intermediate lethal class, ILC), and 24 strains to kill 1 to 0 chick (non-lethal class, NLC) (Table 1). O-serotypes of the strains belonging to LC were O1 (1), O2 (17), O15 (2), O20 (1), O24 (1), O78 (10), O140 (1), and untypable (8), and those belonging to NLC were O2 (4), O8 (2), O9 (1), O14 (1), O18 (2), O78 (4), O101 (1), O152 (5), and untypable (4) (number of strains in parenthesis).

Virulence factors in wild-type E. coli strains: Serum resistance of the 115 strains was classified into 6 patterns as shown in Fig. 1. It was found that, among 41 LC strains, 25 (61.0%) were resistant to serum (grades 5 and 6), 10 (24.4%) displayed intermediate resistance (grades 3 and 4), and only 6 (14.6%) were sensitive (grades 1 and 2) (Table 1). On the other hand, the percentage of serum sensitive strains (45.8 to 50.0%) was significantly higher than that of serum resistant strains (25.0 to 28.0%) among ILC and NLC strains (Table 1). The ratio of strains positive for both aerobactin secretion and ferric aerobactin transport was 61.0% of LC strains, but 12.5 to 20.0% of ILC and NLC strains (Table 2). Congo red binding was found in 10 (24.4%), 3 (6.0%), and 4 (16.7%) strains belonging to LC, ILC, and NLC groups, respectively. Production of β-hemolysin was found in only 1 (2.4%) and 8 (16.0%) strains in LC and ILC strains, respectively, but no strain with α-hemolysin was found. No

Table 1. Correlation between serum resistance and chicken lethality of E. coli strains from chicken septicemia

<table>
<thead>
<tr>
<th>Lethality&lt;sup&gt;a&lt;/sup&gt; class</th>
<th>No. of strains with following serum resistance class&lt;sup&gt;b&lt;/sup&gt;:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistance (grade; 5, 6)</td>
<td>Intermediate resistance (grade; 3, 4)</td>
</tr>
<tr>
<td>LC (n=41)</td>
<td>25 (61.0%)</td>
<td>10 (24.4%)</td>
</tr>
<tr>
<td>ILC (n=50)</td>
<td>14 (28.0%)</td>
<td>11 (22.0%)</td>
</tr>
<tr>
<td>NLC (n=24)</td>
<td>6 (25.0%)</td>
<td>7 (29.2%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chicken lethality was classified by the criteria of Dho and Lafont [11]. LC: lethal class, ILC: intermediate lethal class, NLC: nonlethal class.

<sup>b</sup> Serum resistance was determined by the method of Taylor [35], and classified into 6 grades as shown in Fig. 1.
production of enterotoxins was found in any strain. These results demonstrated that the virulence of wild-type *E. coli* acting on chicks is closely related with its serum resistance and aerobactin-mediated iron uptake.

**Chicken lethality and virulence factors in serotype O2 strains:** The chicken lethality and virulence factors were compared in individual strains of serotype O2 (Table 3). Almost all strains (except for S-77b, S-64, and S-66) with either serum resistance or iron uptake, or both, were virulent and found to have S/5 or 4/5 lethality. These results indicated that serum resistance and iron uptake, alone or together, were important virulence factors in *E. coli* serotype O2 action on chicks.

Analysis of the plasmid profile showed that 19 out of 20 virulent strains, with the exception for S-77b, harbored large plasmid about 100 Mdal in size. These results suggested that the serum resistance and iron uptake are associated with the large plasmid.

**Construction of isogenic strains with or without 100 Mdal plasmid:** Strain S-20 (O2, K⁺, NM, Fmsa⁺) which harbored only 100 Mdal plasmid, designated pKI100, was chosen as representative of the virulent strains, and the isogenic strains with or without the plasmid were constructed. A spontaneous rif² derivative of S-20, AK400, was used as a parent strain. pKI100 of AK400 was tagged by an amp² transposon Tn1 to yield AK422. pKI100::Tn1 in AK422 was then transferred to HB101 nal⁷ strain to generate AK432 by conjugation. pKI100-cured

<table>
<thead>
<tr>
<th>Strains (Pili)</th>
<th>Chicken lethality</th>
<th>Serum resistance pattern</th>
<th>lac/lat</th>
<th>Congo red</th>
<th>Colcin 100 Mdal plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1 (F-I)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-4 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-20 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-30 (-)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-31 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-26 (I)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-62a (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-63 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-71 (-)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-80 (F)</td>
<td>5/5</td>
<td>5</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-89 (F)</td>
<td>4/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-23 (F)</td>
<td>5/5</td>
<td>3</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-88 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-62b (F)</td>
<td>5/5</td>
<td>5</td>
<td>++/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-76 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-77b (F)</td>
<td>4/5</td>
<td>2</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-29 (I)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-50 (F)</td>
<td>5/5</td>
<td>6</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-45 (F-I)</td>
<td>2/5</td>
<td>1</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-67 (-)</td>
<td>2/5</td>
<td>2</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-64 (F)</td>
<td>1/5</td>
<td>5</td>
<td>++/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-66 (-)</td>
<td>1/5</td>
<td>5</td>
<td>++/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-73 (I)</td>
<td>0/5</td>
<td>1</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S-77a (F)</td>
<td>0/5</td>
<td>2</td>
<td>++/-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Presence of Fmsa (F) or type 1 (I) pili was determined by slide glass agglutination test. Chicken lethality was shown by the number of chicks killed/number of chicks inoculated. (+) and (-) indicate positive and negative.
strain, AK1035, was isolated from AK400. pKI100::Tn1 was introduced from AK432 to plasmid-cured AK1035 strain by conjugation to generate AK1061 by selection of amp^r and rif^r. The plasmid profile of strains AK400 (pKI100), AK422 (pKI100::Tn1), AK1035 (pKI100-cured), and AK1061 (pKI100::Tn1-reintroduced) thus obtained is shown in Fig. 2.

Correlation of virulence of S-20 strain with pKI100: AK400, AK422, AK1035, and AK1061 had the same antigenicity (O2 serotype and Fmsha pili^+^), colony morphology (smooth), and colicinogenicity as that of wild-type strain S-20. Both synthesis and secretion of aerobactin, and ferric aerobactin transport were observed in AK400, AK422, and AK1061 (pKI100 or pKI100::Tn1) but not in AK1035 (pKI100-cured). The number of surviving AK400 and AK1061 cells was much greater (2 to 3 times) than that of AK1035 in the medium containing 5 to 10% chicken serum. Similarly, the number of AK432 strain cells with pKI100::Tn1 was 100 to 1,500 times greater than that of the parent HB101 nal^r^ strain without the plasmid, when the medium contained 5 to 10% chicken serum (Fig. 3). C600 strain with pKI100::Tn1 was also found to be more resistant to serum killing than the parent strain without plasmid (data not shown). These results demonstrated that serum resistance and iron uptake, but not colicin production, are determined by pKI100 from the S-20 strain.

Virulence in chickens was examined for the isogenic strains with or without pKI100 as shown in Table 4. The LD_{50} values for AK400, AK422, and AK1061 (10^{-5.7} to 10^{-9.4}) were significantly lower than that for AK1035 (10^{9.0}). These results provided clear evidence that pKI100 is the virulence plasmid of S-20 strain, and plasmid-mediated serum resistance and iron uptake are major virulence factors in _E. coli_ strains with serotype O2 for chicken colibacillosis.
DISCUSSION

Almost all serotype O2 strains with either serum resistance and/or iron uptake were found to kill 4 to 5 chickens (Table 3). A close relationship was therefore found in serotype O2 strains between serum resistance, iron uptake, and virulence. However, there were two types of virulent E. coli strains which did not show both serum resistance and iron uptake in this study. One of the two is exemplified by S-77b, whose higher virulence was dependent on neither serum resistance nor iron uptake. The other type of exceptions is represented by S-64 and S-66, whose serum resistance did not attribute to the virulence. These results suggest that, although serum resistance and iron uptake are the elemental virulence factors, other virulence factors also influence the virulence of avian E. coli. The multifactorial nature of virulence in E. coli has been demonstrated by Smith and Huggins [34]. Alternatively, evidence that S-64 and S-66 strains did not harbor 100 Mdal plasmid may mean that the mechanism of serum resistance expressed by these strains is different from that encoded by 100 Mdal plasmid.

Binns et al. demonstrated that a suitable genetic background is required for serum resistance mediated by ColV, I-K94 plasmid to be fully expressed in E. coli [4, 5]. As shown in Fig. 3, the extent of serum resistance was different in AK1061 and AK432 strains, although both are pKI100::TnI carriages. The former strain resisted serum, and grew in the presence of a 25% serum concentration. However, the latter strain grew only in a lower concentration of serum at 1 to 2%. Similar results were obtained with AK1035 and HB101 nalG. Therefore, serum resistance is a candidate or potential virulence factor and is fully effective only when present in E. coli with the appropriate genetic background.

The proportion of strains positive for ferric aerobactin transport (63.4%) was lower than that positive for aerobactin synthesis and secretion (95.1%) among the 41 strains belonging to LC (Table 2). This was also found to be true of the virulent strains of serotype O2. It was found that S-88, S-62b, and S-76 were able to produce and excrete aerobactin, but not to transport ferric aerobactin across the cell membrane (Table 3). It has been reported that bacterial components such as somatic (O) and capsular (K) antigens impede ferric aerobactin binding to the cloacin DF13 receptor [10, 12, 26]. It is also reported that the affinity of receptor of Shigella for binding to cloacin DF13 is less than that of the receptor expressed from the iut gene of ColV plasmid [12, 25, 26]. Strains S-88, S-62b, and S-76 harbor a large plasmid which is supposed to be closely related to pKI100 of S-20 in size. It is, therefore, likely that the genotype of these strains is iut+, and they express the receptor for cloacin DF13. The receptor activity may be weakened, or lower than other virulent strains.

Epidemiological studies have demonstrated that the majority of E. coli isolated from chicken colibacillosis restricted to certain serotypes [7, 15], indicating a possible correlation between virulence and the O serotype [13]. However, our present study showed that O serotypes of strains belonging to LC were O1, O2, O15, O20, O24, O78 and O140, and those belonging to NLC were O2, O8, O9, O14, O18, O78, O101, and O152. Thus, virulence and the O serotype of avian E. coli are not correlated in our study. We [19] and other groups [7] have demonstrated that the predominant O serotype varies with the area, country, and period of isolation. These results suggested that the frequent isolation of serotype O2 and O78 strains resulted from a clonal dissemination. The 100 Mdal virulence plasmid (pKI100) of S-20 was conjugative with a transfer frequency of $10^{-4}$ to $10^{-5}$ to laboratory strains (C600 and HB101 nalG) or $10^{-9}$ to serotype O2 strain (AK1035). Therefore, virulence factors such as serum resistance and iron uptake mediated by the 100 Mdal plasmid can be transferred to E. coli of various O serotypes. We do not know at the moment whether virulent strains with O serotype other than O2 harbor the 100 Mdal plasmid.

A correlation between plasmid-mediated iron uptake and the virulence of avian E. coli has been proven for ColV plasmid [22, 24, 39], 95 kilobase plasmid of O78 strain [32] and 100 Mdal plasmid of O serotype untypable strain [40]. Plasmid-determined serum resistance was reported for genes iss of ColV, I-K94 plasmid [4], and traT of drug resistance plasmids R6-5 and R100 [28]. Increased survival in serum was correlated with enhanced virulence of E. coli strains carrying plasmid-mediated traT- or iss-gene [1, 5]. S-50 and S-23 strains were found to be virulent, though the former is positive for serum resistance but negative for iron uptake, and the latter is positive for iron uptake but negative for serum resistance. These results suggest
that 100 Mdal plasmid-mediated serum resistance or iron uptake alone can be responsible for the increased virulence of serotype O2 strain. The incompatibility group of pK1100 of S-20 strain was the same as CoV plasmid (inc B1), but different from those of R6-5 and R100 (inc F1I) [41]. pK1100 determined neither drug resistance nor colicin production. Together with these reports and our preliminary physical and genetic mapping (data not shown), it suggests that pK1100 of S-20 is a new type of virulence plasmid responsible for the enhanced virulence of avian E. coli. Further genetic study on pK1100 is now in progress.

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REFERENCES


