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Virulence Factors and Biochemical Characteristics of Serotypes of *Escherichia coli* Serogroup O29

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***Escherichia coli* strains belonging to serogroup O29 were studied. Invasiveness was the most common virulence factor described in this serogroup, but a few papers also reported production of heat-stable (ST) enterotoxin. In the present study invasive ability was found in O29:H$^-$ strains, whereas production of ST-I enterotoxin was observed only in serotype O29:H21 strains, showing that virulence was a characteristic of specific serotypes or bioserotypes within the O29 serogroup. Different serotypes were found among strains that were neither invasive nor toxigenic. Invasive strains were biochemically less active than the toxigenic ones and presented the invasiveness plasmid (pINV) of about 120 to 140 megadaltons, whereas hybridization tests showed that ST-I production was related to a plasmid of about 90 megadaltons. A diffuse adherence pattern to HeLa cells was observed in all ST-I isolates, but the role of this adherence in the pathogenicity of these strains was not determined. Thus, a unique biochemical pattern and plasmid profile may be useful characteristics to distinguish between pathogenic (toxigenic or invasive) and nonpathogenic O29 strains.**

A growing number of studies suggests that the ability of *Escherichia coli* to cause intestinal infections is a characteristic associated with bioserotypes rather than with O serogroups (15, 22, 27, 30, 36). Moreover, it has been observed that the same *E. coli* serogroup may harbor enterotoxigenic and enteroadherent serotypes (14), enterotoxigenic and invasive serotypes (12), cytotoxigenic and enteroadherent serotypes (2), or cytotoxigenic and enterotoxigenic serotypes (21). *E. coli* serogroup O29 was characterized many years ago without any indication of which serotypes could be associated with specific infections. In 1979, Toledo et al. (35) showed that some nonmotile bioserotype O29 strains were invasive as assayed by the Serény test. Furthermore, it was reported in the literature that some serotype O29 strains were able to produce heat-stable (ST) enterotoxin (22). During the last few years we also isolated several O29 strains that produce ST enterotoxin. The purpose of this work was to study the H antigens, virulence factors, and biochemical characteristics of 25 strains of *E. coli* belonging to serogroup O29 that were isolated in our laboratory or received from others.

MATERIALS AND METHODS

Bacterial strains. Twenty-five *E. coli* serogroup O29 strains were studied. Fifteen strains were isolated from feces of children with diarrhea (14 cases in Brazil and 1 in Chile) and were mostly isolated between 1982 and 1984, except for two strains that were isolated in 1976 and 1978. The invasive ability and production of enterotoxins were tested at the time of isolation and monitored throughout the study. The remaining strains were received from the collection of the Centers for Disease Control, Atlanta, Ga.

Serological tests. The study of O-antigen relationship between invasive and toxigenic *E. coli* serogroup O29 strains was carried out with one Serény test-positive strain (4CII-76) and one ST-I producer (28-84FAISA). Antisera were prepared in rabbits by using these strains as vaccines, and cross-absorption tests were performed. The O antigen of all strains studied was confirmed by tube agglutination tests with antiserum against 4CII-76. Motile strains had their flagellar antigen determined by tube agglutination tests with *E. coli* antisera H1 to H50, kindly provided by Paul A. Blake, Centers for Disease Control. The methods used in serological tests were those recommended by Edwards and Ewing (7).

Biochemical tests. Utilization of L-rhamnose, D-xylose, or L-serine, as the sole carbon source was tested as described by Silva et al. (33). Production of β-galactosidase was determined by the method of Lowe (19). All other tests were carried out as described by Edwards and Ewing (7) except for carbohydrate fermentation tests, which were observed for 7 days only.

Invasiveness assay. The strains were tested for invasiveness by the Serény test (32). The test was considered positive if there was evidence of keratoconjunctivitis within 7 days of observation. Exudates from the eyes of guinea pigs with keratoconjunctivitis were cultured on MacConkey agar plates to confirm the presence of the inoculated strain.

Enterotoxin and cytotoxin tests. The strains were tested for production of heat-labile enterotoxins (LT-I and LT-II) with Y1 adrenal cells (5, 13). ST-I toxin production was detected by the infant mouse test (4), and Shiga-like cytotoxins (SLT-I and SLT-II) were assayed by HeLa and Vero cell tests (17, 21). Colony hybridization tests were also carried out with LT-I, LT-II, ST-I, SLT-I, and SLT-II DNA probes. The LT-I probe was the 1.0-kilobase *Bam*HI fragment from plasmid pEWD299 (3). The LT-II probe was the 800-base-pair *Hin*dIII-*Pst*I fragment from plasmid pCP2725 (28). The SLT-I and SLT-II probes were, respectively, the 114-base-pair *Bam*HI and 842-base-pair *Pst*I fragments from plasmids pJN37-19 and pNN111-19 (26). For detection of ST genes we used two probes, ST-Ia and ST-Ib, which are the 157-base-pair *Hin*fI fragment from pRIT10036 (23) and the 510-base-pair *Eco*RI fragment from pNG10 (16), respectively. To localize the enterotoxin genes, hybridization tests were performed with isolated DNA (23) electrophoresed in 0.8%

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agarose gels. The colony hybridization and gel hybridization tests were performed as described by Maas et al. (20).

Adherence assay. Adherence tests were performed with HeLa cells as previously described (31). The presence of genes encoding for localized adherence was also assessed by colony hybridization tests with the 1.0-kilobase *Bam*HI-*Sal*I fragment from plasmid pJPN16 as a probe (24).

Colonization factors. The colonization factor antigens CFA/I and CFA/II were preliminarly detected by mannose-resistant hemagglutination of human group A and bovine erythrocytes (8, 9). Their presence was confirmed by slide agglutination tests with specific CFA/I and CFA/II antisera.

Plasmid DNA profile. Rapid plasmid DNA preparations were carried out by the method of Birnboim and Doly (1). The plasmid DNA profile was assessed by electrophoreses of total DNA content in 0.8% agarose slab gels. To estimate the molecular mass of virulence plasmids the following standard markers were used: pR27 (110 megadaltons [MDa]), p307 (54 MDa), pRP4 (34 MDa), pSa (23 MDa), and pBR322 (2.6 MDa).

RESULTS

All of the *E. coli* strains studied belonged to the O29 serogroup as confirmed by the agglutination tests. The O-antigen identity between the invasive 4CII-76 strain and the toxigenic 28-84FAISA strain was demonstrated by cross-absorption tests. Characterization of flagellar antigen showed that O29 strains were distributed among six different serotypes: O29:H$^-$ (5 strains), O29:H4 (3 strains), O29:H10 (5 strains), O29:H21 (10 strains), O29:H25 (1 strain), and O29:H27 (1 strain).

Virulence tests showed that 5 strains were invasive as assayed by the Serény test and that 10 other strains produced ST-I toxin detected by the infant mouse assay and by hybridization with both ST-Ia and ST-Ib DNA probes. The hybridization with ST-Ib gave a stronger reaction than that seen with ST-Ia. None of the strains produced LT-I or LT-II enterotoxin or SLT-I or SLT-II cytotoxin that could be detected by using cultured cell lines or DNA hybridization with the respective DNA probes. Colonization factors CFA/I and CFA/II were also not detected in any of the strains.

All of the ST-producing strains showed a diffuse pattern of adherence to HeLa cells. On the other hand, the strains that were neither invasive nor toxigenic and did not present any detectable virulence determinant showed no adherence to HeLa cells except for two strains. In this case, the two strains presented an adherence where small chains of bacteria hung from the HeLa cell, a pattern totally distinguishable from that of diffuse or localized adherence.

Specific serotypes were found among invasive and toxigenic strains. These strains belonged to O29:H$^-$ and O29:H21 serotypes, respectively, whereas strains that were neither toxigenic nor invasive presented different flagellar antigens.

In regard to the biochemical behavior, all strains produced gas from glucose, utilized D-xylose as the sole carbon source, were positive for the Jordan tartrate and β-galactosidase (*o*-nitrophenyl-β-D-galactopyranoside) tests, and fermented lactose, xylose, and maltose. All but one fermented rhamnose and sorbitol. The biochemical activity and virulence determinants of the different serotypes in serogroup O29 are presented in Table 1. Comparison of the biochemical behavior within the O29 serogroup showed that nonpathogenic and toxigenic strains have a typical *E. coli* biochemical pattern, whereas the invasive strains are clearly less active.

TABLE 1. Relationship of invasion, ST-I production, adherence and biochemical pattern in O29 *E. coli* serotypes

Tests	Result[a] for serotype					
	O29:H$^-$	O29:H21	O29:H4	O29:H10	O29:H25	O29:H27
Invasion	+	−	−	−	−	−
ST-I production	−	+	−	−	−	−
Type of adherence	NT	DA	− (2)	− (4)	−	−
Biochemical tests						
Sucrose	−	+	−	− (3)	+	−
Salicin	− (4)	+	+	+	+	+
Dulcitol	−	+ (9)	−	− (3)	+	+
Raffinose	−	+	−	+ (3)	+	−
Esculin	−	+	+	+	+	+
Arginine	−	+	+	+ (4)	+	+
Ornithine	+	+	(+) (2)	(+)	+	−
Lysine	−	+	+	+	+	+
Mucate	−	+	+	+	+	+
L-Rhamnose	(+) (3)	+	+	+ (4)	+	+
L-Serine	−	(+)	+	+ (4)	+	+
Sodium acetate	−	+	+	+	+	+
Christensen citrate	(+) (3)	+	− (2)	− (3)	−	−

[a] +, All strains were positive within 1 or 2 days except for the pathogenicity tests, which were evaluated as described in Material and Methods; −, all strains were negative; (+), strains were positive after 48 h. Numbers within parentheses indicate the number of strains presenting that result. NT, Not tested; DA, diffuse adherence.

The study on plasmid DNA profile showed that all of the invasive strains contained a plasmid of 120 to 140 MDa, and all enterotoxigenic strains possessed a DNA band corresponding to a plasmid of about 90 MDa (Fig. 1). In the remaining serogroup O29 serotypes no regular plasmid profile was found except for O29:H4 strains, which did not contain any plasmid.

Hybridization carried out with electrophoresed DNA isolated from all of the strains studied showed that the ST gene is located only in the 90-MDa plasmid DNA band present in enterotoxigenic strains.

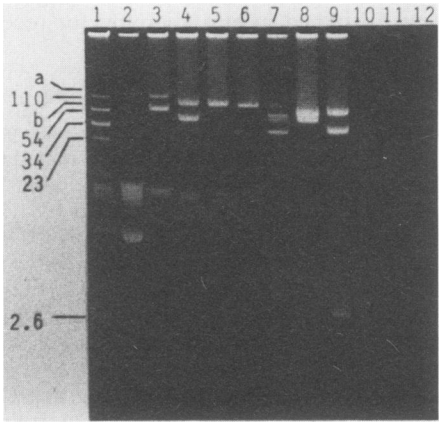

FIG. 1. Plasmid profile of *E. coli* serogroup O29 strains. Lanes: 1, molecular mass markers (megadaltons); 2 and 3, invasive strains; 4 through 7, ST-I-producing strains; 8 through 12, noninvasive, nontoxigenic strains. Bands: a, pINV plasmid; b, plasmid band that hybridizes with the ST-I probe.

DISCUSSION

In the present study it was demonstrated that, within serogroup O29, virulence determinants such as invasiveness, production of ST-I enterotoxin, and adherence are related to specific serotypes. Invasiveness was a characteristic found in all O29:H$^-$ strains, whereas enterotoxigenicity was found in all O29:H21 isolates, which also adhered diffusely to HeLa cells. Moreover, these serotypes presented a characteristic biochemical pattern, suggesting that these may represent specific pathogenic bioserotypes. This is especially true for the invasive strains, which confirmed the observations previously reported that in general enteroinvasive *E. coli* strains are nonmotile and do not produce lysine decarboxylase (33, 36). In regard to plasmid profile, invasive strains confirmed once again the rule that a plasmid DNA band of about 120 to 140 MDa is present in all enteroinvasive *E. coli* and virulent *Shigella* isolates described so far (29, 34).

Enterotoxigenic serotype O29:H21 strains showed a more active biochemical behavior compared with the invasive strains, with no special pattern to be pointed out. Nevertheless, it is noteworthy that all the toxigenic strains showed a plasmid DNA band of about 90 MDa that hybridized with the ST-I probes. Georges et al. (10) reported similar results in Central Africa; they isolated an O29:H21 ST-h-producing strain harboring a 90-MDa plasmid that hybridized with the ST probe. It should be stressed out that the 10 O29:H21 ST-I-producing isolates we studied are not part of a single clone, since they were isolated from different areas at different times and had diverse plasmid DNA profiles.

It was interesting to observe that all of the enterotoxigenic strains showed a diffuse pattern of adherence to HeLa cells. Although some studies demonstrated that diffuse adherence was not associated with diarrhea (11, 25), the role of diffuse adherence adhesin in pathogenesis has not been elucidated. In fact we do not know whether this characteristic, associated with a virulence factor such as production of ST-I enterotoxin, could contribute to the pathogenicity of a strain as happens with the colonization factors (18). Studies are under way in our laboratory to verify this hypothesis.

In our study only 10 strains coming from the Centers for Disease Control were not tested for virulence by the time of isolation. Among those, one strain belonging to serotype O29:H21 still produces enterotoxin ST-I. The remaining nine strains belong to serotypes other than O29:H$^-$ or O29:H21 and are avirulent. We have no reason to believe that all nine strains have lost the virulence plasmid during storage, since that did not happen with any of the virulent strains that were isolated and stored for as long as 5 years. As far as we know no other invasive serotype has been isolated within the O29 serogroup besides the O29:H$^-$ serotype. In regard to ST-I-producing serogroup O29 strains there are very few reports. Merson et al. (22) found only two strains, and Georges et al. (10) found only one, all of them identified as O29:H21, the same serotype we have found. Only one report mentions an ST-producing strain identified as O29:H35 (6), a serotype not found in our study. These results suggest that would be interesting to do further work in different geographical areas to confirm whether serotype and plasmid profile are definite markers to identify O29 ST-I-producing strains, as they are for invasive strains.

Demonstration that invasive and toxigenic *E. coli* strains may occur in a same serogroup was previously reported by Gross et al. (12) for serogroup O167. The fact we have found another O serogroup presenting enteroinvasive and enterotoxigenic *E. coli* strains associated with specific serotypes or bioserotypes reinforces the concept that a strict correlation between virulence, biochemical behavior, plasmid profile, and serotype may exist for other pathogenic bacterial groups.

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