Expression of cytotoxicity by potential pathogens in the standard *Escherichia coli* collection of reference (ECOR) strains

Xin-He Lai,† Su-Yan Wang and Bernt Eric Uhlin

Author for correspondence: Xin-He Lai. Tel: +46 90 785 6735. Fax: +46 90 772630. e-mail: Lai.X-H@micro.umu.se

Department of Microbiology, Umeå University, Umeå S-90187, Sweden

The standard *Escherichia coli* collection of reference (ECOR) strains was examined for ability to exert cytotoxicity towards mammalian cells. A group of strains with functional haemolysin expression caused strong cytotoxicity and detachment in J774 macrophage cells as measured by lactate dehydrogenase release and as observed under a microscope. The expression of haemolysin was monitored by using antisera recognizing the *E. coli* α-haemolysin, the HlyA protein, and by quantitative haemolysis assays. The presence of the hlyA gene, which may be part of a pathogenicity island, was also confirmed. These analyses revealed that different ECOR strains express quantitatively different levels of haemolysin. One putative enteroaggregative *E. coli* (EAEC) strain was also found in the ECOR collection. The EAEC strain was characterized by the clump formation assay, PCR amplification of the EAEC DNA probe sequence and confirmative sequence analysis of the amplified fragment. The EAEC heat-stable enterotoxin 1 gene, astA, was found in 14% (10/72) of the ECOR strains and a consensus sequence for astA was proposed by comparing these sequences with those from pathogens. The astA gene appeared to be plasmid-located. Based on evidence from the work of other laboratories and from the present findings, it is concluded that the ECOR collection contains strains that may represent pathogenic *E. coli*. It is noted that caution is necessary when handling or disposing of those potentially pathogenic ECOR strains.

Keywords: cytotoxicity, haemolysin, enteroaggregative *Escherichia coli*, enteroaggregative heat-stable enterotoxin, ECOR

INTRODUCTION

*Escherichia coli* is a normal inhabitant of the intestine of humans and various animals. However, pathogenic *E. coli* strains can cause intestinal and extraintestinal infections due to the production of virulence factors.

Pathogenic *E. coli* strains are able to cause infections of the intestine (enterovirulent *E. coli*), urinary tract (uropathogenic *E. coli*) and the brain (extraintestinal *E. coli*). Among the many pathogens causing diarrhoeal infections there are six well-characterized groups (Nataro & Kaper, 1998): enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC). A new category of diarrhoeagenic *E. coli* typified by detachment of epithelial cells is closely associated with the production of haemolysin (Gunzburg et al., 1993; Marques et al., 1995; Nataro & Kaper, 1998). EAEC is special for its aggregative adherence (AA) to HEp-2 cells; most of the EAEC strains are AA-probe-positive (Nataro & Kaper, 1998) and this probe has been sequenced from pCVD432 (Schmidt et al., 1995b). The EAEC heat-stable entero-
toxin 1 (EAST1) gene astA was initially cloned and sequenced from the prototype EAEC strain 17-2 (Savarino et al., 1993). Pathogenicity islands are large segments of chromosomal DNA containing virulence gene clusters (Blum et al., 1994). The pap, kps, sfa and bly genes/gene clusters are associated with pathogenicity islands of uropathogenic E. coli isolates (Blum et al., 1995). The pap and sfa gene clusters encode P and S fimbriae that are associated mostly with uropathogenic E. coli. Two α-haemolysin loci contain the genes for α-haemolysin production. The kps gene cluster contains genes for type II capsule which is absent in strain K-12 (Boyd & Hartl, 1998). Approximately half of the E. coli isolated from patients with cystitis or pyelonephritis produce the pore-forming haemolysin (Johnson, 1991). Haemolysin is an important virulence factor in both extraintestinal and intestinal infections caused by E. coli (Savarino et al., 1993b; Welch et al., 1981), and among them HlyA is the best characterized. The E. coli standard collection of reference (ECOR) strains is a set of 72 wild-type E. coli isolates (Ochman & Selander, 1984b) from humans and 16 other mammalian species from a large collection of approximately 2600 isolates (Milkman, 1973). The collection is thought to broadly represent genotypic variation in E. coli (Ochman & Selander, 1984a). Using results from multilocus enzyme electrophoresis (MLEE) the major lineages of the ECOR collection are divided into five groups: A, B1, B2, D and E (Herzer et al., 1990). Although it has been stated that none of the ECOR strains is pathogenic (Milkman & McKane, 1993), it is evident that different pathogenic E. coli may be grouped among the ECOR strains on the basis of MLEE (Pupo et al., 1997). Furthermore, phylogenetic studies demonstrate the existence of genes encoding typical pathogenicity determinants for uropathogens, such as pap, hly, kps and sfa, among some of the ECOR strains (Bingen et al., 1998; Boyd & Hartl, 1998; Marklund et al., 1992), though it is unclear whether or not these genes are active. Moreover, none of the ECOR strains gives a hybridization signal with an elbxA probe from the EHEC bly sequence (Boyd & Hartl, 1998).

In this work, we examined the blyA genotype, phenotype and expression of some ECOR strains, assessed these strains for their cellular toxicity towards different host cells, determined the existence of EAEC in the ECOR collection and conducted a sequence comparison of the EAST1 (astA) genes from these enteropathogenic strains.

**METHODS**

**Bacterial strains.** The ECOR collection was kindly provided by Dr Howard Ochman, University of Rochester, New York, USA. Uropathogenic E. coli J96 and the 536 mutant 536-21 were used in this study as haemolytic and non-haemolytic controls, respectively (Blum et al., 1994, 1995). E. coli K-12 strain HB101 was also included as a non-cytotoxic control. All strains were routinely stored at −80 °C in 15% (v/v) glycerol. For the detection of the targeted sequences by PCR, E. coli strains grown on TYS agar overnight at 37 °C were suspended in sterile water at a concentration of approx. 5 × 10^7 cells ml⁻¹ and boiled for 4 min. A 1 µl portion of the DNA solution was subjected to PCR. Plasmid preparations from those astA-positive strains were also used as PCR templates for astA amplification.

**Detection of haemolysis by liquid- and solid-phase assays.** Bacteria were routinely diluted 1:100 from overnight cultures and grown with shaking. Supernatants (50 µl) of exponential-phase (about 100 Klett units) or overnight bacterial cultures were incubated with 50 µl 2% (final concentration) suspension of horse erythrocytes (RBCs) at 37 °C for 3 h (Bauer & Welch, 1996). The amount of lysis was determined by measuring released haemoglobin spectrophotometrically at A410. RBCs were incubated in distilled H₂O to measure total lysis (100%), and background lysis was determined with RBCs incubated in saline (Bauer & Welch, 1996). Percentage lysis was calculated from A410 measurements as follows: 100 x ([A410 of sample – A410 of background]/[A410 of total – A410 of background]). Blood agar plates with washed or unwashed RBCs were also used to check the haemolytic activity of both the supernatants and colonies of some of the ECOR strains carrying or not carrying haemolysin gene(s). A clear haemolytic zone of at least 1 mm around an isolated colony was scored as positive.

**Western blot analysis of haemolysin.** Exponential-phase or overnight bacterial cultures were centrifuged and TCA added to 20 ml of the supernatants at 10% final concentration. Samples were kept on ice for at least 60 min. Precipitated proteins were pelleted by centrifugation at 4 °C (Bauer & Welch, 1996) and resuspended in SDS-PAGE loading buffer. Proteins were separated on 10% SDS-PAGE gels and transferred to a 0.2 µm pore size PVDF membrane with a semi-dry transfer cell Trans-Blot system (Bio-Rad). The membrane was blocked in PBS (80 mM NaH₂PO₄, 20 mM NaH₂PO₄, 100 mM NaCl, pH 7.5)/0.1% (v/v) Tween 20/5% milk powder overnight at room temperature. The primary antibody was rabbit polyclonal antisera raised against HlyA (a kind gift from Dr A. Juarez, Universidad de Barcelona, Spain) and used at 1:100 dilution as described by Balsalobre et al. (1996). All the remaining procedures were according to the instructions from the ECL kit (Amersham Pharmacia Biotech).

**Macrophage cytotoxicity by lactate dehydrogenase (LDH) assay and cell detachment.** The murine macrophage cell line J774 was routinely grown, infected, and assayed for cytotoxicity and detachment as described by Lai et al. (1999), Vanmaele et al. (1995) and Oscarsson et al. (1999). These experiments were repeated twice and representative results are shown. Briefly, 1 d before infection with bacteria, about 2 × 10⁴ J774 cells were seeded into each well of flat-bottom 96-well plates for cytotoxicity assays and about 1.25 x 10⁵ cells into each well of 24-well tissue culture plates for detachment experiments. J774 cells were infected with bacteria at a multiplicity of infection (MOI) of 100. At 2 h post-infection, cells were washed and incubated with medium containing 100 µg gentamicin ml⁻¹. Supernatants of the infected macrophages from designated time points were sampled and assayed for the activity of the released intracellular enzyme LDH (Korzewniewski & Callewaert, 1983) using the Cytotox 96 kit (Promega) according to the manufacturer’s instructions. The percentage cytotoxicity was calculated as described previously (Lai et al., 1999) and total release (100%) was taken as the activity in macrophage lysates after treating with Triton X-100 provided with the kit. HB101 and ECOR strains do not have endogenous LDH activity when grown aerobically. For detachment assay at 24 h post-infection, the monolayers were washed three times with PBS to remove nonadherent cells. J774 cells remaining in each well were fixed for 10 min.
with 70% methanol and then stained with Giemsa stain for 30 min. The monolayers were then washed three times with water to remove excess Giemsa stain, and the stained cells were lysed with 2% SDS. A portion of the lysates were transferred to 96-well microtitre plates and the absorbance of the contents of each well was recorded using a multiscan plate reader at 620 nm. The percentage of monolayer detachment was calculated as follows: 100 – [(A_{290} of un inoculated well/A_{290} of inoculated well) x 100]. At least four independent experiments were performed for each strain. Values of wells containing uninfected J774 cells were taken as zero detachment (100% attachment).

Clump formation assay. Bacterial clump formation at the surface of liquid culture was assayed as described by Albert et al. (1993). Each of the 72 ECOR strains was inoculated into 5 ml Luria broth in glass test tubes and incubated at 37 °C in a shaker incubator at 140 r.p.m. for 16–20 h. EAEC strain JM221, which forms clumps visible as a thick scum in liquid overnight cultures, was used as positive control (Albert et al., 1993).

PCR. Primers for the whole hlyA gene were from Boyd & Hartl (1998) and can amplify a product of 2930 bp size using a programmable thermal controller (MiniCycler, M J Research). Amplified products were analysed by electrophoresis in agarose gels of appropriate concentrations. DNA ladder containing uninfected J774 cells were taken as zero detachment (100% attachment).

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PCR. Primers for the whole hlyA gene were from Boyd & Hartl (1998) and can amplify a product of 2930 bp size using a protocol (1 cycle of 94 °C for 30 s; 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 68 °C for 6 min; 1 cycle of 72 °C for 10 min). Primers for the AA probe were from Schmidt et al. (1995b); this primer set generated a 630 bp fragment using the original procedure. PCR primers for astA were as described by Itoh et al. (1997) and Savarino et al. (1993). astA was amplified by 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min followed by 1 cycle of 72 °C for 7 min, generating a 124 bp product. PCR analysis was performed with a programmable thermal controller (MiniCycler, M J Research). Amplified products were analysed by electrophoresis in agarose gels of appropriate concentrations. DNA ladder markers of 100 bp and/or 1 kb (Bio-Rad) were used as molecular size standards.

Nucleotide sequencing and sequence analysis. To sequence the AA probe and the astA gene, the PCR products using bacterial templates were purified with GeneClean III kit (Bio101). A total of 50–150 ng purified PCR product was subjected to Taq cycle-sequencing reactions with the dye terminator cycle sequencing Ready Reaction kit (PE Applied Biosystems) according to the manufacturer’s instructions. Both 3’ and 5’ primers were used to crosscheck the sequencing results. Electrophoresis of sequencing products was performed on 4% polyacrylamide gels with an automated sequencer (model 377XL; Applied Biosystems). The nucleotide sequences were analysed with the analysis program version 3.0 (Applied Biosystems).

RESULTS
Presence of hlyA gene, expression of HlyA protein and haemolytic phenotypes
From the work of Bingen et al. (1998) and Boyd & Hartl (1998), we learned that some of the ECOR strains contain the hlyA gene for z-haemolysin production. It has not yet been reported whether these strains can express active haemolysin. We verified the presence of the hlyA gene in nine of the ECOR strains by PCR (Fig. 1 and Table 1, haemolysin genotype). Our results are somewhat different from those of the two previous studies (Bingen et al., 1998; Boyd & Hartl, 1998), which suggested that 12 and 8 strains of the ECOR collection have this gene by using DNA hybridization or PCR amplification corresponding to most of the blyC gene and to the 5’ end of the blyA gene, respectively.

Our 3 h quantitative liquid haemolysis studies indicated that there were different levels of haemolytic activity in the supernatant of blyA-positive ECOR cultures (Fig. 1c). The haemolysis phenotype is Ca2+-dependent since we were unable to see any haemolysis without addition of Ca2+ in this assay (data not shown). Further results from our Western blot analysis using the same preparation of supernatants used in the liquid haemolysis assays revealed that the secretion and extracellular properties of HlyA were different for individual ECOR strains (Fig. 1a, b). The immunoblot signals from samples prepared at exponential phase (Fig. 1a) were generally stronger than those from overnight cultures (Fig. 1b). The exceptional case was ECOR51 which was strongly haemolytic (Fig. 1c) but had no detectable immunoblot signal at exponential phase and only a weak signal from an overnight sample (Fig. 1a, b). ECOR56 appeared only marginally haemolytic (about 30%) by the quantitative liquid assay (Fig. 1c) but it showed the greatest cytotoxicity of all towards mammalian cells (Table 1). We therefore consider that there is some unidentified virulence factor(s) in ECOR56 that contribute(s) to its high cytotoxicity. Although ECOR63 was hlyA-positive in our PCR experiment we were unable to detect its secretion by immunoblot or its haemolytic phenotype by either blood agar plate or liquid assay (Fig. 1). In some strains (ECOR48, 51, 60) we observed a strong band of about 60 kDa (data not shown), which might be the breakdown product of HlyA and might be the reason for their weak Western blot signals at the 110 kDa position. Furthermore, we did not see a clear band of 110 kDa in the Western blot using total bacterial cell lysates, suggesting that the HlyA produced by each individual ECOR strain was largely not membrane-associated (data not shown). We also monitored hlyA expression with blood agar plates and checked the whole collection. Consistent with our liquid haemolysis assay results, not all the described strains carrying this gene were haemolytic. Note that all the clearly haemolytic strains were within the B2 subgroup (see Table 1), and some were isolated from the urine of women with urinary tract infections (Ochman & Selander, 1984b).

Macrophage cytotoxicity and detachment
All the 72 ECOR strains were tested for expression of cytotoxicity towards J774 cells as monitored by LDH release and cell detachment (see Methods). Results from cytotoxicity and cell detachment experiments were closely related to haemolytic activity for those strains of strong haemolytic phenotype (Table 1, Fig. 1c). We found that ECOR strains with strong haemolytic activity (ECOR51–54, 56 and 60 in Table 1 and Fig. 1c) were extremely cytotoxic to J774 cells and caused substantial cell detachment. The weakly haemolytic strain ECOR48...
Table 1. Expression of haemolytic phenotype and cytotoxicity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Serotype†</th>
<th>Haemolysin A</th>
<th>Detachment (%)§</th>
<th>Cytotoxicity (%)‖</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Genotype</td>
<td>Phenotype‡</td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>A</td>
<td>Rough</td>
<td>–</td>
<td>–</td>
<td>1.29 ± 0.50</td>
</tr>
<tr>
<td>ECOR8</td>
<td>A</td>
<td>O86:HN</td>
<td>–</td>
<td>–</td>
<td>11.13 ± 2.15</td>
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<tr>
<td>ECOR24</td>
<td>A</td>
<td>O15:NM</td>
<td>+</td>
<td>+</td>
<td>13.07 ± 3.00</td>
</tr>
<tr>
<td>ECOR43</td>
<td>E</td>
<td>ON:HN</td>
<td>–</td>
<td>–</td>
<td>7.26 ± 3.62</td>
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<tr>
<td>ECOR48</td>
<td>D</td>
<td>ON:HM</td>
<td>+</td>
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<td>56.55 ± 2.03</td>
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<tr>
<td>ECOR51</td>
<td>B2</td>
<td>O25:HN</td>
<td>+</td>
<td>+</td>
<td>37.23 ± 2.45</td>
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<tr>
<td>ECOR52</td>
<td>B2</td>
<td>O25:HN</td>
<td>+</td>
<td>+</td>
<td>68.79 ± 3.80</td>
</tr>
<tr>
<td>ECOR53</td>
<td>B2</td>
<td>O4:HN</td>
<td>+</td>
<td>+</td>
<td>69.34 ± 1.72</td>
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<tr>
<td>ECOR54</td>
<td>B2</td>
<td>O25:HN</td>
<td>+</td>
<td>+</td>
<td>64.75 ± 2.78</td>
</tr>
<tr>
<td>ECOR56</td>
<td>B2</td>
<td>O6:HN</td>
<td>+</td>
<td>+</td>
<td>64.56 ± 2.16</td>
</tr>
<tr>
<td>ECOR57</td>
<td>B2</td>
<td>ON:NM</td>
<td>–</td>
<td>–</td>
<td>14.30 ± 3.61</td>
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<tr>
<td>ECOR60</td>
<td>B2</td>
<td>O4:HN</td>
<td>+</td>
<td>+</td>
<td>67.83 ± 1.34</td>
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<tr>
<td>ECOR63</td>
<td>B2</td>
<td>ON:NM</td>
<td>+</td>
<td>–</td>
<td>16.80 ± 1.69</td>
</tr>
<tr>
<td>ECOR65</td>
<td>B2</td>
<td>ON:H10</td>
<td>–</td>
<td>–</td>
<td>8.14 ± 3.03</td>
</tr>
</tbody>
</table>

*From Herzer et al. (1990).
†From http://www.bio.psu.edu/Faculty/Whittam/ECOR.
‡–, No haemolysis; +, weak haemolysis; +++, strong haemolysis.
§Cell detachment was measured as described in Methods.
‖Cytotoxicity was measured as described in Methods.

Fig. 1. Western blot analysis and quantitative haemolytic activity of the secreted HlyA, and PCR detection of the hlyA gene in some ECOR strains. Proteins from 2 ml of exponential-phase (a) or overnight (b) culture supernatants precipitated with 10% TCA were detected in Western blots with anti-HlyA antibody. Lane M contains Rainbow (RPN 800) protein molecular mass markers. (c) Haemolytic activity was assayed by incubating 50 µl 2% RBCs with 50 µl exponential-phase LB broth culture supernatants for 3 h. Each reported value for percentage haemolysis represents the mean from duplicate samples. The hlyA PCR results of some ECOR strains and the haemolytic scores of these supernatants on blood agar plates are also shown for comparison. These tests were repeated at least twice and representative results are shown. In all experiments, strains J96 and 536-21 were included as positive and negative controls, respectively.

displayed higher cytotoxicity than ECOR24 for unknown reason(s). In spite of their equally weak positive haemolysis on blood agar plates, ECOR48 gave a higher haemolytic value than ECOR24 in the quantitative liquid assay (Fig. 1c). It remained unclear what factors contribute to the marginal cytotoxicity of ECOR43 and
ECOR37, ECOR43 happened to harbour astA (Fig. 2, lane 9), but it was nonhaemolytic (Fig. 1 and Table 1). However, cells infected with other ECOR strains without the haemolysin gene (ECOR8 and 65 in Fig. 1 and Table 1) or even with this gene but without haemolytic activity at all (ECOR63 in Fig. 1 and Table 1) were still alive and did not display a significant cytotoxic or detaching activity after being cultured for 24 h.

PCR detection of the AA probe sequence and the astA gene among ECOR strains

Bacterial clump formation has been suggested as a rapid test for identification of EAEC (Albert et al., 1993). At the surface of ECOR8 liquid cultures we observed obvious bacterial clumps (data not shown), which we interpreted as an indication that ECOR8 might be a representative of EAEC strains in the ECOR collection. This observation prompted us to further screen this collection by PCR designed specifically for the detection of the AA probe sequence (Schmidt et al., 1995b). The AA probe sequence was only found in strain ECOR8 (see below for sequence analysis). We subsequently used the PCR primers for astA (Itoh et al., 1997; Savarino et al., 1993) to amplify the respective fragment. Some of the astA PCR-positive strains are shown in Fig. 2. EAEC strain 17-2 and the E. coli K-12 strain MC1061 were used as positive and negative controls for astA, respectively.

Nucleotide sequences of the AA probe and the astA from ECOR

Comparing the original EAEC AA probe sequence (Schmidt et al., 1995b) with that of ECOR8 from this work (data not shown), we found only 2 nucleotides changed at positions 387 (T → C) and 484 (G → A). It is not yet known if that region is within the translated part and whether or not these two changes lead to predicted amino acid changes.

All the previous astA sequences were derived from diarrhoeagenic E. coli (Savarino et al., 1993; Yamamoto & Echeverria, 1996; Yamamoto & Nakazawa, 1997; Yamamoto et al., 1997). We sequenced the astA gene from strains in the ECOR collection. Comparison of the published astA sequences and our data from this study is shown in Fig. 3. The astA sequences of strains ECOR3, ECOR43 and ECOR68 were identical to the published common astA sequences of EAEC strain 042 (Yamamoto et al., 1997), DAEC (Yamamoto et al., 1997), EPEC (Yamamoto et al., 1997), DAEC (Yamamoto et al., 1997), EPEC (Yamamoto & Echeverria, 1996; Yamamoto & Nakazawa, 1997), and ECOR5, 10, 43, 44 and 68 is shown in the first (top) line of the pile-up nucleotide sequences; nucleotides that are identical to the consensus sequence are shown as dashes. The deduced amino acid sequence of EAST1 is shown above and the respective substitutions below the nucleotide sequences. The residues within parentheses and square brackets represent the synonymous base changes at the third base of ETEC strain 887, and of ECOR 10 and 44, respectively, as compared with the consensus sequence. The astA nucleotide sequences of EAEC strain 17-2 and EPEC-related E. coli (EPEC-r) are from Savarino et al. (1993) and Yamamoto et al. (1997), respectively.
Table 2. Calculated percentage identity among deduced EAST1 amino acid and (in parentheses) astA nucleotide sequences

<table>
<thead>
<tr>
<th></th>
<th>Consensus*</th>
<th>17-2†</th>
<th>ECOR33</th>
<th>ECOR32, 35</th>
<th>EPEC-r‡</th>
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<td>Consensus</td>
<td>100 (100)</td>
<td></td>
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<tr>
<td>17-2</td>
<td>97-37 (99.15)</td>
<td>100 (100)</td>
<td></td>
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<tr>
<td>ECOR33</td>
<td>97-37 (97.44)</td>
<td>94-74 (96.58)</td>
<td>100 (100)</td>
<td></td>
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</tr>
<tr>
<td>ECOR32, 35</td>
<td>94-74 (95.73)</td>
<td>92-11 (94.87)</td>
<td>97-37 (98.29)</td>
<td>100 (100)</td>
<td></td>
</tr>
<tr>
<td>EPEC-r</td>
<td>92-11 (96.58)</td>
<td>89-47 (95.73)</td>
<td>92-11 (94.02)</td>
<td>89-47 (95.73)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

* Including DAEC, EAEC, EPEC, ETEC and ECOR strains 5, 10, 30, 43, 44, 68 and 71.
† EAEC variant from Savarino et al. (1993).
‡ EPEC-related E. coli strain N1 from Yamamoto et al. (1997).

that these two strains had two more base changes at position 23 (GCG → GTC), leading to an amino acid change (Ala → Val). Besides these three bases, they also differed from EAEC strain 17-2 (Savarino et al., 1993) by one more base at codon position 21 (GCA → ACA), resulting in a total of two deduced amino acid changes at positions 11 (Ala → Thr) and 21 (Thr → Ala). ECOR strains 30 and 71 also differed in their astA sequences from the consensus sequence in the third bases at either positions 8, 10 or 23 (see Fig. 3 for details).

The percentages of identity both in nucleotide and in predicted amino acid sequences (Table 2) were calculated to determine the approximate genetic distance among the various astA and EAST1 sequences. At the amino acid level, the sequence from EPEC-related E. coli (Yamamoto et al., 1997) appeared to be the most distant one known so far as compared with the consensus deduced protein sequence.

Within the astA alleles from the ten ECOR strains, there were eleven polymorphic sites in the 117 bp segment. The astA coding sequence has a GC content of approximately 54 mol%, which is a little higher than the mean value for genes of E. coli (50.8%).

Distribution of astA among ECOR strains

astA was sparsely distributed among four groups but absent from group B2, with two strains in group A, five strains in group B1, two strains in group D and one strain in group E. Preliminary studies of the location of the astA sequences suggested that the gene may be plasmid-located. We obtained amplified fragments of the same size using plasmid DNA preparations as template from all astA-positive strains (data not shown). One interesting finding was that two isolates from the same giraffe in Washington Zoo (Ochman & Selander, 1984b), ECOR32 (07:H21) and ECOR68 (0N:NM), were both found in this study to harbour astA, but their nucleotide sequences differed from each other by five bases, causing two deduced amino acid changes (Fig. 3). This result may indicate that astA has been subjected to some genetic exchanges, such as intragenic recombination, rather than horizontal transfer. Most of the astA-positive strains (8/10) were from North America (Canada, 1; USA, 7), and the remaining two from Europe (2 out of the 22 strains from Sweden) whose sequences were identical to the common astA sequence.

**DISCUSSION**

In this study we provide evidence showing that the ECOR collection includes at least one putative EAEC strain (ECOR8) and some potential pathogenic strains as indicated by their carrying various virulence factors and displaying distinct cytotoxic effects on different host cells (macrophages and erythrocytes). These haemolytic strains resemble a minor category of E. coli called cell-detaching E. coli which are potentially diarrhoeagenic (Gunzburg et al., 1993; Marques et al., 1995; Nataro & Kaper, 1998). Considering the relatively low G + C content (about 40 mol%) of blyA (Felmlee et al., 1985) and its close linkage with pap, sfa and cnf (Blum et al., 1995), one could speculate that blyA may have been recently acquired through horizontal transfer in these ECOR strains as one of these pathogenicity island elements.

We found that obvious cell detachment and toxicity correlated well with the expression of highly active α haemolysin, but not with the existence of the astA gene or the silent blyA gene under the conditions used. Possible explanations for the differential expression of HlyA and haemolytic activity of these blyA-positive strains are that some strains (ECOR24, 48, 63) have an incomplete blyCABD operon (Stanley et al., 1998) or that the potential amino acid substitutions deduced from their partially sequenced blyA genes (Boyd & Hartl, 1998) lead to a decrease (or loss) of haemolytic activity. ECOR51 has a strong haemolytic phenotype but seems to express less of the mature 110 kDa HlyA. It is feasible that the HlyA from ECOR51 either has higher specific activity than those from other ECOR strains or that its partially degraded HlyA (i.e. 60 kDa) product confers this phenotype. Earlier studies have shown that this breakdown product of HlyA recognized by HlyA antibodies may have haemolytic activity (Noegel et al., 1979; Goebel & Hedgepeth, 1982; Nicaud et al., 1985).
ECOR is a complex collection and evidently some of the strains expressed active haemolysin and are potential pathogens. The bly operon is commonly present in multiple copies in the chromosome of extraintestinal E. coli isolates (Blum et al., 1995). The linkage between genes for haemolysin and P fimbriae is estimated to be 4–15 kbp for several O6 and O4 isolates (Low et al., 1984), which are also the serotypes of ECOR strains 53, 56 and 60 (Table 1). Elliott et al. (1998) demonstrated that z haemolysin confers virulence to a nonpathogen in the RITARD animal model, causing inflammation. Island et al. (1998) reported that haemolytic and CNF1-negative E. coli show cytotoxicity in T24 human bladder cells. Results from this study further prove the relatedness between target cell toxicity and detachment and the expression of highly active haemolysin. The hallmark of infections due to EPEC and EHEC is the attaching-and-effacing (A/E) histopathology (Law & Chart, 1998; Nataro & Kaper, 1998). A recombinant plasmid clone containing the entire locus for enterocyte effacement (LEE) region is sufficient to confer the A/E phenotype when cloned into K-12 or E. coli from the normal flora (McDaniel & Kaper, 1997). Using a PCR-based assay, it was found that ECOR37, a healthy animal isolate of untypable serotype, contains a LEE island at the selC locus (Berghorsson & Ochman, 1998), which is consistent with MLEE results repeatedly showing this strain most closely linked to EHEC O157:H7 strains (Perna et al., 1998; Pupo et al., 1997) and with a newly proposed evolutionary model where an EPEC-like strain with LEE is the ancestor of O157:H7 (Feng et al., 1998). One study shows that the chromosomes of some ECOR strains are several hundred kilobases larger than others; thus they could possibly harbour additional pathogenicity islands (Berghorsson & Ochman, 1998).

It was reported that the PCR assay with primers derived from the AA probe sequence shows similar sensitivity (89%) and specificity to those of the AA probe (Schmidt et al., 1995b), which is used as an alternative standard to define EAEC (Law & Chart, 1998; Nataro & Kaper, 1998). ECOR8 (O86:NM, one of the EAEC typical serotypes) was positive with this PCR assay and we further confirmed by sequence analysis that it contained the AA probe sequence. Previous work showed that ECOR8 carries genes for PapI and Kps, which are absent in E. coli K-12 (Boyd & Hartl, 1998), and harbours a single copy of IS200 (Bisercic & Ochman, 1993). The pathogenicity potential of ECOR8 remains to be determined.

Since the first astA sequence from EAEC strain 17–2 was published (Savarino et al., 1993), Yamamoto & Echeverria (1996) and Yamamoto et al. (1997) subsequently sequenced its homologues from other diarrhoeagenic E. coli and found that all of their astA genes were nearly identical to each other but differed from the original one from 17–2. Comparison of the astA sequences from EAEC (Yamamoto et al., 1997), ETEC (Yamamoto & Echeverria, 1996; Yamamoto & Nakazawa, 1997), EPEC (Yamamoto et al., 1997), EPEC-related E. coli (Yamamoto et al., 1997) and ECOR strains (this report) made it possible to propose the common nucleotide sequence shared by EAEC, ETEC, EPEC and ECOR strains as the astA consensus sequence, whilst those which differed in deduced amino acid sequence were variants (including the astA sequence from strains 17–2, N1 and ECOR strains 32, 33 and 35) (Savarino et al., 1993; Yamamoto et al., 1997; and this study). Savarino et al. (1993) first designed a synthetic peptide spanning the region of EAST1 from residue 8 to 29 with enterotoxic activity. From Fig. 3 one can clearly see that within the functional domain, positions 8–11, 20, 21, 23 and 33 are hot spots for nucleotide changes. Interestingly, sequence analysis of astA from ECOR strains reveals that all the astA nucleotide variations are located in this functional region. At the eighth and ninth (arginine) residues, both changes in the third base do not cause the deduced amino acid substitution, whilst the alanine residue at positions 11 and 23 leads to substitutions with threonine and valine, respectively. It will be interesting to further investigate the biological significance of the amino acid change from the tiny, hydrophobic, non-polar alanine to the small, less hydrophobic, polar threonine or to the small, aliphatic, less hydrophobic valine. Also interesting would be to check the expression of astA in vivo and in vitro when specific antiserum becomes available, and its activity with the Ussing chamber (Savarino et al., 1993).

The pathogenesis of EAEC infection is not well understood. The significance of EAST1 in pathogenesis is unknown although E. coli categories other than EAEC, notably the EHEC, ETEC and EPEC strains, have been shown to produce this toxin with high frequency (Savarino et al., 1996). According to the three-stage model of EAEC pathogenesis proposed by Nataro & Kaper (1998), EAST1 might be involved at the third stage where the elaboration of an EAEC cytotoxin could damage the intestinal cells. It was also suggested recently that EAST1 toxin is closely associated with an adherence factor, CS31A, among pathogenic bovine E. coli (Bertin et al., 1998). We are more in favour of the notion that astA may confer an ecological advantage to intestinal E. coli isolates simply by contributing to pathogenesis (Savarino et al., 1996), although this hypothesis remains to be verified.

Due to the heterogeneous status of the ECOR collection for carrying various virulence factors, it seems appropriate to conclude that some of the ECOR strains of both human and animal origin are potential pathogens with features of cell-detaching E. coli. Caution should be taken when handling or disposing of these strains.

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Expression of cytotoxicity by ECOR strains


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