

Haemolysin production by strains of Verocytotoxin-producing *Escherichia coli*

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Twenty-one strains of Verocytotoxin-producing *Escherichia coli* (VTEC) that hybridized with DNA probe CVD419 were examined for the ability to produce haemolysin. With solid media, all strains produced most haemolysin when grown in blood agar tubes and least when grown on blood agar plates incubated in air. Haemolysin production was increased considerably by incubating blood agar plates in an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52% nitrogen at 37 °C for 16 h, followed by 6 h at 21 °C in air. Haemolysin production was also increased when strains were grown on L-agar containing the iron chelator ethylenediamine-di(o-hydroxyphenylacetic acid) prior to subculture on blood agar. Intracellular haemolysin was detected in five out of the 21 strains of *E. coli* grown on L-agar in the atmosphere described above, but haemolysin was not detected in L-broth culture supernatants. The haemolysins lysed guinea pig, mouse and ferret erythrocytes, but not human, rabbit, rat, turkey or chicken erythrocytes. Also, the addition of calcium ions to culture media was not required for haemolytic activity. It was concluded that haemolysins produced by VTEC appear to be quite distinct from *E. coli* α -haemolysin and resemble a form of β -haemolysin.

Keywords: Verocytotoxin-producing *Escherichia coli*, haemolysin, enterohaemolysin, growth conditions, iron

INTRODUCTION

Haemolysins form a family of lytic toxins which can play a key role in the pathogenesis of disease. Strains of haemolytic *Escherichia coli* produce either α - or β -haemolysins which are secreted and cell-associated, respectively. Both types of haemolysin are expressed in the exponential phase of growth, but only α -haemolysin requires calcium ions for lytic activity. Recently, strains of Verocytotoxin-producing *E. coli* (VTEC), belonging to serotypes O26:H11, O111:H— and O157:H7, were shown to produce a plasmid-encoded haemolysin termed ‘enterohaemolysin’ (Beutin *et al.*, 1988, 1989, 1994). The extent to which strains of VTEC produce haemolysin has not been elucidated fully; however, a DNA probe (CVD419) devised by Levine *et al.* (1987) to detect enterohaemorrhagic *E. coli* was used by Schmidt *et al.* (1995) to detect VTEC producing ‘enterohaemolysin’.

The haemolysin described by Schmidt *et al.* (1995) as enterohaemolysin was shown to be genetically related to *E. coli* α -haemolysin (Schmidt *et al.*, 1995), having a similar molecular mass of approximately 110 kDa and a requirement for calcium ions for haemolytic activity (Beutin *et al.*, 1989). Enterohaemolysin activity has been detected most frequently with washed sheep erythrocytes, but unlike α -haemolysin, enterohaemolysin does not lyse horse erythrocytes (Cavaliere *et al.*, 1984; Scotland *et al.*, 1990).

The role of haemolysins in the pathogenesis of VTEC infections is not known. However, patients with haemolytic uraemic syndrome, caused by strains of *E. coli* O157, have been shown to produce serum antibodies to enterohaemolysin (Schmidt *et al.*, 1995), suggesting that this haemolysin is probably expressed during pathogenesis.

With the aim of improving our understanding of the expression of haemolysin by VTEC, we examined strains for production and mode of action of haemolysin.

Abbreviations: EDDA, ethylenediamine-di(o-hydroxyphenylacetic acid); VTEC, Verocytotoxin-producing *Escherichia coli*

METHODS

Bacteria. All 21 strains of *E. coli* used in the present study (Table 1) were isolated from human cases of diarrhoea, bloody diarrhoea or haemolytic uraemic syndrome. Except for *E. coli* strain E20513, all strains produced Verocytotoxin VT1, VT2 or both and hybridized with the gene probe CVD419 (Scotland *et al.*, 1990). *E. coli* strains E92356 and E101402 were α -haemolysin-producing control strains. Strains were stored on Dorset's egg agar slopes at room temperature and were obtained from the culture collection held by the Laboratory of Enteric Pathogens (London, UK).

Culture media. Bacteria were grown in L-broth (37 °C, 6 h) prior to inoculation of experimental media. One experimental medium used was L-agar (Oxoid) with and without the addition of CaCl₂·2H₂O (10 mM), and with and without 5% (v/v) erythrocytes from chicken, ferret, guinea pig, human (AB⁺, A⁺, B, O⁺, O⁻), mouse, rabbit, rat, sheep or turkey. Erythrocytes were washed ($\times 3$) in PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) with and without 10 mM CaCl₂·2H₂O. Blood agar was also used to make agar plates and agar tubes (8 \times 1 cm) and bacteria were also grown in L-broth or on L-agar containing 100 μ g EDDA (Sigma) ml⁻¹, with and without the addition of 5 mM FeCl₃. EDDA was made iron-free by the method of Rogers (1973). The ability of bacteria to produce gas was determined by

growing strains in glucose/peptone broth containing a Durham tube.

Growth conditions. Bacteria were grown in air, in 5% carbon dioxide in air (British Oxygen) and in atmospheres comprising: (i) 12% oxygen, 7% carbon dioxide, 35% hydrogen and 46% nitrogen (Oxoid Gas Generating kit BR38 without catalyst); (ii) 8% carbon dioxide, 40% hydrogen and 52% nitrogen (Oxoid Gas Generating kit BR38 without catalyst); (iii) 13% carbon dioxide and 87% nitrogen (Oxoid Gas Generating kit BR38); and (iv) 5% oxygen, 5% carbon dioxide, 3% hydrogen and 87% nitrogen (variable atmosphere incubator; Don Whitley Scientific). Bacteria were grown in these various atmospheres at 37 and 21 °C for a range of incubation times.

Extracellular/intracellular haemolysin. Strains of *E. coli* were examined for extracellular haemolysin using L-broth culture supernatants (see below). For the detection of intracellular haemolysin, bacteria were grown on L-agar in an atmosphere of 8% carbon dioxide, 40% hydrogen and 52% nitrogen (Oxoid Gas Generating kit BR38 with catalyst). For agar-grown bacteria, the combined cell mass from three plates was harvested. Bacteria were suspended in 5 ml of either PBS alone or PBS containing 1% (w/w) BSA (Sigma), prior to sonication (30 s) using a Heat Systems W-225 sonicator (Ultrasonics). Bacterial debris was sedimented by centrifugation (15 000 g,

Table 1. Strains of *E. coli* used in the present study

Strain no.	Serotype	Intracellular haemolysin*	Contact haemolysin†	Reference/source‡
E20513§	O111:H2	ND	0.00	Chart <i>et al.</i> (1988)
E68446	O?:H-	ND	7.66	Thomas <i>et al.</i> (1996)
E41787	O5:H-	ND	9.33	Willshaw <i>et al.</i> (1992)
E113378	O26:H11	2.00	41.50	LEP (unpublished)
E105991	O26:H11	0.86	10.90	LEP (unpublished)
E107943	O26:H11	1.50	143.59	LEP (unpublished)
E108848	O26:H11	ND	62.18	LEP (unpublished)
E113372	O26:H11	ND	19.77	LEP (unpublished)
E45037	O91:H21	ND	18.02	Willshaw <i>et al.</i> (1992)
E43549	O105ac:H19	ND	12.11	Willshaw <i>et al.</i> (1992)
E55391	O111ac:H-	1.04	36.44	Willshaw <i>et al.</i> (1992)
E52849	O111ac:H-	1.35	17.18	Willshaw <i>et al.</i> (1992)
E75647	O121:H19	ND	30.50	LEP (unpublished)
E113371	O128ab:H2	ND	2.51	LEP (unpublished)
E113375	O128ab:H2	ND	31.50	LEP (unpublished)
E41509	O128ab:H2	ND	110.17	Willshaw <i>et al.</i> (1992)
E40135	O128ab:H25	ND	1.84	Willshaw <i>et al.</i> (1992)
E92568	O134:H25	ND	17.51	Thomas <i>et al.</i> (1996)
E42772	O145:H25	ND	51.40	Willshaw <i>et al.</i> (1992)
E31708	O163:H19	ND	7.67	Willshaw <i>et al.</i> (1992)
E46091	O165:H25	ND	32.00	Willshaw <i>et al.</i> (1992)
E80184/1	O173:H2	ND	42.41	Thomas <i>et al.</i> (1996)

* Measured at 430 nm; ND, extracellular haemolysin not detected.

† OD₄₃₀/bacterial cell mass.

‡ LEP, Laboratory of Enteric Pathogens.

§ Haemolysin-negative control.

|| Strains which did not produce gas.

15 min) and supernatants were filtered (0.45 µm filter; Millipore). Culture supernatant or sonicated preparation (5 ml) was mixed with 10 µl guinea pig blood, washed ($\times 3$) with PBS and incubated at room temperature for 6 h. Whole erythrocytes were sedimented (4000 g, 5 min) and haemolysis was quantified by measuring the OD₄₃₀ (Unicam SP600 spectrophotometer; Pye Unicam).

Quantifying intracellular haemolysin. Haemolysin production was quantified by determining the wavelength at which maximum absorbance (λ_{\max}) of lysed guinea pig erythrocytes was observed. Intracellular haemolysin was mixed with guinea pig erythrocytes (as described above) and the λ_{\max} for lysed erythrocytes was identified using cell-free supernatant as 430 nm.

Contact haemolysin. Contact haemolysin was detected by the method of Haque *et al.* (1994), except that calcium ions were omitted from the PBS. Strains of *E. coli* were grown on L-agar in an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52% nitrogen at 37 °C for 16 h, followed by 6 h in air at 21 °C. Bacterial cell mass was transferred to pre-weighed screw-capped Eppendorf tubes and reacted with guinea pig erythrocytes.

Oxygen scavengers. Guinea pig blood agar plates and tubes were prepared containing: 5% (v/v) DMSO; 5 mM sodium hydrosulphite; 5 mM 1,3-diphenylisofuran; or 5 mM DTT (all from Sigma).

RESULTS

Haemolysin production

Strains of *E. coli* 'streaked' onto the surface of L-agar containing washed (PBS/CaCl₂) sheep erythrocytes produced zones of haemolysis which extended approximately 1 mm beyond the edge of the bacterial cell mass but not around individual colonies. However, when bacteria were inoculated into blood agar with a straight wire the zones of haemolysis were noticeably larger, although zones around individual colonies were very narrow. In contrast, control strains of α -haemolysin-producing *E. coli* gave clear zones of haemolysis under these growth conditions.

Inoculating bacteria deep into sheep blood agar tubes with a straight wire resulted in complete clearing of the blood agar down to the point reached by the inoculating wire. For 16 strains, the agar tubes became fragmented due to production of gas (Table 1); these strains were subsequently shown to produce gas when growing in glucose/peptone broth with an inverted Durham tube. *E. coli* strains E41787, E46091, E113378, E105991 and E107943 were non-gas producing and were used for further studies.

Role of calcium in haemolysis

The role of calcium ions in the haemolytic process was examined using the blood agar tube method. Washing sheep erythrocytes ($\times 3$) in PBS was essential for haemolysis; however, the addition of CaCl₂ to either erythrocyte washing solutions or blood agar was not required for haemolytic activity. In contrast, the α -haemolysin-producing control strains required sheep



Fig. 1. Strains of *E. coli* were most haemolytic when grown in an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52% nitrogen at 37 °C for 16 h, followed by 6 h in air at 21 °C.

cells to be washed with PBS/CaCl₂ for haemolysis to occur.

Haemolysin specificity

Using blood agar tubes, strains of *E. coli* lysed guinea pig, mouse and ferret erythrocytes from, but not those from human, rabbit, rat, turkey or chicken.

Oxygen scavengers

The role of oxygen in haemolysin production was examined by growing strains in the presence of oxygen scavengers. Addition of DMSO, sodium hydrosulphite, 1,3-diphenylisofuran or DTT to blood agar did not influence haemolysin production.

Growth atmosphere

Strains of *E. coli* produced maximum zones of haemolysis when grown on L-agar plates containing guinea pig erythrocytes in an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52% nitrogen at 37 °C for 16 h, followed by 6 h in air at 21 °C (Fig. 1). Haemolysin production was reduced if the 6 h incubation period was carried out at either 37 °C in air or 21 °C in 8% carbon dioxide, 40% hydrogen and 52% nitrogen.

Iron and haemolysin production

Bacteria grown on L-agar containing EDDA were used to inoculate L-agar plates containing 5% guinea pig erythrocytes prior to culture in an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52%

nitrogen at 37 °C for 16 h, followed by 6 h in air at 21 °C. Bacteria grown on blood agar produced noticeably larger zones of haemolysis compared with bacteria grown on L-agar containing EDDA with added FeCl₃.

Extracellular/intracellular haemolysin

Haemolysin activity was not detected in broth culture supernatants prepared from any of the strains examined. However, intracellular haemolysin was detected in five of the 21 test strains when bacteria were grown on agar and sonicated (Table 1). For the detection of intracellular haemolysin, it was essential to grow strains on L-agar plates incubated in an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52% nitrogen; haemolysin activity was detected only when BSA was present in the PBS used for sonication. Extracellular α -haemolysin was detected with control strains of *E. coli*.

DISCUSSION

The production of haemolysins by strains of VTEC was influenced considerably by environmental growth conditions. The maximum observed haemolysin production was achieved by growing bacteria in agar tubes, suggesting that either component gases in air were inhibiting haemolysin production or the gas mixture generated by bacteria growing deep in agar columns was particularly suitable for the expression of haemolysin. With blood agar plates, maximum observed haemolysin activity was detected with an atmosphere comprising 8% carbon dioxide, 40% hydrogen and 52% nitrogen, suggesting that a component of air was inhibiting haemolysin production. However, the use of oxygen scavengers showed that oxygen did not inhibit haemolysin production, suggesting that expression of the haemolysin may have been influenced by the specific ratio of carbon dioxide:hydrogen:nitrogen.

Maximum observed haemolysin production was detected when bacteria were grown in a specific atmosphere at 37 °C for 16 h, followed by 6 h in air at 21 °C. The reasons why this temperature regime should increase haemolysin production are unknown; however, other workers (Beutin *et al.*, 1995) have also employed a two-step incubation temperature of 37 °C followed by 30 °C to maximize haemolysin production. It would seem unlikely that a two-step temperature regime was essential for the expression of haemolysin during pathogenesis.

The observation that haemolysin was not detected in broth culture supernatants but could be detected intracellularly suggested that haemolysin was made but not exported from the cell. This resembles the situation in strains of *Edwardsiella tarda* which are haemolytic although haemolysin cannot be detected in culture supernatants (Janda & Abbott, 1993).

The haemolytic action of haemolysin did not require the addition of calcium ions, which are essential for α -

haemolysin activity (Beutin, 1991). This suggests that either the culture media contain sufficient calcium for haemolysis to take place or the observed haemolysis was calcium-independent. Calcium-independent haemolysins have been described for bacteria such as *Proteus* spp. and *Serratia marcescens* (Welch, 1987; Koronakis *et al.*, 1987; Braun *et al.*, 1985). It is possible that the haemolysin described herein may belong to the calcium-independent family of haemolysins and therefore be quite distinct from α -haemolysin.

Growing strains of VTEC on iron-restricted L-agar, prior to inoculation of blood agar plates, increased haemolysin production, suggesting that this haemolysin was iron-regulated. Certain haemolysins, including α -haemolysin, are iron-regulated with the expression of toxin controlled by the chromosomally located *fur* gene (Grunig *et al.*, 1987). Haemolysis during pathogenesis has been postulated as a means of providing pathogenic bacteria with iron (Lebek & Gruening, 1985); the iron-restricted conditions existing in host tissues may form the switch for the expression of haemolysin. A feature of human infections with VTEC, particularly with *E. coli* O157:H7, is haemolytic anaemia, which may be caused by a haemolysin. However, since our studies showed that haemolysin did not lyse human erythrocytes, its role in the pathogenesis of VTEC infections remains to be resolved. Beutin *et al.* (1989) described an α -like haemolysin, termed enterohaemolysin, which lysed human erythrocytes; we failed to observe this with our panel of VTEC using human erythrocytes belonging to a range of blood groups. We conclude that VTEC haemolysins are unlike *E. coli* α -haemolysin in not being actively secreted and not requiring calcium ions for activity.

ACKNOWLEDGEMENTS

This study was funded in part by a grant from the Department of Health.

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Received 4 July 1997; revised 25 August 1997; accepted 2 September 1997.