Acid-Induced Autoagglutination Found in Chicken Pathogenic *Escherichia coli* Strain

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ABSTRACT. Chicken pathogenic *Escherichia coli* strains were found to autoagglutinate in a static culture of trypticase soy broth (TSB). One strain, designated PDI-386, was further studied for its autoagglutinating property. Acidity in the cultured medium caused by glucose degradation induced the autoagglutination. The bacterial cells grown in a glucose-free L-broth could be aggregated by adding acid, which suggests a potentiality of autoagglutination of the strain grown in the L-broth. The autoagglutinating parent (Agg) formed small colonies with irregular edges like rough colonies on the TS agar, whereas its non-autoagglutinating variant (Nag) formed larger smooth colonies with a perfectly round edge. The Nag colony was easily generated from the Agg colony on the TS agar. The autoagglutinating property was very unstable when the bacteria was passed in the TSB, but rather stable in the L-broth. Under electron microscope, the Agg were found to possess pili of more than 20 μm in length. However, the phenotypic expression of autoagglutination did not correlate with that of mannose-sensitive hemagglutination against guinea pig erythrocytes. Incubation of the Nag in the L-broth at room temperature for more than 10 days provoked the reversion of the autoagglutination. There was no difference between the Agg and the Nag in terms of surface hydrophobicity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of membrane proteins and LPS, and plasmid profiles. The virulence of the Agg was higher than that of the Nag. The autoagglutinating property is, however, so unstable that the pathogenicity of *E. coli* isolates from chickens should be carefully evaluated.—**KEY WORDS:** autoagglutination, *Escherichia coli*, virulence.

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In domestic birds, colibacillosis is usually caused by strains of *E. coli* belonging to serotypes O1, O2 and O78. Recent studies have implicated that ability to adhere to the epithelial respiratory cells of birds mediated by type-1 like pili [3, 10, 11, 27] and an ability to grow under iron-limiting conditions mediated by aerobactin [11, 24] would be involved in the virulence of avian isolates.

However, since most *E. coli* strains have type-1 pili [9, 12], these structures do not by themselves confer virulence evidently and their role as an adherence factor in the pathogenesis is uncertain. On the other hand, some of the *E. coli* isolates which seemed to be avirulent by experimental infection actually possessed the iron-sequestering system mediated by aerobactin [11, 24]. Moreover, the experimental reproduction of the disease is usually difficult and gives ambiguous results [16]. These inconsistencies can be explained if there are some other virulence factors that are very unstable under *in vitro* culture conditions.

During the characterization of field isolates of chicken pathogenic *E. coli*, we found that more than 60% of the field isolates were found to autoagglutinate after the static culture in TSB at 37°C. One of

the autoagglutinating isolates, designated as PDI-386, was further investigated for the autoagglutinating property. In this communication, we propose that the autoagglutinating property could be a novel *in vitro* marker for distinguishing virulent strains from avirulent strains of chicken *E. coli*.

MATERIALS AND METHODS

Bacterial strains: Chicken pathogenic *E. coli* strains were isolated from chickens suffering from colisepticemia in 1983 in Japan. Among them, more than 60% of the field isolates were found to show the autoagglutination. The autoagglutinating isolates were checked for their pathogenicity by the experimental infection using specific-pathogen-free chickens. One autoagglutinating isolate designated PDI-386 (O78: H?: K-), which was highly pathogenic for chicken, was further investigated in this study.

Media: The media used in this experiments were as follows: L-broth [8], TSB (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Maryland), TS agar (BBL), brain heart infusion broth (Difco Laboratories, Detroit, Michigan), heart infusion broth (Difco), antibiotic medium 3 (AM3) (Difco), M9 minimal salts medium [8]

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supplemented with 0.2% glucose and 0.0005% thiamine, and Eagle's minimal essential medium (Nissui Seiyaku Co., Ltd.).

Stability of autoagglutinating property during subculturing: The *E. coli* strain, PDI-386, was plated on TS agar and incubated at 37°C for 48 hr. A colony of the autoagglutinating clone of PDI-386 was picked up and suspended in 1 ml of L-broth. Fifty μ l of this was inoculated into 5 ml of L-broth or TSB, and grown at 37°C up to the stationary phase (about 10^9 /ml). Each culture was then diluted 10^7 -fold with sterilized saline, inoculated into each media, and grown again. This subculturing was repeated once a day for 15 days. The percentage of the small colonies at each subculture was measured by plating the appropriately diluted culture on TS agar and incubating it at 37°C for 48 hr.

Generation time: 10^3 CFU of testing bacteria were inoculated into tubes containing 10 ml of TSB or L-broth previously warmed in a water bath at 37°C. One hundred μ l of samples were removed from each tube every 30 min at intervals from 0 min to 7 hr. Samples were serially diluted ten-fold and plated on AM3 agar for colony counting. The generation time (doubling time) was calculated for the exponential phase of growth.

Microscopic observation: A drop of the bacterial culture was placed on a collodion-coated 400-mesh grid (Nisshin EM Co., Ltd.) and allowed to stand for 5 min. Excess suspension was removed by dipping the grid into distilled water and excess water was blotted off with the edge of a filter paper. The samples were immediately stained with 0.5% uranyl acetate for 15 seconds. Electron microscopy was performed on HS-9 microscope operated at 75 kV.

Hemagglutination (HA) test: Guinea pig and chicken erythrocytes were obtained by cardiac puncture from anesthetized animals. Packed erythrocytes were suspended in a volume of 0.8% NaCl solution sufficient to give a 2% final erythrocyte concentration. The bacteria to be tested were incubated in L-broth for 48 hr at 37°C. HA test was carried out on ice with small glass test tubes (10 mm in diameter, 100 mm in length) by using equal volumes of bacterial culture and the erythrocytes. The inhibitory effects of mannose on the HA reaction was monitored by performing the reactions in the presence of 0.5% D-mannose.

Test for hydrophobicity: The surface hydrophobicity of the bacteria was measured by the "salting-out" method and by adsorption to *p*-xylene as

described by Ferreirós and Criado [14].

Analysis of membrane proteins: Total membrane and the outer membrane proteins were prepared as previously described [2]. Total and outer membrane proteins were examined by SDS-PAGE [23] using 10% acrylamide in the separating gel and 3% in the stacking gel.

LPS analysis by SDS-PAGE: The method of Kido *et al.* [21] was used to extract LPS. SDS-PAGE was performed as described by Tsai and Frasch [33]. Gels were stained by the method of Hichcock and Brown [17].

Detection of siderophore: Production of aerobactin was examined by both chemical [4] and biological [7] methods.

Plasmid analysis: The method of Birnboim and Doly [5] was used to analyze the plasmid content of the different strains.

Test for virulence in chickens: Specific-pathogen-free chickens (white leghorn, 3 to 4 week-old) were used. About 10^8 bacterial cells of each clone were inoculated into the air sac of 5 chickens. Chickens were observed for 14 days after inoculation. Surviving birds were anesthetized and sacrificed after the 14th day. Dead and sacrificed birds were examined for lesions of colisepticemia [16]. The mortality were statistically estimated by Fishers' *t*-test.

RESULTS

Correlation between the autoagglutination and pH of the medium: Fifty μ l of overnight shaking culture of PDI-386 in L-broth was inoculated in 5 ml of TSB in a test tube, and the growth of bacteria was followed in static culture at 37°C. The bacteria autoagglutinated after an incubation period of about 5 hr. This was easily seen by the naked eye as a formation of small aggregates in the previously homogeneous bacterial suspension. Consequently the supernatant of the culture cleared, and almost all bacterial cells aggregated to form a large ball on the bottom of the test tube (Fig. 1). The other culture media listed in the Materials and Methods did not induce autoagglutination.

The pH value of the TSB after overnight culture of the PDI-386 was found to be 4.8, whereas those of the other media were found to be in a neutral range (pH 6.8–7.2). The TSB used in this experiment contained glucose and did not contain any substances which had a buffer function. On the other hand, L-broth did not contain glucose, and the

Fig. 1. Difference in growth properties of chicken pathogenic *E. coli* strain PDI-386. The tube on the right side shows autoagglutination of bacterial cells in trypticase soy broth. The tube on the left side shows the isogenic non-autoagglutinating derivative as control. The presence of sedimented large ball on the bottom of the test tube, clear supernatant (right) contrasts with the uniform turbidity of the control (left).

Fig. 2. Colony morphology of autoagglutinating cells. Chicken pathogenic *E. coli* strain PDI-386 was streaked on trypticase soy agar and incubated at 37°C for 48 hr. The small irregular edged colonies show autoagglutination in trypticase soy broth; the larger, round, smooth colony (indicated by an arrow) shows the non-autoagglutinating derivative.

other media contained phosphate buffer. Thus, the bacterial growth in L-broth containing 1% glucose was examined. As expected, the PDI-386 was found to autoagglutinate in the medium.

To test the potentiality of autoagglutination of bacterial cells grown in a glucose-free L-broth, we added one tenth volume of 3 M sodium acetate (pH 4.8) to homogeneous suspension of the bacteria grown in the L-broth. The bacterial cells subsequently aggregated and sedimented on the bottom of the test tube in a few minutes.

The autoagglutination of the PDI-386 strain was temperature-dependent. It was observed only at high temperature (35°C–43°C). The cells incubated at 25°C did not show autoagglutination even with an addition of acid.

Colony morphology of autoagglutinating bacteria: Colony morphology of the PDI-386 formed on L agar revealed typical wild type colonies of *E. coli*. However, the colonies appeared heterogeneous on the TS agar and the L agar containing 1% glucose. Most of the colonies were rather small and exhibited smooth surface with an irregular edge (designated small colonies, Fig. 2). Among them, larger smooth colonies with a perfectly round edge, which are usually found in the *E. coli* wild isolates, were also found (about 10^{-1} - 10^{-2} of the parent population, Fig. 2). Such large smooth colonies, designated a large colony, could easily be generated from a single small colony by plating them on the TS agar. On the other hand, 20 large colonies selected independently

did not generate any small colony on the TS agar. Each of 50 small and large colonies was selected and checked for its characteristics. All the 50 small colonies showed autoagglutination after the static culture in TSB, whereas none of the large colonies did. Although the difference in colony morphology was not always clear when compared with several lots of TSB, reproducible results could be obtained with TSB supplemented with 1% glucose which gave sufficient amount of acid production.

Stability of autoagglutination in bacterial population: The difference in their colony morphologies on TS agar enabled us to examine the stability of agglutinating property. A small colony of PDI-386 was picked up and passaged either in L-broth or TSB, and the percentage of the small colony at each passage was measured as described in Materials and Methods. After 5 or 6 passages in TSB, more than 90% of the cells were converted to large colonies. On the other hand, small colony morphology was rather stable after several passages in L-broth (Fig. 3). Average generation time of the bacteria in the two medium clearly described the difference in their stability. In TSB, the average generation time of the autoagglutinating parent (designated Agg) was 23 min, whereas that of the non-autoagglutinating derivative (designated Nag) was 17 min. In L-broth, those of the Agg and the Nag were 12 min and 23 min, respectively. This suggests that the Nag could easily overgrow the Agg in TSB.

Electron microscopic observation: The Agg was

Fig. 3. Kinetics of loss of autoagglutinating ability. Autoagglutinating parent of PDI-386 was inoculated in L-broth (○—○) and Trypticase soy broth (△—△). At each subculture, they were examined for colony morphology on trypticase soy agar. Small irregular edged colonies (Fig. 2) considered to be autoagglutinating clones and percentages of autoagglutinating clones at each time were expressed as % small colonies.

found to possess numerous fibril structures on the surface (Fig. 4A). The fibril structure was 6–8 nm in diameter and was very similar to that of type-1 somatic pili [6]. However, it was much longer than type-1 pili [6], (Fig. 4A). Some of them were more than 20 μm in length. Most of the bacterial cells seemed to link to each other by this pili, but some of them attached directly to one another. On the other hand, even in a high magnification, no such structure except the flagella was seen in the Nag (Fig. 4B).

Reversion to autoagglutination and relationship between autoagglutination and type-1 pili: The Agg showed no HA reaction against chicken erythrocytes. However, mannose-sensitive HA reaction was shown against guinea pig erythrocytes, implies the existence of functional type-1 pili. Therefore, 60 independent Nag were selected and checked for their HA activity against guinea pig erythrocytes. Among them, 37 showed mannose-sensitive HA (HA^+Nag) while the remaining 23 did not (HA^-Nag). The latter 23 HA^-Nag were further studied for their ability to autoagglutinate and HA reaction. The incubation was continued in the same culture at room temperature for more than 10 days. Samples were removed from the cultures every day and used for the HA test and for the autoagglutina-

Fig. 4. Electron microscopic observation of negatively-stained bacteria. A, the autoagglutinating parent, PDI-386. B, the non-autoagglutinating derivative. Bar, 100 nm.

tion test by inoculating into TSB. Four of the 23 samples started to show HA activity after 48 hr-incubation but have not yet exhibited autoagglutination at that time (HA^+Nag) while the remaining 19 showed neither autoagglutination nor HA activity (HA^-Nag). The 4 HA^+Nag samples reverted to autoagglutinating cell types on the 12th day (HA^+Agg). The other 19 HA^-Nag samples reverted to autoagglutinating cell types on the 14th day but still did not show HA activity just after reversion. They finally exhibited HA activity (HA^+Agg) only after two to three more days of incubation.

The relationship between autoagglutination and mannose-sensitive HA was further investigated by inhibition test. Autoagglutination was not inhibited by adding D-mannose (1%) before or after cultivation in TSB.

Characterization of the Agg and the Nag: No remarkable difference was noted between the Agg and the Nag in hydrophobicity and SDS-PAGE patterns of membrane proteins and LPS (data not shown). Both the Agg and the Nag produced aerobactin and contained five sizes of plasmids (molecular sizes of 128 kilobases (kb), 95 kb, 78 kb, 14 kb, and 7.5 kb).

Virulence of autoagglutinating bacteria: Three Nag were independently isolated from the Agg by virtue of their colony morphology and were purified by repeating single colony isolation three times in order to generate three independent Nags. The Agg and its isogenic three Nags were examined for their virulence in chickens. All the chickens inoculated with Agg died within 3 to 5 days while the chickens inoculated with the two Nags survived and one of the chickens inoculated with the one Nag died within 10 days. All dead chickens showed typical lesions of colisepticemia, i.e., fibrinous perihepatitis, pericarditis, airsacculitis, and joint involvement. There was no significant difference in severity of lesions among chickens inoculated with the Agg and the Nag. The difference in the mortality between the Agg and the Nag was statistically significant ($P < 0.02$).

DISCUSSION

The present study demonstrated autoagglutination as a novel property of chicken pathogenic *E. coli*. The presence of acid in the culture was found to be a prerequisite to the induction of autoagglutina-

tion. This was shown by the observation that medium containing phosphate buffer, such as heart infusion broth and tissue culture media, did not induce autoagglutination. Although numerous type-1 pili were found on the Agg bacteria, autoagglutination was not inhibited by D-mannose and the expression of autoagglutination was not correlated with mannose-sensitive HA reaction. These pieces of evidences suggest that autoagglutination was distinct from the mutation or modification of type-1 pili [29, 31] and from two previously reported virulence-associated autoagglutination found in other genera [18–20, 22, 25, 26, 32].

The Agg bacteria were found to form rough-like colonies on TS agar and L-broth agar supplemented with 1% glucose but the expression of autoagglutination was very unstable during the passage in TSB, i.e., the medium is frequently used for clinical microbiology. Therefore, the Agg bacteria might often be neglected between the time of initial isolation and the testing at a later date or alternatively it might be misidentified as a rough variant or a kind of undesirable contamination.

Many bacterial virulence determinants have recently been characterized to be encoded on virulence-associated plasmids [30]. The plasmid content of the Agg and the Nag, however, were found to be identical so that no negative conversion from autoagglutinating cell type was caused by a deletion or segregation of the plasmids. This was also supported by the fact that the Nag could be reverted back to Agg phenotypes after the prolonged incubation at room temperature.

The essential components which affect the ability to autoagglutinate of the bacteria may exist on the surface [13, 18, 19]. However, comparison of surface properties by hydrophobicity test and SDS-PAGE patterns could not specify the difference between the Agg and the Nag in this study although the choice of measuring method including physical conditions may have greatly influenced the results of surface hydrophobicity. Therefore, the bacterial component which contribute to the expression of autoagglutination is still unknown. Moreover, the optimum condition for autoagglutination is also uncertain. The bacterial cells of the Agg suspended in sodium citrate buffered saline (pH 4.8) did not give sufficient autoagglutination (unpublished data). Since citric acid chelates divalent cations, one or more kinds of divalent cations such as Mg^{2+} or Ca^{2+} may be needed to induce autoagglutination. A

required condition for autoagglutination is now under investigation.

The expression of the type-1 pili is known to be conducted by a phase variation-type genetic regulation that controls between on and off states of expression [1, 15]. The expression of the autoagglutination was also changeable in high frequency and phenotypic changes were always alternating between two phenotypes; i.e., the Agg and the Nag. Since the phenotypic expression of autoagglutination was not correlated with mannose-sensitive HA reaction, there might be another regulatory gene which was similar to the phase variation-type genetic control found in type-1 pili gene region.

Comparison of virulence between the parent and three independent Nag suggests the importance of autoagglutinating property although further studies are needed to confirm linkage between virulence and autoagglutination. This finding may also explain the discrepancy in the previous observation that some of the strains expressing the aerobactin and the type-1 pili were avirulent by experimental infection [11, 16, 24]. Although the biological role of autoagglutination is still unknown, it is proposed that this phenotype would contribute to bacterial survival in intracellular vacuoles such as lysosomes. The normal pH inside macrophage lysosomes was estimated to be 4.7 to 4.8 [28], which was optimum for induction of autoagglutination. The Bacteria taken into intracellular vacuoles via endocytosis would aggregate and would be more resistant against lysosomal enzymes than a single bacterial cell. However, since the autoagglutination observed was very unstable *in vitro*, one should be careful to evaluate its pathogenic roles. The phenotypic expression of autoagglutination in the bacteria to be tested should be controlled or monitored during the experiment. As an initial step to elucidate the function of autoagglutination, it is necessary to analyze the genetic background of this phenotype. The molecular cloning of this determinant *in vitro* was recently accomplished (unpublished data) and determination of its polynucleotide sequence as well as analysis of the gene product by the amplified gene will confirm the regulation of its expression as well as the structure of the bacterial component. This may also lead to an understanding of the precise mechanism of autoagglutination and its role in pathogenicity.

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