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Plasmid-Mediated Antimicrobial Resistance in *Salmonella enterica*

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Abstract

The selective pressure imposed by the use of antimicrobials in both human and veterinary medicine promotes the spread of multiple antimicrobial resistance. The dissemination of antimicrobial resistance in *Salmonella enterica* strains, causing severe enteritis in human, has been reported worldwide and is largely attributed to conjugative DNA exchange. In the present review, the relevance of plasmids to the dissemination of antimicrobial resistance in *S. enterica* is discussed. Recent examples of plasmid-mediated resistance to expanded-spectrum cephalosporins are reported to illustrate the severity of current situation in enteric pathogens.

The exchanges between plasmid(s) and the bacterial chromosome and the integration of resistance genes into specialised genetic elements, called *integrons*, play a major role in acquisition and dissemination of resistance genes. The evolution of a plasmid through the acquisition of integrons is reported, describing novel mechanisms for short-term accumulation of resistance determinants in plasmids circulating in *Salmonella*.

Introduction

Antimicrobial resistance is the best-known example of rapid adaptation of bacteria to a new ecosystem. The ability of bacteria to expand their ecological niche, also in presence of antibiotics, can be explained by accumulation of point mutations leading to the modification of existing genes and/or by the acquisition of resistance genes by horizontal gene transfer. Resistance genes are often located on extrachromosomal genetic elements or in segments inserted within the chromosome that originate from other genomes. The acquisition of a new gene may occur by genetic transformation, but when resistance genes are located on plasmids –self-replicating double-stranded circles of DNA–, they can be mobilised by conjugative transfer. The latter may occur at high frequency and efficiency, and several resistance genes can be acquired simultaneously (Waters, 1999). In this process, mobilisable DNA molecules can be transferred from a donor to a recipient cell, *via* a contact-dependent transmission and energy-driven processes. Plasmids encoding the transfer functions (*tra* genes) can mobilise *in trans* smaller, non-self-transmissible plasmids (Waters, 1999) (Figure 1).

Plasmids contain genes that are essential for the initiation and control of replication. Some plasmids also contain genes that ensure stable inheritance, such as equipartitioning during cell division or conjugal transfer. Plasmids are usually classified in incompatibility (Inc) groups, defined as the inability of two plasmids to be propagated stably in the same cell line. Incompatibility is a manifestation of relatedness, sharing common replication controls or equipartitioning elements (Couturier *et al.*,

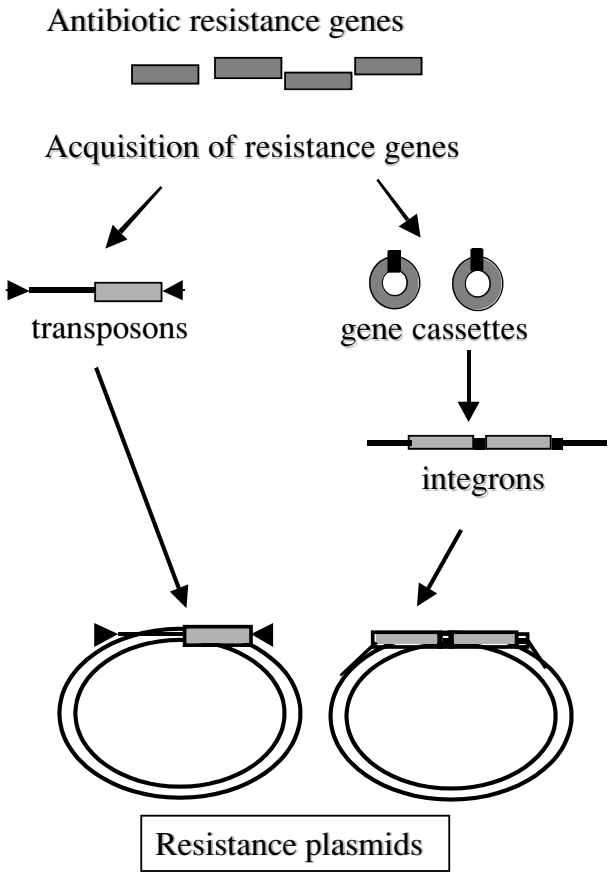

Figure 1. Schematic representation of plasmid-mediated horizontal transfer of antibiotic resistance. Resistance genes are indicated by shadowy boxes. Resistance genes are often found located within transposons and can be transposed into the bacterial chromosome or incorporated within plasmids, indicated by white circles. Resistance genes can also be located as gene cassettes within integrons. A gene cassette consists of one complete open reading frame followed by a recombination site (black box). Gene cassettes exist free as covalently closed circular molecules generated by the integrase and the circular intermediate participates in the integrase-mediated process of insertion. As many as seven different gene cassettes have been described within a single integron.

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1988). Four major incompatibility groups have been defined on the basis of genetic relatedness and pilus structure: the IncF group, including IncF, IncS, IncC, IncD, IncJ, the IncP group, including IncP, IncU, IncM, IncW, the Ti plasmid group, including IncX, IncH, IncN, IncT and the IncI group, including IncI, IncB and IncK (Waters, 1999).

Resistance genes encoded in plasmids are often located within genetic elements called transposons. These elements include the transposase function that enables the transposon to recombine into the bacterial chromosome or plasmids (Figure 1).

Among genetic determinants responsible of capturing and expression of resistance genes, DNA elements called *integrons* have been described. Since late 1980s', integrons have been recognized as naturally occurring gene expression elements responsible for the recruitment and assembly of antibiotic resistance genes in clusters (Figure 1). Integrons encode a site-specific recombinase, the integrase, which efficiently promotes the acquisition of exogenous genes (Martinez and de la Cruz, 1990; Hall and Collis, 1995). Four classes of integron have been defined based on the homology of the integrase proteins (40-60 % amino acid identity). Class 1 integrons, carrying the *intI1* integrase gene, represent the must common structure found in bacterial pathogens (Carattoli, 2001). Most of these integrons have been described to be located within transposons that contribute to vertical transmission, favouring their mobilisation between plasmids and the bacterial chromosome by transposition events. In particular, the Tn*21* transposon, which is a member of the Tn*3* family of transposable elements, has been associated to a class 1 integron, named In2. Tn*21* and In2 have been found widely distributed in Gram-negative bacteria (Liebert *et al*., 2000).

Class 2 integrons are also diffused and have been described within the Tn*7* family of transposons (Radstrom *et al*. 1994), while only one class 3 integron has been reported, not associated with transposons, but containing the bla_{IMP} gene cassette conferring resistance to carbapenems (Arakawa *et al*., 1995). A fourth class of integrase has been described in the small chromosome of *Vibrio cholerae* associated to virulence genes (Rowe-Magnus *et al*., 1999).

A resistance gene that has emerged on a plasmid, located within a transposon or an integron, may be transferred to other strains and species, enabling it to penetrate into niches not accessible to its original host strain (O'Brien, 2002).

In this review I report some relevant findings describing the role of plasmid-mediated resistance in *Salmonella enterica* to antimicrobials of relevance for human therapy.

S. enterica includes nontyphoidal *Salmonella* belonging to different serotypes on the basis of the flagellar and somatic antigens, and represents one of the most important food-borne pathogens causing gastroenteritis in humans (Neidhardt, 1996). Nontyphoidal *S. enterica* strains are easily passed from animals to humans and are thus classified as zoonotic pathogens. They can colonize or infect humans as well as a variety of domesticated and wild animals ranging from mammals to birds and reptiles. Most infections are related to ingestion of contaminated food products rather than person-to-person transmission or direct fecal-oral transmission (Mead *et al*., 1999).

Several *S. enterica* isolates are characterized by the presence of host-adapted virulence plasmids encoding genes contributing to colonization and resistance to complement killing, such as the *spvA*, *spvB* and *spvC* (salmonella plasmid virulence) and the *rck* (resistance to complement killing) genes (Guiney *et al*., 1994).

As assessed in the last few years, *Salmonella* shows increasing antimicrobial resistance rates in isolates obtained from both food animals and humans. *S. enterica* strains belonging to different serotypes and showing multiple antibiotic resistance (to four or more antimicrobials) are now widespread in both developed and developing countries, most of these strains are zoonotic in origin, acquire their resistance in the food-animal host and cause human infections through the food chain (Threlfall, 2002). *Salmonella* infections have been associated with the ingestion of poultry, meat, milk and dairy products (Bean *et al*., 1996). Most infections result in self-limiting diarrhea and do not require antimicrobial treatment. However, severe, life-threatening bacteremias and other deep-seated infections do occur, particularly in children and immunocompromised hosts and in these cases an antimicrobial therapy is recommended (Blaser and Feldman, 1981). Good drugs for *Salmonella* infections include fluoroquinolones, ampicillin, trimethoprim-sulfamethoxazole or third-generation cephalosporins. Rising rates of resistance to ampicillin and trimethoprim-sulfamethoxazole have significantly reduced their efficacy and fluoroquinolones are not approved for the use in children. Consequently, extended-spectrum cephalosporins have become the current drugs of choice for the treatment for invasive infections in children (Hohmann, 2001). The emergence of *Salmonella* species that are resistant to extended-spectrum cephalosporins (Herikstad *et al*., 1997; Threlfall *et al*., 1997) is cause of worldwide concern.

Importance of Plasmids in the Dissemination of Resistance in *S. enterica*

Since the aminoglycoside antibiotic apramycin was licensed for veterinary use in the 1980s, resistance to apramycin and the related antibiotic gentamicin, one the most frequently used aminoglycoside in human therapy, was reported. In the United Kindom, during the period 1982-84, the incidence of resistance to apramycin in salmonellas increased from 0.1% in 1982 to 1.4% in 1984 (Wray *et al*., 1986). Resistance to both apramycin and gentamicin was detected in different *Salmonella* serotypes, as well in *Escherichia coli*. In particular, the incidence of *S. enterica* (*S*.) Typhimurium definitive type 204c (DT204c) from calves dramatically increased (Wray *et al*., 1986). In *S.* Typhimurium DT204c the gentamicin resistance was specified by three types of plasmids of the I1 incompatibility group, which also conferred resistance to apramycin (Threlfall *et al*., 1986). Most of these plasmids produced the enzyme aminoglycoside 3-N-acetyltransferase IV and the resistance was transferable by conjugation in most of the strains examined. The increasing incidence of the

gentamicin-resistant *S.* Typhimurium DT204c was also observed in humans, providing the first evidence that the use of apramycin in animal husbandry gave rise to resistance to gentamicin, an antimicrobial used for human therapy (Threlfall *et al*., 1986).

In the 1990s, the increasing frequency of *E. coli* and *Salmonella* with plasmids conferring resistance to gentamicin and apramycin was reported in other European countries (Chaslus-Dancla *et al*., 1991; Pohl *et al*., 1993). Gentamicin- and apramycin- resistant strains were isolated from both humans and cattle in France and Belgium and six different types of replicons were identified (Pohl *et al*., 1993).

During the 1990-1997 alarming reports pointed out the rapid development in several countries of resistance to β-lactam antibiotics in *Salmonella* (Threlfall *et al*., 1997). A survey conducted between 1987 and 1994 in France, demonstrated a dramatic increase (from 0 to 42.5%) in the prevalence of β-lactam resistance among *Salmonella* isolates. Several types of β-lactamases were found on plasmids belonging to different incompatibility groups Q, P, F and HI (Llanes *et al*., 1999).

In 1994, ampicillin-resistant clinical strains of *S.* Enteriditis were reported to significantly increase in Greece. The β-lactamase was located on the bacterial chromosome or on plasmids belonging to the IncN group, easily transferable to *E. coli* by conjugation (Vatopoulos *et al*., 1994).

Resistance to β-lactams in Gram-negative bacteria is mediated predominantly by two major types of β-lactamases: the chromosomally-encoded enzymes of the Amber class C (*e.g.* AmpC β-lactamase in *Citrobacter*, *Enterobacter*, and *Serratia* spp, *Morganella morganii* and *Pseudomonas aeruginosa*) or by plasmid-encoded enzymes of the Amber class A, in species that do not produce AmpC β-lactamases, such as *E. coli*, *Salmonella* spp., and *Shigella* spp. (Bauernfeind *et al*., 1998a). Extended-spectrum β-lactamase (ESBL), evolved from the bla_{TEM-1}, bla_{TEM-2}, and bla_{SHV-1} genes, extending resistance to new third-generation cephalosporins. During the last decade, infections caused by *S. enterica* carrying ESBLs have been reported, and most of the ESBL-producing strains were found to carry plasmids encoding the bla_{TEM-1}, and bla_{SHV-1} gene derivatives (Hammani *et al*., 1991; Morosini *et al*., 1995; Tassios *et al*., 1997; Villa *et al*., 2000; Mulvey *et al*., 2003).

Is there any relationship between *Salmonella* plasmids and plasmids circulating in bacteria causing hospital-acquired infections? Several examples are here reported, strongly indicating that ESBL-carrying plasmids in *Salmonella* may originate from bacterial pathogens of nosocomial origin. An example of plasmid transfer was documented in *S.* Othmarschen isolated from patients involved in an outbreak in a hospital in Madrid. Strains carried a plasmid-encoded ceftaxidime resistance conferred by a novel ESBL, designated TEM-27. TEM-27 was encountered in *Salmonella* strains as well as in *E. coli* and *Enterobacter cloacae* strains isolated in the same hospital, suggesting that similar plasmids coding for the production of TEM-27, circulated among different pathogens within the hospital (Morosini *et al*., 1996).

In Southern Italy, during the period 1990 to 1998, several epidemiologically unrelated *S.* Enteriditis isolates showing resistance to expanded-spectrum cephalosporins were recurrently isolated from ill patients. Most of these strains carried the bla_{SHV-12} gene located on conjugative plasmids (Villa *et al*., 2002a). Notably, this was the first case of acquisition of the bla_{SHV-12} gene by *Salmonella* in Italy and worldwide. However, SHV-12-encoding plasmids were previously encountered in *K. pneumoniae* isolated from hospitals throughout Italy (Laksai *et al*., 2000; Pagani *et al*., 2000). Therefore, it is plausible that SHV-12-encoding plasmids originated from nosocomial bacterial pathogens and were horizontally transmitted to *S.* Enteriditis strains.

In 1999, the spread of a *S.* Typhimurium clone resistant to third generation cephalosporins has been reported in Russia, Hungary and Greece. In this case non-distinguishable institutional outbreak and community *S.* Typhimurium isolates harbored a transferable plasmid containing the bla_{CTX-M} gene (Tassios *et al*., 1999).

The relatedness of resistance plasmids harbored by strains of various origins can be demonstrated by incompatibility grouping, restriction fragmentation pattern analysis and identification of specific resistance determinants located within the plasmids. These analyses may allow a better understanding of how resistance plasmids propagate, helping to trace their evolution.

The *S.* Typhimurium pSEM plasmid has a very similar restriction pattern to that of the *K. oxytoca* plasmid, pACM1. The pSEM plasmid was identified in 1997 in *S.* Typhimurium strains isolated from children in Albania (Villa *et al*., 2000), while pACM1 was isolated from *K. oxytoca* strains responsible of a nosocomial outbreak in the USA (Preston *et al*., 1997; Preston *et al*., 1999). Both plasmids belonged to the same IncL/M group and conferred resistance to expanded-spectrum cephalosporins by the bla_{SHV-5} gene. Both plasmids carried a class 1 integron conferring aminoglycoside resistance by the *aacA4*, *aacA1* and *aadA1* resistance gene cassettes (Villa *et al*., 2000; Preston *et al*., 1997). Thus, these plasmids could be members of a family of broad-host-range replicons widely spreading among Gram-negative pathogens. Other plasmids of the IncL/M group showing similar restriction profiles, and carrying the bla_{SHV-5} gene and a class 1 integron, were previously described in several countries in Europe from clinical isolates of *P. aeruginosa* and *K. pneumoniae* (de Champs *et al*., 1991; Petit *et al*., 1990; Preston *et al*., 1997, Prodinger *et al*., 1996). The identification of a family of related plasmids has serious public health implications, since it demonstrates that broad-host-range plasmids carrying resistance to clinically relevant antibiotics, can spread worldwide among bacteria responsible of both nosocomial and community-acquired infections. The fact that conserved plasmids can be identified in a wide variety of pathogens isolated in different countries, illustrates the important role of plasmids in the dissemination of antimicrobial resistance among Gram-negative bacteria.

Recently, a case of treatment failure due to ceftriaxone-resistant *S.* Anatum has been reported in Taiwan (Su *et al*., 2003). In this study, ceftriaxone-susceptible *S.* Anatum was initially isolated from the urine of a 70-year-old diabetic patient hospitalized for the treatment of a large pressure

sore in the sacral area and urinary tract infection. The unexpected emergence of the resistance during the treatment with ceftriaxone led to systemic bacteremia by *S.* Anatum and to the fatal outcome in the patient. The emergence of the resistance has been linked to the *in vivo* acquisition of a resistance plasmid carrying the CTX-M3 β-lactamase by the susceptible *S.* Anatum strain. In the same hospital this β-lactamase has been previously identified in clinical isolates of *E. coli*, *K. pneumoniae* and *Enterobacter cloacae*, suggesting that such bacteria may have acted as reservoirs of the resistance plasmid (Su *et al.*, 2003).

Plasmid-Mediated Resistance to Expanded-Spectrum Cephalosporins Encoded by the CMY-2 AmpC β-Lactamase

The *ampC* genes were regarded as exclusively chromosomal until 1989, when an AmpC-type β-lactamase was found for the first time on transmissible plasmids (Bauernfeind *et al.*, 1998a). Plasmid-mediated AmpC β-lactamases belong to the homogeneous group of genes related to the chromosomal *ampC* gene of *Citrobacter freundii* (*cmy-2*, *bil-1* and *lat* genes), the *cmy-1*, *fox* and *mox* family, or originate from the *Morganella morganii* AmpC β-lactamase (DHA-1) (Bauernfeind *et al.*, 1998a). The latter was identified on a plasmid in *S.* Enteriditis (Barnaud *et al.*, 1998; Verdet *et al.*, 2000).

The CMY-2 AmpC β-lactamase, described for the first time in 1990 in *K. pneumoniae* (Bauernfeind *et al.*, 1998b), is now emerging, becoming one of the most frequent plasmid-mediated cephalosporinase in *Salmonella* and *E.coli* (Table 1).

The first case of plasmid-mediated CMY-2 in *Salmonella* was reported on a conjugative plasmid of *S.* Senftenberg recovered in 1994 from stool of an Algerian child (Koeck *et al.*, 1997).

Review of 1996 data from the National Antimicrobial Resistance Monitoring System (NARMS) in the United States identified only 1 (0.1%) *Salmonella* isolate among 1272 human *Salmonella* isolates showing expanded-spectrum cephalosporin resistance (Dunne *et al.*, 2000). However, in 1999, NARMS reported the emergence of domestically acquired broad-spectrum cephalosporin-resistant *Salmonella*, most of them producing the CMY-2 AmpC β-lactamase (Dunne *et al.*, 2000).

S. Typhimurium strains carrying indistinguishable CMY-2-encoding plasmids were isolated in Nebraska from a patient and cattle during a local outbreak of salmonellosis, demonstrating that the *Salmonella*-resistant strain evolved primarily in livestock (Fey *et al.*, 2000).

Since 1998, *Salmonella* and *E. coli* of human and animal origin, showing resistance to expanded-spectrum cephalosporins, have been recurrently isolated in Iowa. Molecular studies demonstrate the emergence of plasmid-encoded CMY-2 β-lactamase in most cephalosporin-resistant isolates. During the 1998-1999 period, nearly 16% of *E.coli* isolates and 5.1% of *Salmonella* isolates from clinically ill animals in Iowa, produced CMY-2. The rates in human isolates were much lower: 0.6% for *Salmonella* and 0.2% for *E. coli* (Winokur *et al.*, 2000; Winokur *et al.*, 2001). Similar plasmids, carrying the *cmy-2* gene were also reported in *Salmonella* isolated from animals in Illinois [Odeh *et al.*,2002].

The spreading of the CMY-2-carrying plasmids in the USA was confirmed by molecular analysis of domestically-acquired *Salmonella* strains of human origin isolated in nine different States, representing the 87% of the total expanded-spectrum cephalosporin resistant *Salmonella* collected by the Center for Disease Control and Prevention (CDC) during the 1996-1998 surveillance period. The isolates were distinguishable by their chromosomal DNA patterns, thus demonstrating that they did not represent the epidemic spread of a clonal strain (Dunne *et al.*, 2000). Molecular analysis demonstrated the presence of plasmid-encoded CMY-2 β-lactamase in all strains. The sequence of the *cmy-2* genes revealed no sequence divergence among strains (Carattoli *et al.*, 2002). The DNA sequence found in the USA isolates was identical to a *cmy-2* sequence described in *K. pneumoniae* (Bauernfeind *et al.*, 1996), yet it was different in three base pair from the *cmy-2* sequence described in the *S.* Senftenberg strain isolated in Algeria (Koeck *et al.*, 1997), suggesting that the *cmy-2* gene disseminating throughout the USA was distinct from that found in Algeria. Plasmids carrying the *cmy-2* gene were divided in three groups on the basis of restriction patterns and *cmy-2* gene hybridisation profiles. Plasmids

Table 1. Plasmid-encoded CMY-2 β-lactamases reported in *S. enterica* and *E. coli*.

Organism	Years of isolation	Country (reference)
S. enterica Senftenberg	1994	Algeria (Koeck *et al.*, 1997)
E.coli	1997	North Africa (Bauernfeind *et al.*, 1998b)
*E. coli**	1998	United Kindom (Stapleton *et al.*, 1999)
S. enterica Typhimurium	1998	Nebraska, USA (Fey *et al.*, 2000)
S.enterica	1996-1998	USA (Carattoli *et al.* 2002)
S.enterica and *E.coli*	1998-1999	Iowa, USA (Winokur *et al.*, 2000; Winokur *et al.*, 2001)
S.enterica	1999	Canada (Allen and Poppe, 2002)
S. enterica and *E.coli*	1997-2000	Taiwan (Yan *et al.*, 2000; Yan *et al.* 2003)
S. enterica and *E.coli*	1999-2000	Spain (Navarro *et al.*, 2001)
S. enterica and *E.coli**	1998-2000	USA (Zhao *et al.*, 2001)
E.coli	2001	Illinois, USA (Odeh *et al.*, 2002)
S.enterica	2000-2001	Romania (Miriagou *et al.*, 2002)
S.enterica Newport	2002	Pennsylvania, USA (Rankin *et al.*, 2002)
E.coli	2002	Cleveland, USA (Hoyen *et al.*, 2002)

*Referred to as CMY-4, which differs from CMY-2 by one amino acid residue at position 221 in the deduced amino acid sequence, the result of one nucleotide difference in the *cmy-2* gene coding sequence.

of type A and C showed restriction patterns very similar to those of plasmids characterised in Iowa, and transferred resistance to at least four antibiotic (streptomycin, chloramphenicol, tetracycline and sulphonamides), in addition to the ceftriaxone resistance conferred by CMY-2 β-lactamase. Type B plasmids transferred resistance only to β-lactam antibiotics by CMY-2 β-lactamase (Carattoli *et al.*, 2002).

During January-April 2002, CDC highlighted the emergence of broad-spectrum cephalosporin-resistant *S.enterica* serotype Newport, with exposure to raw or undercooked ground beef as the putative vehicle of transmission (Zansky *et al.* 2002). Plasmid-encoded CMY-2 has been identified in *S.* Newport strains from animals in Pennsylvania (Rankin *et al.*, 2002). The occurrence of CMY-2 has been recently reported outside the USA, in *Salmonella* and *E. coli* of human origin from Taiwan (Yan *et al.*, 2000; Yan *et al.*, 2003) and Spain (Navarro *et al.*, 2001), in *Salmonella* isolated from food-animals in Canada (Allen and Poppe, 2002), and in human isolates of *Salmonella* from Romania (Miriagou *et al.*, 2002).

It has been suggested that the increased prevalence of cephalosporin-resistance in *Salmonella* strains of both human and animal origin may be related to the use of ceftiofur in food animals. Ceftiofur is an expanded-spectrum cephalosporin approved in the USA in 1991 for therapeutic veterinary use in cattle, and in 1995 for use in swine. Ceftiofur is used only in veterinary medicine for respiratory tract infections, metritis, foot rot, and abscess prophylaxis (Winokur *et al.*, 2000). The CMY-2 β-lactamase confers resistance to ceftiofur, as well as to other expanded-spectrum cephalosporins used in human therapy such as ceftriaxone, and the veterinary use of ceftiofur may have positively selected the *cmy-2*-carrying *Salmonella* and *E. coli* strains (Carattoli *et al.*, 2002).

Evolution of Plasmids Conferring Multidrug-Resistance

Integrons and Transposons

As previously mentioned, the DNA exchanges mediated by conjugative plasmids and the integration of resistance genes into specialised genetic elements play a major role in the acquisition and dissemination of resistance genes.

A very efficient genetic mechanism by which bacteria can acquire resistance genes is the integrase-mediated site-specific recombination. Class 1 integrons promote the capture of one or more gene cassettes within a specific attachment site (*attI1*), thereby forming composite clusters of antibiotic resistance genes (Figure 2). Their essential components are the integrase gene (*intI1*) and the promoter, which drives the expression of any suitably, oriented array of gene cassettes integrated at the *attI1* site. These functions are contained in the so-called 5'-conserved segment (5'-CS) of the integron (Hall and Collis, 1995). Integrons belonging to class 1 are also characterized by the presence of the *sul1* gene, conferring resistance to sulfonamides, located in the 3'-conserved segment (3'-CS) (Hall and Collis, 1995). This segment also includes the *qacEΔ1* gene, conferring resistance to quaternary ammonium compounds, and the open reading frame (ORF) 5, endowed with a still unknown function (Figure 2). The mobile gene cassettes are integrated between the 5'-CS and 3'-CS (Bissonette and Roy, 1992). The gene cassette is defined as a discrete unit consisting of one complete ORF followed by a recombination site named 59-base element (Recchia and Hall, 1995) (Figure 2).

Expanding Drug Resistance Through Integrons Acquisition in IncFI Plasmids of S.enterica

During the early 1970s plasmids of the IncFI group, encoding multiple antimicrobial resistance, were frequently

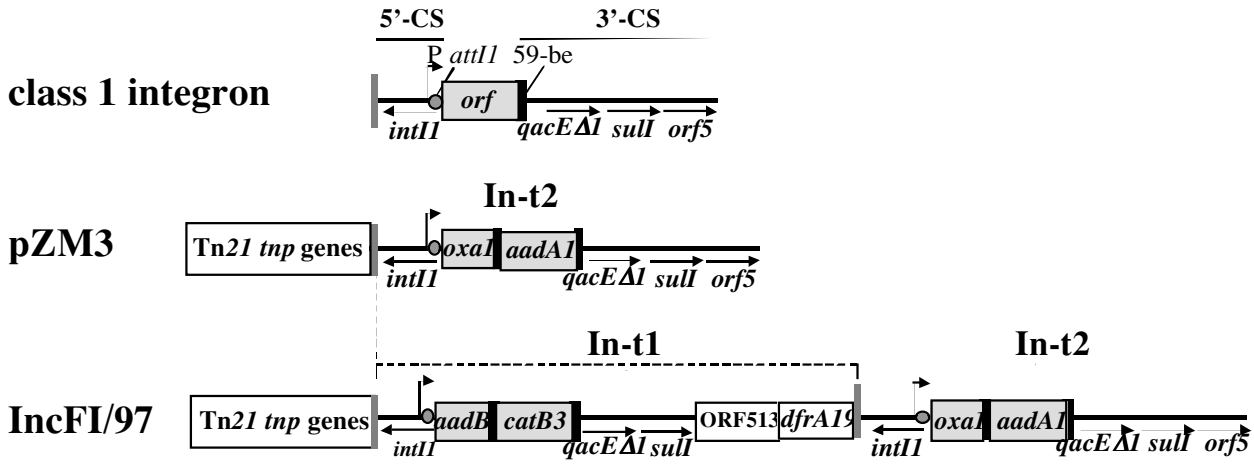

Figure 2. Schematic representation of a class 1 integron, and integron region in plasmids pZM3 and IncFI/97 (Villa *et al.*, 2002b). pZM3 was isolated from *S. enterica* Wien in 1970, and IncFI/97 was isolated from *S. enterica* Typhimurium in 1997. White boxes represent the *tnpA*, *tnpR*, *tnpM* genes of Tn*21*, ORF513 and *dfrA19* genes. The integrase (*intI1*), the *qacEΔ1*, the *orf5* and the *sul1* genes are underlined by arrows showing the direction of transcription. The shaded boxes represent gene cassettes inserted into integrons. Black bars are the 59-base elements. Vertical shaded bars indicate the IRi sites. Dashed lines show the In-t1 insertion occurred within the IncFI/97 plasmid.

reported in *Salmonella* isolated in Europe, Middle East and North Africa (Anderson *et al.*,1977). Epidemiological and genetic data supported the view that acquisition of IncFI plasmids contributed to the epidemic spread of *S.* Wien causing protracted outbreaks. Most of these plasmids conferred resistance to ampicillin, chloramphenicol, streptomycin, spectinomycin, sulfonamides, tetracycline, and kanamycin, although a minority of IncFI plasmids, particularly those of Mediterranean origin, lacked the streptomycin, spectinomycin and sulfonamides resistance (Anderson *et al.*, 1977; Colonna *et al.*, 1988). Several IncFI plasmids also carried virulence determinants such as aerobactin iron transport system (Colonna *et al.*, 1988). The presence of both virulence and antimicrobial resistance determinants on the same plasmid, probably contributed to the virulence properties of *S.* Wien carrying IncFI plasmids. *S.* Wien epidemics declined in early 1980s', but outbreaks linked to IncFI-carrying *Salmonella* were reported in the 1990s (Mohan *et al.*, 1995).

Twenty-nine multiple-drug-resistant *S.* Typhimurium strains isolated in 1997 in Albania from sporadic cases of infantile gastroenteritis, carried the IncFI plasmids (Tosini *et al.*, 1998). These strains showed a wide repertoire of resistance, being resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin, sulfonamides, tetracycline and trimethoprim. Four different resistance genes were located within two class 1 integrons, both located in the IncFI plasmid: In-t1 carrying the *aadB* and *catB3* genes, conferring kanamycin and chloramphenicol resistance, and In-t2 carrying the *oxa1* and *aadA1* genes, conferring β-lactams and streptomycin-spectinomycin resistance (Tosini *et al.*, 1998).

To gain insight into the evolution of these plasmids, one of the recently isolated IncFI plasmid, the IncFI/97, was compared with IncFI plasmids representative of those identified in epidemic strains during the 1970-78 period. Molecular analysis demonstrated that IncFI/97 was quite well conserved respect to its ancestor (Carattoli *et al.*, 2001). IncFI/97 retained the overall structural organization of IncFI plasmids isolated in early 1970s. The presence of the In-t2 integron, carrying the *oxa1-aadA1* genes, was found in all IncFI ancestor plasmids showing streptomycin, spectinomycin and sulfonamide resistance (pZM3 plasmid in Figure 2 was isolated in 1970). This observation indicated that In-t2 was maintained unaltered in IncFI plasmids for nearly thirty years (Carattoli *et al.*, 2001). IncFI/97 was supposed to be a new plasmid variant, since it contains a new integron, In-t1. Recently, plasmids carrying In-t1 and In-t2 have been identified in *S.* Typhimurium from Europe and Africa (Lindstedt *et al.*, 2003), suggesting that the IncFI plasmid could have re-emerged.

The integration site of In-t1 within the IncFI/97 plasmids was identified revealing a close physical link between the In-t1 and In-t2 integrons (Figure 2). In-t1 was localized downstream the transposase genes of Tn*21*, and In-t2 was located downstream the 3'-CS of In-t1, in a head-to-tail configuration (Figure 2) (Villa *et al.*, 2002b).

In-t1 shows a peculiar 3'-CS, containing an open reading frame known as ORF513, or ORF341E, and a novel trimethoprim resistance gene, designated *dfrA19* (formerly *dfrA18*) (Villa *et al.*, 2002b).

Several mechanisms of integration may have generated the In-t1/In-t2 configuration. A plausible mechanism of In-t1 insertion within In-t2 could be the recombination between the extended homology regions at the 5'-CS of both integrons. Such event might have occurred by homologous recombination between the Tn*21*-target and a circular molecule containing all the sequence ascribed to In-t1. This event would have occurred very recently in the evolutionary time-scale of IncFI plasmids.

The fact that integration of In-t1 occurred in the same site containing the resident In-t2 also suggests that specific integration sites may have been active in Tn*21*. It has been postulated that integrons could be ancestral transposable elements, since they are often flanked by inverted repeats and carry accessory genes encoding for transposition functions (Radstrom *et al.*, 1994). However, In-t1 does not seem to contain accessory genes, except the ORF513-encoded protein. The ORF513 has been previously reported in other class 1 integrons and is always followed by a resistance gene (Stokes *et al.*, 1993; Verdet *et al.*, 2000; Sabate *et al.*, 2002; Di Conza *et al.*, 2002; Doi *et al.*, 2002; Arduino *et al.*, 2002; Partridge and Hall, 2003).

The ORF513 predicted product shows substantial similarity with the predicted product of ORF2, identified in the multidrug resistance locus of *S.* Typhimurium definitive type 104 and on a plasmid found in *E. coli* (Cloekaert *et al.*, 2000; Boyd *et al.*, 2001). The weak similarity of ORF513 and ORF2 proteins with the transposase of IS*801* suggests that these proteins could be transposase-related proteins involved in the incorporation of novel antibiotic resistance genes (Partridge and Hall, 2003), and contributing to the integron mobilization, as hypothesized in the case of In-t1 (Villa *et al.*, 2002b).

In conclusion, although the data available do not provide a unequivocal explanation for In-t1 acquisition, the evolution of the IncFI plasmids through the recruitment of a second integron constitutes a novel example of integron mobilization and acquisition of resistance genes by plasmids in bacterial pathogens.

The 30-year retrospective investigation of old and recent IncFI plasmids provides the evidence that their evolutionary story combines the maintenance of pre-existing antimicrobial determinants with the acquisition of new resistance genes and represents a good example of how plasmids can evolve as evolutionary units through the sequential acquisition of multiple resistance determinants.

Conclusions

Recent reports support the hypothesis that the versatility of plasmids together with the usage of antimicrobials in human medicine and animal husbandry, may largely contributed to the spread of antimicrobial resistance.

Many reports point out the rapid development of resistance to β-lactams by related-plasmids circulating in unrelated *Salmonella* strains. The presence of ESBL genes in *Salmonella* can be explained by horizontal transfer of resistance from bacteria of nosocomial origin. This phenomenon is a disturbing development for public health, since *Salmonella* carriage of such transmissible plasmids may facilitate the spread of a variety of resistance

determinants to other community-acquired pathogens. In addition, further dissemination of such strains may drastically reduce therapeutic options for severe *Salmonella* infections.

In the USA the emergence of CMY-2-encoding plasmids has been implicated with *Salmonella* and *E. coli* strains transmitted by food animals. The homology between the *cmy-2* alleles in animals and humans, and the high rates of carriage in food animals, strongly indicate that the CMY-2-producing organisms have been transmitted to humans through food sources and animal contacts (Winokur *et al*., 2001). The increased prevalence of the CMY-2-encoding plasmids may be related to the use of ceftiofur in veterinary medicine. Ceftiofur is an antibiotic used exclusively in veterinary medicine but it can cross-select resistance to cephalosporins largely used in human therapy.

These observations imply that plasmid-mediated antimicrobial resistance is a global problem that does not respect any boundaries, either between animals and humans, or bacterial species and genera, demonstrating the strong capacity of plasmids to be horizontally transmitted.

The story of the IncFI plasmids can help in understanding molecular mechanisms responsible of the evolution of resistance plasmids. Antimicrobial drug resistance in IncFI plasmids evolved through the acquisition of multiple resistance determinants in few steps, by homologous and site-specific recombination mediated by transposons and integrons. The IncFI7/97 evolution seems to have proceeded through acquisition of resistance genes in a specific site, leading to the assembly of a complex configuration of clustered resistance determinants, whose expression is guaranteed by promoters located within the integrons. The presence of multiple physically linked resistance genes on the same plasmid, conferring resistance to different classes of antibiotics, may confer a selective advantage to the host when several antimicrobials are simultaneously administered. Such synergy between different co-expressed resistance genes would allow the recipient host to be positively selected by each individual class of antibiotics.

Is there any relationship between plasmid-mediated resistance and virulence? The simultaneous presence in the same bacterial cell of a resident virulence plasmid and a resistance plasmid has been frequently reported in *Salmonella*. Since most of the resistance genes are localised on transferable elements it is plausible that such events could represent occasions for the capture of resistance determinants by virulence plasmids. The linkage of virulence and resistance on the same plasmid could represent a relevant step to bacterial adaptation and evolution. For bacteria carrying virulence and resistance linked determinants, the selection of an infective population will select for antimicrobial resistance, and antimicrobial resistance pressure will select the virulence traits. However, once those determinants have been selected in the bacterial host, they can evolve further and eventually be transferred to other bacterial population (Martinez and Baquero, 2002). Several resistance plasmids, including IncFI plasmids, have been described to carry genes encoding virulence factors, such as bacteriocins, siderophores, cytotoxins, or adhesion factors (Martinez and Baquero, 2002). It could be suspected that many current resistance plasmids also contain genes with a role in bacterial virulence. Recently, a plasmid of the IncFII group, carrying the *spvA*, *spvB* , *spvC* and *rck* genes, as well as integrons and transposons conferring multidrug-resistance has been found in *S.* Typhimurium strains isolated in Spain (Guerra *et al*., 2002). The acquisition of resistance on virulence plasmids, could represent a novel tool in the bacterial evolution, implementing adaptive strategies to explore and colonise novel hosts and environments (Martinez and Baquero, 2002).

Many questions remain unanswered about mechanisms driving the dissemination of plasmids along the food chain, or mediating hospital/community exchanges. The exact contribution of antimicrobials use for animal and human therapy, on the one hand, and animal growth promotion and prevention of infection in humans, on the other, to the positive selection of specific resistance genes also remains uncertain. However, further research extending the knowledge of antimicrobial resistance mechanisms will facilitate the development of effective preventive and control strategies against this phenomenon.

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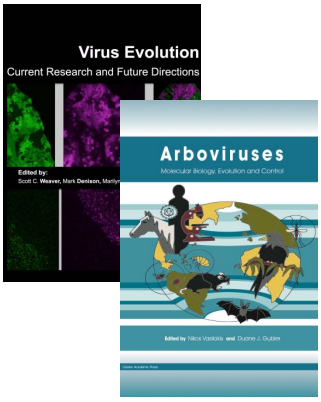

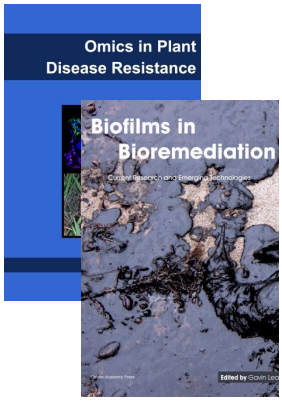

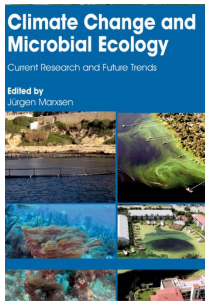

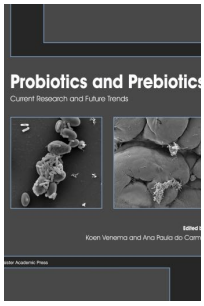

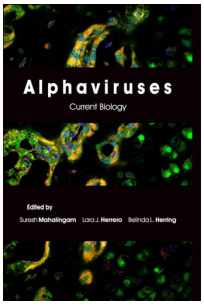

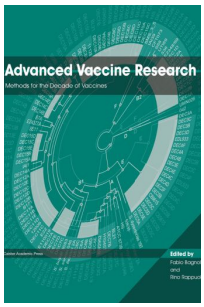

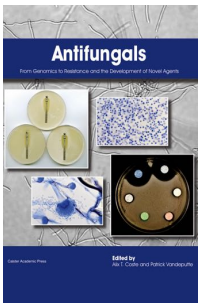

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