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International Journal of Medical Microbiology 295 (2005) 99–107
IJMM
www.elsevier.de/ijmm

The detection of salmochelin and yersiniabactin in uropathogenic *Escherichia coli* strains by a novel hydrolysis-fluorescence-detection (HFD) method

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Received 15 October 2004; received in revised form 20 January 2005; accepted 3 February 2005

Abstract

Escherichia coli strains produce a variety of structurally different siderophores of which enterobactin, aerobactin and yersiniabactin have been reported earlier to occur in strains of extraintestinal infections. In uropathogenic *E. coli* (UPEC) strains novel siderophores, named salmochelins, have recently been identified which contain C-glucosylated 2,3-dihydroxybenzoyl-L-serine (glucosyl-DHB-serine) residues connected in a linear (mono-, di- , trimeric) or cyclic form. We report here on a fast and simple hydrolysis-fluorescence-detection (HFD) method, based on identification of C-glucosylated dihydroxybenzoic acid (glucosyl-DHB). Salmochelin containing culture filtrates were bound to DEAE cellulose spin columns, hydrolyzed and the breakdown products were subsequently identified by HPLC or thin layer chromatography (TLC). The hydrolysis products can be easily detected by their fluorescence, either during HPLC separation connected to a fluorescence detector or after TLC on cellulose plates viewed under a UV_{254} or UV_{365} lamp. While DHB originates from the hydrolysis of enterobactin and salmochelin, glucosyl-DHB is only found as a characteristic hydrolysis product of salmochelins (S1, S2, S4). The HFD method allows detection of salmochelin in the presence of other siderophores, such as enterobactin, aerobactin and yersiniabactin. Several clinical UPEC isolates containing the iro*N* gene cluster were analyzed by this procedure, showing that all isolates were glucosyl-DHB positive indicating salmochelin production, while a collection of other pathogenic *E. coli* strains (EHEC, EIEC, ETEC, EAggEC and EPEC) were glucosyl-DHB negative. In addition, the HFD method allowed the identification of yersiniabactin due to a fluorescent salicylate-containing degradation product.

Keywords: Siderophores; Enterobactin; Salmochelin; Yersiniabactin; UPEC; ExPEC; *E. coli*

Introduction

Iron acquisition systems in bacteria comprise the biosynthesis of siderophores and their cognate transport systems residing in the inner and outer membranes. In enterobacteria three different types of siderophores have been described, the catecholates (enterobactin, salmochelins, serratiochelins), hydroxamates (ferrioxamines) and mixed ligand type siderophores (aerobactin, yersiniabactin) which are optimally designed for their iron transport function in various ecological environments

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doi:10.1016/j.ijmm.2005.02.001

(Winkelmann, 2001, 2002). Growth of pathogenic bacteria depend on the ability to acquire iron in the presence of iron-withholding proteins like transferrin (Evans and Oakhill, 2002), lactoferrin (Ekins et al., 2004) and the siderophore-binding lipocalins (Goetz et al., 2002; Fluckinger et al., 2004; Flo et al., 2004). Although both, saprophytic and pathogenic bacteria produce siderophores, it is the environmental suitability of the siderophores and the regulation of the receptors under conditions of extra- and intraintestinal growth that makes them a virulence factor. Besides enterobactin, other siderophores occur in enterobacteria of which aerobactin and yersiniabactin have frequently been linked with pathogenicity in host fluids and tissues. The encoding genes are often found on plasmids or so-called pathogenicity islands (PAI) together with operons encoding fimbriae and other virulence properties. The pathogenicity island encoding the yersiniabactin system has been termed high pathogenicity island (HPI) due to its prevalence in highly pathogenic *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* biotype IB strains (Carniel et al., 1996; Carniel, 2001; Schubert et al., 1998, 2000). The yersiniabactin HPI has been further identified by PCR in *Salmonella*, *Serratia*, *Klebsiella*, *Enterobacter*, and *Citrobacter* (Bach et al., 2000; Schubert et al., 2000; Zaharik et al., 2002), and in particular in various extraintestinal pathotypes of *Escherichia coli* (ExPEC) isolated from blood cultures and urine samples (Russo and Johnson, 2000; Johnson et al., 2000; Schubert et al., 2002) as well as in some non-O157 serovars of shigatoxin producing *E. coli* (Karch et al., 1999). This distribution suggests that yersiniabactin may be important in certain sites of infection. Virulence of *E. coli* might be differently expressed depending on their extraintestinal or fecal ecology (Johnson et al., 2000). Recent reports suggest that also the salmochelin system encoded by the *iroBCDEN* genes may play a role in the pathogenicity of *Salmonella* and uropathogenic *E. coli* strains (Bäumler et al., 1998; Hantke et al., 2003). The present investigation was mainly designed to assess the production of salmochelins under iron-restricted growth by using a novel hydrolysis-fluorescence-detection (HFD) method with subsequent chromatographic assignment of the fluorescent hydrolysis products.

Materials and methods

Strains and growth conditions

A representative collection of *E. coli* strains originating from the stock of the Robert Koch-Institut (RKI) which had previously been grouped into EHEC, EIEC, ETEC, EAEC, and UPEC strains was analyzed for their ability to produce catecholate siderophores. UPEC isolates were from the Department of Biology, University of Ljubljana, Slovenia and were characterized besides other markers for carrying the *iro*N gene (Starcic-Erjavec et al., submitted). Strains were grown in low-iron M63 medium (Hantke et al., 2003).

In addition, the following strains were used for comparison: *E. coli* SK22, also known as strain Nissle 1917, was used as a producer of aerobactin, enterobactin, salmochelin, and yersiniabactin. The *E. coli* K-12 derivative H1443 *malT tsx aroB* was used to demonstrate enterobactin-dependent growth stimulation while *Yersinia enterocolitica* H1804 WA-1 *ybt*-1 (serotype: O8) and *Y. enterocolitica* H5030 *ybt*-30 (serotype: O8) were stimulated by yersiniabactin in their growth on a low-iron medium.

Isolation and hydrolysis of siderophores

From low-iron culture supernatants samples (2 ml) were removed to which $FeSO_4$ (2 mM) was added to allow iron-complex formation. This solution was pipetted into a 2 ml polypropylene tube containing a centrifuge tube filter (cellulose acetate, 0.22 μm, Costar Corning Inc.) filled with 0.5 ml of DEAE cellulose (bed volume) to bind negatively charged siderophores including enterobactin, salmochelin, yersiniabactin, and aerobactin. After washing with water (3×0.5 ml) and centrifugation for 2 min at 13,000*g* the bound siderophores were eluted by centrifugation with 2×0.5 ml NH_4Cl (2 M). The eluate containing catecholates, yersiniabactin and aerobactin was hydrolyzed in H_2SO_4 (3 M final concentration) by mixing 700 μl NH_4Cl eluate with 300 μl H_2SO_4 (10 M) and heating in a boiling waterbath (2 h) in a closed vial. The hydrolyzate was then transferred to another centrifuge tube containing 0.5 ml Amberlite XAD-16 as an adsorbing resin. After centrifugation (2 min, 13,000*g*) the resin was treated with distilled water (2×0.5 ml), the bound catecholate fragments consisting of 2,3-dihydroxybenzoic acid (DHB), glucosyl-DHB and degradation products of yersiniabactin (YBD) were eluted with 1 ml methanol.

HPLC separation of enterobactin, salmochelin and its degradation products

HPLC separation of siderophores was performed on a Shimadzu HPLC equipped with two LC-10AT pumps, system controller SCL-10A, autoinjector SIL-10AXL, UV-VIS spectrophotometric detector SPD-10AV, and a fluorescence detector RFXL. The siderophores were separated on a C18 reversed phase column (ReproSil-pur 120, ODS-3, 5 μm, 250 mm × 4 mm, Dr. Maisch, Ammerbuch) using a gradient of 6–40% acetonitrile (ACN) in water (+0.1% trifluoroacetic acid (TFA)) within 20 min using a fluorescence detector (excitation 320 nm, emission 420 nm). The resulting peaks were