Plasmid 69 (2013) 127–137

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

The IncF plasmid pRSB225 isolated from a municipal wastewater treatment plant's on-site preflooder combining antibiotic resistance and putative virulence functions is highly related to virulence plasmids identified in pathogenic *E. coli* isolates

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ARTICLE INFO

Article history:
Received 25 September 2012
Accepted 18 November 2012
Available online 2 December 2012
Communicated by Prof. R. Chalmers

Keywords:
IncF plasmid
Wastewater treatment plant
Antibiotic resistance
Host range
Mobile genetic elements
Conjugative transfer

ABSTRACT

The IncF antibiotic resistance and virulence plasmid pRSB225, isolated from an unknown bacterium released with the purified wastewater from a municipal sewage treatment plant into the environment has been analysed at the genomic level by pyrosequencing. The 164,550 bp plasmid comprises 210 coding sequences (cds). It is composed of three replicons (RepFIA, RepFIB, and RepFII) and encodes further plasmid-specific functions for stable maintenance and inheritance and conjugative plasmid transfer. The plasmid is self-transmissible and shows a narrow host range limited to the family *Enterobacteriaceae*. The accessory modules of the plasmid mainly comprise genes conferring resistance to ampicillin (bla_{TEM-1b}), chloramphenicol (*catA1*), erythromycin (*mphA*), kanamycin and neomycin (*aphA1*), streptomycin (*strAB*), sulphonamides (*sul2*), tetracycline (*tetA*(B)) and trimethoprim (*dfrA14*), as well as mercuric ions (*mer* genes). In addition, putative virulence-associated genes coding for iron uptake (*iutA*/*iucABCD*, *sitABCD*, and a putative high-affinity Fe^{2+} uptake system) and for a toxin/antitoxin system (*vagCD*) were identified on the plasmid. All antibiotic and heavy metal resistance genes are located either on class 1 (Tn*10*-remnant, Tn*4352B*) and class 2 transposons (Tn*2*-remnant, Tn*21*, Tn*402*-remnant) or a class 1 integron, whereas almost all putative virulence genes are associated with IS elements (IS*1*, IS*26*), indicating that transposition and/or recombination events were responsible for acquisition of the accessory pRSB225 modules. Particular modules of plasmid pRSB225 are related to corresponding segments of different virulence plasmids harboured by pathogenic *Escherichia coli* strains. Moreover, pRSB225 modules were also detected in entero-aggregative-haemorrhagic *E. coli* (EAHEC) draft genome sequences suggesting that IncF plasmids related to pRSB225 mediated gene transfer into pathogenic *E. coli* derivatives.

1. Introduction

The emergence of multidrug resistant pathogenic bacteria belonging to the genera *Escherichia*, *Salmonella* and *Klebsiella* poses a serious problem for human and animal health. Highly resistant representatives of these genera

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0147-619X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.plasmid.2012.11.001

were identified in different habitats (Anjum et al., 2011; Antunes et al., 2011; Peirano and Pitout, 2010; Samuelsen et al., 2011; Uekötter et al., 2011). Moreover, it seems that there is a tendency from single-drug towards multi-drug resistant microorganisms indicating their rapid adaptation to changing prescription practices (French, 2010; Sengstock et al., 2010; Torres et al., 2001).

The extensive use of antimicrobial compounds has been recognized as one of the reasons for the selection of resistant bacteria and their dissemination. Moreover, mobile genetic elements like insertion sequence elements (IS elements), transposons and plasmids facilitating horizontal movement of antibiotic resistance genes contribute to the spread of these determinants. In addition, the presence of heavy metal resistance genes on transposons and plasmids promotes dissemination of antibiotic resistance, since both kinds of determinants are often linked on the same element. Accordingly, presence of heavy metal pollutions selects for the maintenance of these elements (Dröge et al., 2000; Schlüter et al., 2003; Tennstedt et al., 2003). Furthermore, genes coding for putative virulence-associated proteins like hemagglutinin, proteases, hemolysins and iron acquisition systems were identified on antibiotic resistance plasmids isolated from human and avian pathogenic bacteria (Fricke et al., 2008; Herrero et al., 2008; Périchon et al., 2008; Venturini et al., 2010). Linkage of antibiotic and virulence functions on an individual plasmid can enhance the virulence of a microorganism by improving its chance for successful invasion and survival within the infected host. Presence of antibiotic resistance determinants might complicate treatment of infections caused by pathogens harbouring these kind of virulence plasmids.

Plasmids belonging to the incompatibility group F (IncF) play a major role in the dissemination of antibiotic resistance and virulence determinants among members of the family *Enterobacteriaceae*. Conjugative transfer of most IncF plasmids is efficient and they are stably maintained in their hosts since they encode partitioning, stabilization and addiction systems (Lang et al., 2010; Pecota et al., 1997). IncF plasmids encoding antibiotic resistance and virulence functions were previously identified in human pathogens like *Escherichia coli* (Périchon et al., 2008; Venturini et al., 2010), *Klebsiella pneumoniae* (Yi et al., 2010), and *Salmonella enterica* (Fricke et al., 2009). In addition, genomic islands harbouring antibiotic resistance and/or virulence genes are present in *Salmonella*, *Escherichia*, and *Pseudomonas* strains (Dobrindt et al., 2004; Ho Sui et al., 2009; Izumiya et al., 2011). Frequently, plasmids are involved in integration of resistance and virulence clusters into the chromosome of the host bacterium. Moreover, homologous recombination *via* insertion sequence elements seems to play an important role for integration of plasmids or parts thereof into the bacterial chromosome (Izumiya et al., 2011), probably resulting in a more stable state of former plasmid-encoded determinants within the bacterial cell. Such recombination events, also between plasmids within the same host cell (Boyd et al., 1996; Schlüter et al., 2003), can contribute to the diversification and further dissemination of genetic determinants.

In this study, the complete nucleotide sequence of the IncF antibiotic resistance and virulence plasmid pRSB225 isolated from bacteria of a municipal sewage treatment plant's on-site preflooder was analysed. Comparative genomics of pRSB225 and highly related elements provides new insights into the evolution of IncF resistance/virulence plasmids and their dissemination among environmental and pathogenic bacteria including entero-aggregative-heamorrhagic *E. coli* (EAHEC) strains.

2. Materials and methods

2.1. Isolation of plasmid pRSB225 from bacteria of a municipal wastewater treatment plant

Plasmid pRSB225 was isolated from bacteria of a municipal wastewater treatment plant's on-site preflooder during the year 2003 applying the direct isolation method (Tennstedt et al., 2003). For this purpose, 1 l of water obtained from the on-site preflooder was centrifuged (16.000 *g*) and the pellet was resuspended in 10 ml Lysogeny Broth (LB) medium. The suspension was plated in three replicates in a serial dilution (100 µl of the dilutions 10^0, 10^{-1}, 10^{-2}) on LB agar medium containing 25 µg kanamycin ml^{-1} to select for kanamycin resistant bacteria. After over-night incubation at 37 °C, grown bacteria were harvested and subjected to total plasmid isolation using the Macherey–Nagel Nucleobond Kit PC100 on AX100 columns (Macherey–Nagel, Düren, Germany) following the manufacturer's manual. Aliquots of the plasmid preparations (containing approx. 300 ng plasmid-DNA) were applied to electroporate the *E. coli* laboratory strain DH5α (Grant et al., 1990). *E. coli* DH5α competent cells were prepared as described previously (Tauch et al., 1994) and electroporation was carried out using the Gene Pulser device (Bio-Rad Laboratories GmbH, München, Germany) according to the manufacturer's instructions. Putative transformants were plated in serial dilutions (100 µl of the dilutions 10^0, 10^{-1}, 10^{-2}) on LB agar medium containing 25 µg kanamycin ml^{-1} and incubated over night at 37 °C.

2.2. Standard DNA techniques and resistance pattern assessment

Putative transformants were checked for their plasmid content by Eckhardt-gel analysis (Hynes et al., 1985). Plasmid DNA was purified from selected transformants using the Macherey–Nagel Nucleobond Kit PC100 on the AX100 columns (Macherey–Nagel, Düren, Germany). Restriction enzyme digests and gel electrophoresis were carried out as described previously (Sambrook, 2001). Determination of the antibiotic resistance pattern mediated to *E. coli* DH5α host cells by the plasmid and replicon typing were carried out as described previously (Tennstedt et al., 2003) and according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). To test the conjugative transfer properties of pRSB225, the *E. coli* DH5α strain containing the plasmid was mated with *Pseudomonas* sp. B13 GFP1 (Dröge et al., 2000), *Cupriavidus necator* GFP3 (formerly known as *Ralstonia eutropha* GFP3) (Tennstedt et al., 2005) and *E. coli* CV60 (Szczepanowski et al., 2004) as described previously (Dröge et al., 2000).