Characterization of class I integrons in clinical strains of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis from Norwegian hospitals

Bjørn-Arne Lindstedt,¹ Even Heir,¹ Irene Nygård¹ and Georg Kapperud^{1,2}

Correspondence

Bjørn-Arne Lindstedt
bjorn-arne.lindstedt@folkehel
sa.no

¹Norwegian Institute of Public Health, Division of Infectious Diseases Control, N-0403 Oslo, Norway

²Department of Pharmacology, Microbiology and Food Hygiene, Norwegian School of Veterinary Sciences, N-0033 Oslo, Norway

The characterization of integrons and their promoters in 156 antibiotic-resistant clinical isolates of the important zoonotic pathogens *Salmonella enterica* subsp. *enterica* serovars Typhimurium (*S. Typhimurium*) and Enteritidis (*S. Enteritidis*) from Norwegian hospitals were performed. Integrons were found in 64 of 66 *S. Typhimurium* isolates (97 %) and in 20 of 90 *S. Enteritidis* isolates (22.2 %) with the following sizes; 650, 1000, 1200, 1500, 1600, 1700, 2000 and 2100 bp. The integrons were further sequenced and the *aadA1*, *aadA2*, *aadA5*, *aadB*, *pse-1*, *catB3*, *oxa1*, *dfrA1*, *dfrA12* and *dfrA17* genes, as well as a fragment of the *sat1* gene, were found embedded in cassettes. An internal fragment of the *purG* gene was additionally found as an artefact PCR amplicon.

Received 7 May 2002

Accepted 9 October 2002

INTRODUCTION

Integrons are genetic units that include components of a site-specific recombination system enabling them to capture and mobilize genes. Integrons contain a gene of the λ family of integrases that carries out recombination between two distinct target sites; the *attI* site and the 59-base element where *attI* is the target site for cassette integration (Hall & Collis, 1995; Hall & Stokes, 1993). The genes captured by this system are packaged as small discrete units with essentially all flanking sequences removed, but with the 59-base element included, and are named 'gene cassettes' (Hall & Collis, 1995; Hall & Stokes, 1993). Integrons are believed to be ancient structures (Rowe-Magnus *et al.*, 2001), although the mechanism that created these cassettes is at present unknown. The majority of known gene cassettes are antibiotic resistance genes, but it is likely that any gene could be included in a cassette and several uncharacterized ORFs have been found in cassettes. Integrons are classified by comparison of the amino acid sequence of their integrases, encoded by the *intI* genes. Four integron types have been identified, but in recent studies additional integron classes have been reported (Rowe-Magnus *et al.*, 2001; Nield *et al.*, 2001). The majority of integrons found in clinical isolates of enterobacteria are of class I. More than 60 different antibiotic resistance genes have been identified within cassette structures either alone or in combination (Mazel & Davies, 1999).

A promoter region is located in the 5'-conserved segment of the integron and contains two promoters named P_c (P_{ant}) and

P_2 . Four different P_c and two different P_2 promoters have been described in the literature (Stokes & Hall, 1989; Bunny *et al.*, 1995) and their relative strength as compared to the *Escherichia coli* *tac* promoter has been reported (Lévesque *et al.*, 1994; Collis & Hall, 1995). Two of the P_c promoters are designated strong (TTGACA- N_{17} -TAAACT) and weak (TGGACA- N_{17} -TAAGCT), and two are named hybrid promoters, with the strong promoter six times more effective than the *E. coli* *tac* promoter. The *tac* promoter is, however, more efficient than the weak cassette promoter and the two hybrid promoters, TGGACA- N_{17} -TAAACT and TTGACA- N_{17} -TAAGCT (Lévesque *et al.*, 1994; Collis & Hall, 1995). The P_2 promoter has an active version TTGTTA- N_{17} -TACAGT, but when the -35 and -10 regions are separated by 14 nt, TTGTTA- N_{14} -TACAGT, there is no P_2 promoter activity.

Integrons are widespread and have been studied in great detail among the *Enterobacteriaceae*, with a particular focus on the multi-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) definitive type (DT) 104, while studies on integrons among *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) isolates are limited. The incidence of *S. Enteritidis* infections has increased in many countries, and in Europe this serotype is now predominant among human *Salmonella* isolates. The increase began as early as the mid-1970s and *S. Enteritidis* displaced *S. Typhimurium* as the primary cause of infection by 1990 (Kingsley & Baumler, 2000; Baumler *et al.*, 2000;

Guard-Petter, 2001). While other *Salmonella* serotypes have been shown to experimentally infect eggs, *S. Enteritidis* is the only human pathogen that causes frequent human illness associated with egg contamination (Guard-Petter, 2001). *S. Enteritidis* is therefore an important zoonotic pathogen in which increased resistance to several antibiotics will pose an increased threat.

This study presents the result of characterization of integrons and their promoters in 156 multi-resistant strains of the important zoonotic pathogens *S. Typhimurium* and *S. Enteritidis* from human clinical isolates (hospitalized patients) submitted to the Reference Laboratory for enteropathogenic bacteria at the Norwegian Institute of Public Health from hospitals throughout Norway.

METHODS

Strain selection

S. Typhimurium. In all 66 antibiotic-resistant isolates of *S. Typhimurium* from 22 cases of domestically acquired infections and 44 cases of infections acquired abroad, were obtained from the strain collection at the reference laboratory for enteropathogenic bacteria at the Norwegian Institute of Public Health, Oslo, Norway. The strains were isolated from 1996 to 2001. A total of 53 isolates were phage-typed as DT104 and 22 of these had the ACSSuT (see below for definition of abbreviations) resistance profile (Tables 1 and 2).

S. Enteritidis. The strain collection of the National Reference Laboratory had 1114 *S. Enteritidis* isolates from 2001 of which 27 (2.4%) were domestically acquired. A total of 54 (4.8%) were resistant to two or more antibiotics. In 2000 the strain collection contained 794 *S. Enteritidis* isolates where 10 (1.3%) were domestically infected and 36 (4.5%) were resistant to two or more antibiotics. All the 90 isolates of *S. Enteritidis* that were resistant to two or more antibiotics were obtained from the strain collection at the reference laboratory. Table 3 lists the integron-positive *S. Enteritidis* isolates.

Antimicrobial resistance. Antimicrobial susceptibility was tested by a tablet diffusion method according to the manufacturer's guidelines (Rosco Diagnostics). The isolates were screened for resistance to ampicillin (A), ciprofloxacin (Cx), tetracycline (T), chloramphenicol (C), nalidixic acid (Na), trimethoprim-sulfamethoxazole (Ts), sulphadiazine (Su), streptomycin (S), gentamicin (Gn) and doxycycline (Dx). Only *S. Enteritidis* strains that were resistant to two or more antibiotics, and *S. Typhimurium* strains resistant to four or more antibiotics were included in the study.

Integron PCR. A number of PCR primers were used for amplification of gene cassettes and genes known to be associated with integrons as well as a gene associated with transposons, including Tn21, Tn501 and Tn1696. These genes include *intI1*, *intI2*, *intI3*, *sulI*, *qacEΔ1*, *merA* and *tniA*. All strains were tested with markers for the integrase gene *intI1*, the quaternary ammonium compound resistance gene *qacEΔ1* and the sulphonamide resistance gene *sulI*, as well as for the integrated gene cassettes. Integrons have been located within transposons and all isolates were additionally tested for the presence of the transposase gene *tniA* that can be associated with integron In2 (Partridge *et al.*, 2001) and the mercury resistance gene *merA* associated with the transposon Tn21 as well as others. Primers for amplification of the antibiotic resistance gene *floR* were included (Table 4). To examine the cassette promoters, an upper primer situated at the 3' end of the *intI1* gene coupled with lower primers situated in the *aadA1*, *aadA2*, *aadB*, *pse-1*, *oxa1*, *dfrA1*, *dfrA12* and *dfrA17* genes were used. See Figs 1 and 2 for the location of the most used primer sets. The primer in the *intI1* gene was placed such that the P_C and the P2 promoter sequences, as well as the binding site for the integron cassette-A primer, would be included in the amplified PCR product. Furthermore, primers for fully sequencing the middle regions of the longer cassettes were designed from the end sequences generated by the vector-localized T7 and T3 universal primers.

The temperature profile was: 94 °C denaturation for 5 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, then a 7 min extension step at 72 °C on a Perkin-Elmer GeneAmp PCR system 9700. The primers in the *floR*, *sulI*, *merA* and *tniA* genes had the same profile but with annealing at 60 °C.

Cloning and sequencing. The PCR amplicons from the cassette primers were cloned directly into the vector PCR-Script Amp using the

Table 1. Characteristics of the *S. Typhimurium* DT104 isolates

All isolates carry the two integrons containing *aadA2* in one and *pse-1* in the other, or *aadA2* containing integron alone. +, Present; –, absent.

<i>merA</i>	<i>intI1</i>	<i>qacEΔ1</i>	<i>floR</i>	<i>sulI</i>	Resistance	No. of isolates
–	+	+	+	–	ACST	2
–	+	+	+	+	ACSSuT	22
–	+	+	+	+	ACSuT	2
–	+	+	+	+	ACSSuTGn	1
–	–	+	+	–	ACST	1
–	+	+	+	+	ACSSuTCx	1
–	+	+	+	+	ACSSuTTm	2
–	+	+	+	+	ACSSuTDx	14
–	+	+	–	+	ACSSuTDx	2
–	+	+	+	+	ACSSuTNaDx	2
–	–	–	–	+	ACSTDx	1
–	–	+	+	+	ACSSuT	1
+	–	+	–	+	ACSSuTDx	1
–	–	+	+	+	ACSSuTDx	1

Table 2. Characteristics of the *S. Typhimurium* non-DT104 isolates

+, Present; —, absent; NR, no reaction; NDA, not domestically acquired, but country of origin uncertain.

Isolate	Year of isolation	Phage type	<i>intI1</i>	<i>qacEΔ1</i>	<i>merA</i>	Resistance	Location	Genes in cassettes	Plasmid
1864/96	1996	NR	+	+	—	CSuTmGn	India	<i>aadB-catB3</i>	Yes
2428/96	1996	NR	+	+	—	ACSSuT	NDA	<i>aadA2-pse1</i>	No
1875/96	1996	DT120	+	+	+	ACSSuT	Spain	<i>oxa1-aadA1</i>	Yes
1550/96	1996	DT120	+	+	+	ACSSuT	Spain	<i>oxa1-aadA1</i>	Yes
1176/96	1996	DT120	+	+	+	ACSSuT	Norway	<i>oxa1-aadA1</i>	Yes
1203/97	1997	DT120	+	+	+	ACSSuT	Spain	<i>oxa1-aadA1</i>	Yes
1974/98	1998	U302	+	+	+	ACSSuT	Portugal	<i>aadA2-pse1</i>	No
900/98	1998	NR	+	+	—	ACSSuTTmGn	Ethiopia	<i>aadB-catB3-oxa1-aadA1</i>	Yes
3428/98	1998	NR	+	+	+	ACSSuTTmGn	Africa	<i>aadB-catB3-oxa1-aadA1</i>	Yes
2339/99	1999	DT120	+	+	+	ACSSuT	Spain	<i>oxa1-aadA1</i>	Yes
5180/00	2000	DT204	+	+	+	CSuTTmDx	Greece	<i>dfrA17-aadA5</i>	Yes
7542/00	2000	DT204	+	+	+	ACSSuTTmNaDx	Belgium	<i>dfrA1-aadA1</i>	Yes
10403	2001	DT104a	—	+	—	ACSSuTTmNaCx	Australia	<i>dfrA12-orf-aadA2</i>	Yes

Table 3. Characteristics of the *S. Enteritidis* isolates containing integrans

All the integron-positive isolates were positive for *intI1*, *qacEΔ1* and *sulI*. All the integrans were localized on plasmids. NDA, Not domestically acquired, but country of origin uncertain.

Isolate	Year of isolation	Origin*	Resistance	Genes in cassettes
7228/00	2000	Indonesia	ATNaTs	<i>dfrA1-aadA1</i>
4084/00	2000	Rwanda	ACTs	<i>dfrA1-aadA1</i>
4305/00	2000	India	ATCTs	<i>dfrA1-aadA1</i>
9843/00	2000	NDA	ATNaTs	<i>dfrA1-aadA1</i>
4027/00	2000	Kenya	ATCTs	<i>dfrA1-aadA1</i>
3635/00	2000	NDA	TNaTs	<i>dfrA1-aadA1</i>
9417/01	2001	NDA	TTs	<i>sat1Δ</i>
9997/01	2001	Italy	TTs	<i>dfrA1-aadA1</i>
7508/01	2001	Asia	ATs	<i>dfrA12-orf-aadA2</i>
7224/01	2001	NDA	TTs	<i>dfrA1-aadA1</i>
1694/01	2001	Spain	TTs	<i>dfrA1-aadA1</i>
5191/01	2001	Thailand	TNaTs	<i>dfrA1-aadA1</i>
1731/01	2001	Iran	ATTs	<i>dfrA1-aadA1</i>
9430/01	2001	Greece	Ts	<i>dfrA1-aadA1</i>
2712/01	2001	Thailand	ATNaTs	<i>dfrA1-aadA1</i>
3121/01	2001	Asia	TNaTs	<i>dfrA1-aadA1</i>
775/01	2001	Spain	TTs	<i>dfrA1-aadA1</i>
3120/01	2001	South America	TNaTs	<i>dfrA12-orf-aadA2</i>
5693/01	2001	Spain	TTs	<i>dfrA1-aadA1</i>
4947/01	2001	France	TTs	<i>dfrA1-aadA1</i>

PCR-Script Amp Electroporation-competent Cell Cloning Kit (Stratagene) after purification with the QIAquick PCR purification kit (Qiagen). In some cases bands were cut out from the agarose gel and DNA was extracted with the QIAquick gel extraction kit (Qiagen) before cloning. The ligated vector + amplicon construct was transformed into Epicurian Coli XL-1 Blue MRF' electroporation competent cells

(Stratagene) with a Gene Pulser II (Pulse Controller Plus; Bio-Rad) set at 400 Ω resistance, 2.5 kV voltage and 25 μF capacitance using a 0.2 cm cuvette. The transformed cells were incubated for 1 h at 37 °C in SOC medium before plating on IPTG/X-Gal plates with 50 μg ampicillin ml⁻¹ for blue-white screening of positive inserts. The plates were incubated overnight at 37 °C and cooled 1 h at 4 °C to intensify the blue-

Table 4. PCR primer pairs

Primer	Sequence (5'–3')	Reference
cassette-A	GGCATCCAAGCAGCAAG	Lévesque <i>et al.</i> (1995)
cassette-B	AAGCAGACTTGACCTGA	Lévesque <i>et al.</i> (1995)
intI1-F	GCCTTGCTGTTCTTCTACGG	Lévesque <i>et al.</i> (1995)
intI1-R	GATGCCTGCTTGTTCTACGG	Lévesque <i>et al.</i> (1995)
intI2-F	CACGGATATGCGACAAAAAGGT	Mazel <i>et al.</i> (2000)
intI2-R	GTAGCAAACGAGTGACGAAATG	Mazel <i>et al.</i> (2000)
intI3-F	GCCTCCGGCAGCGACTTTCAG	Mazel <i>et al.</i> (2000)
intI3-R	ACGGATCTGCCAAACCTGACT	Mazel <i>et al.</i> (2000)
sulI1-F	CTTCGATGAGAGCCGGCGGC	Sandvang <i>et al.</i> (1997)
sulI1-R	GCAAGGCGGAAACCCGCGCC	Sandvang <i>et al.</i> (1997)
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	Sandvang <i>et al.</i> (1997)
qacEΔ1-R	CAAGCTTTTGCCCATGAAGC	Sandvang <i>et al.</i> (1997)
merA-F	ATCGGCGGCACCTGCGTCAATGTC	This study
merA-R	CGGCCAGGCGCTTAGGAATCGTCT	This study
floR-U	ACCCGCCCTCTGGATCAAGTCAAG	This study
floR-L	CAAATCACGGGCCACGCTGTATC	This study
tniA-U	GCTCGCGCCGGGCAATCCAC	This study
tniA-L	CCCACCAGGCCAGCCGCACTACG	This study
IPUR-U	CGGTGTGGCGGGCTTCGTGATG	This study
IPUR-L	CCTCGCCGCTGTCGCTGGTGATT	This study
pse-1-R	GGGGCTTGATGCTCACTCCA	Huovinen & Jacoby (1991)
pse-1-L	AATGGCAATCAGCGCTTCCC	Huovinen & Jacoby (1991)
aadA2-L	TGTTGGTTACTGTGGCCGTA	Bito & Susani (1994)
aadA2-R	GCTGCGAGTTCATAGCTTC	Bito & Susani (1994)

Fig. 1. Location of the PCR primers used to amplify the integron variable regions containing the inserted gene cassettes (cassette-A and cassette-B), the primer set to amplify the disinfectant resistance gene *qacEΔ1* and the primer set for the *sulI1* gene. Black circles denote the location of the 59-base element, *attI* is the cassette integration site and P denotes promoters. **Fig. 2.** Location of the PCR primers used to amplify genes associated with the *S. Typhimurium* DT104 integron region. The genes are *aadA2*, *floR* and *pse-1*.

coloured colonies. White colonies were picked and incubated overnight in LB medium with 50 µg ampicillin ml⁻¹ added. Plasmids were then isolated with the Wizard Plus SV Minipreps DNA Purification System (Promega). All the isolated plasmids were digested for 1 h at 37 °C with the restriction endonucleases *Pst*I and *Not*I (New England Biolabs) and separated on a 1 % GTG agarose gel to confirm insert sizes. Plasmids containing inserts were subjected to cycle sequencing with the BigDye Terminator kit (ABI biosystems) from both ends with the T7 and T3 universal sequencing primers. The sequence reaction was separated by capillary electrophoresis on an ABI-310 Genetic Analyser (ABI biosystems) with POP-6 polymer. Some of the amplicons from the cassette primers were in addition subjected to direct sequencing after purification with the QIAquick PCR Purification kit (Qiagen) using the BigDye Terminator kit (ABI biosystems) with 3.2 pmol of either the cassette-A or cassette-B primer (Table 4). All sequencing reactions were purified with the DyeEx Spin Kit (Qiagen) to remove unincorporated dye terminators prior to electrophoresis. The promoter regions for all the found cassette amplicons were subjected to direct sequencing of both strands with 3.2 pmol of the appropriate primer sets.

Sequence analysis. The sequence raw data were exported from the ABI-310 into the Lasergene software package (DNASTAR). All sequence comparisons and alignments were done using this software which additionally performed on-line searches for related sequences via the National Center for Biotechnology Information (NCBI) BLAST server.

Plasmid isolation and hybridization. Plasmids were isolated from all strains as detailed by Kapperud *et al.* (1989) and separated by 1 % agarose gel electrophoresis. The gels were photographed under UV light after ethidium bromide staining. Plasmid DNA was transferred to Hybond-N+ membranes (Amersham-Pharmacia Biotech) by vacuum blotting and hybridized at 42 °C using synthetic oligonucleotide probes directed at the *intI1*, *sulI* and *qacEΔ1* genes with the ECL 3'-oligolabeling and detection system (Amersham-Pharmacia Biotech).

RESULTS AND DISCUSSION

Integron markers

Sixty-six antibiotic-resistant *S. Typhimurium* and 90 *S. Enteritidis* isolates, all of human origin, were tested for the presence of known markers for integrans by PCR amplification. The integrase *intI1* gene was detected in 91 % (60/66) of the *S. Typhimurium* isolates; only strains 10403, 700/98, 5031/00, 6347/00, 4805/00 and 5790/00 failed to give an amplicon after repeated attempts with the *intI1* primer set. The isolates were tested with primers for two additional known integrin integrases, *intI2* and *intI3*, which both failed to amplify a fragment. In the *S. Typhimurium* isolates 5031/00 and 6347/00 no amplicons from the gene cassette primers were detected. In isolates 10403, 700/98, 4805/00 and 5790/00 gene cassettes were found with the cassette primers, indicating a possible deletion in the *intI1* gene upstream from the 5' conserved segment cassette primer-binding site, or the presence of a different class of integrase. The most common amplicons were 1000 and 1200 bp that predominantly occurred in combination and were detected in 51 of the 66 (77.3 %) *S. Typhimurium* isolates. The 1000 bp amplicon occurred alone in two *S. Typhimurium* isolates (3 %) and the remaining *S. Typhimurium* amplicons had sizes of 200, 1500, 1600, 1700, 2000 or 2100 bp. A notable feature is that the vast majority of *S. Typhimurium* isolates

carried two gene cassettes; only 10 *S. Typhimurium* isolates (15.2 %) displayed single bands from the cassette primers. The disinfectant resistance gene *qacEΔ1* was absent in one *S. Typhimurium* isolate (1.5 %) and the sulphonamide resistance gene *sulI* was absent in seven *S. Typhimurium* isolates (10.6 %). Only one *S. Typhimurium* (1.5 %) isolate (2755/97) was positive for the *tniA* gene which has been associated with the In0, In2, In5 and Tn402(In16) integrans (Radstrom *et al.*, 1994; Brown *et al.*, 1996; Hall *et al.*, 1994).

All the integrin-positive *S. Enteritidis* isolates (Table 3) contained the *intI1*, *qacEΔ1* and *sulI* genes. The *merA* marker was found in all the *S. Enteritidis* isolates with the *dfrA1*-*aadA1* integrin, but none of the *S. Enteritidis* isolates were positive for the *tniA* marker.

S. Typhimurium gene cassettes

All the 1000 bp amplicons were shown to contain the *aadA2* gene that confers resistance to streptomycin and spectinomycin with a perfect match (100 % similarity) to the *aadA2* gene from *S. Typhimurium* DT104 (accession no. AF071555; Briggs & Fratamico, 1999). The 1200 bp amplicons contained the *pse-1* β-lactamase gene with a perfect match to the *pse-1* gene from *S. Typhimurium* DT104 (accession no. AF071555; Briggs & Fratamico, 1999). The *aadA2* gene cassette was seen alone in two strains (4380/00 and 7192/00) which also failed to amplify products with *pse-1*-specific primers. The *pse-1* cassette thus appears to be lost in these strains. No isolates carried the *pse-1* gene cassette alone without an accompanying *aadA2* cassette. The 1500 bp amplicon was found to carry two gene cassettes in tandem, consisting of the *aadB* and the *catB3* genes with a perfect match to the integrin In-t1 (accession no. AJ009818; Tosini *et al.*, 1998). The 2000 bp amplicon carried two known genes, *dfrA12* and *aadA2*, as well as an ORF and was present in a DT104a isolate. This integrin is discussed below in the *S. Enteritidis* section. The 2100 bp amplicon carried two tandemly inserted gene cassettes consisting of the *oxa1* and the *aadA1* genes with a perfect match to the In-t2 integrin (accession no. AJ009819; Tosini *et al.*, 1998). Tosini *et al.* (1998) found a third integrin (In-t3) of 3200 bp that was localized to an IncL/M plasmid. This 3200 bp integrin was absent in our isolates, which appear to contain only the IncFI plasmid. The 1700 bp amplicon revealed two gene cassettes consisting of the *dfrA17* and the *aadA5* genes. The 1600 bp amplicon contained gene cassettes with the *dfrA1* and *aadA1* genes and was found in one isolate (7542/00) from Belgium. This *dfrA1*-*aadA1*-containing integrin was confirmed to be plasmid-located.

S. Typhimurium integrin-combinations and structure

The most common feature in *S. Typhimurium* was the presence of two integrans, one containing *aadA2* and the other the *pse-1* gene. This combination has been observed several times and is characteristic for the genetically homogeneous *S. Typhimurium* DT104 strain which has emerged

to become the most common type of *S. Typhimurium* isolate in several countries and is predominantly associated with resistance to multiple antibiotics. We failed to get any integron variable region amplicons for only two isolates (5031/00 and 6347/00) using the cassette primers, but isolate 6347/00 was positive for both *aadA2* and *pse-1* when primers directed at these genes were used, and isolate 5031/00 was positive for *aadA2*. It is likely that these genes are located as cassettes, but deletions in the 5' integron structures that affect the upper cassette primer possibly prevent PCR amplification. These two strains also carried the integron-associated *qacEΔ1* gene and one strain (6347/00) also carried the *sulI* gene, which may further indicate the presence of a 5' deleted integron structure. Another possible, but less likely explanation would be that the *aadA2* and *pse-1* genes in these isolates are not localized within integrons. Isolate 5031/00 (DT104) is additionally negative for the *sulI* and the *floR* genes, which again suggest extensive deletions in the integron region of this isolate. Both domestically acquired isolates and isolates from patients that have been abroad show the same dominance of the DT104 integron combination as would be expected from the known frequency of this phage type worldwide. The 1600 bp amplicon from isolate 7542/00 with the *dfrA1-aadA1* gene combination, conferring resistance to trimethoprim and streptomycin, has recently been seen in Irish *S. Typhimurium* animal and food isolates (Daly & Fanning, 2000) as well as in Norwegian animal isolates (Sunde & Sørum, 1999). Two isolates (3428/98 and 900/98, Table 2) from infants that had returned from travelling in Africa showed two recently published integron structures, In-t1 and In-t2 (Tosini *et al.*, 1998) with sizes of 1500 and 2100 bp, respectively. These two integrons have previously been identified in *S. Typhimurium* isolates from Albanian infants with acute gastroenteritis (Tosini *et al.*, 1998). The 1500 bp In-t1 amplicon contained the *aadB* gene that has been shown to confer resistance to kanamycin and low levels of gentamicin (Shaw *et al.*, 1993), and the *catB3* gene that encodes the enzyme chloramphenicol acetyltransferase (CatB3) (Bunny *et al.*, 1995). The 2100 bp In-t2 amplicon contained the *oxa1* gene that encodes a β -lactamase and the *aadA1* gene that encodes the aminoglycoside adenyltransferase AAD(3'')-Ia enzyme which confers streptomycin resistance. These two integrons were both located on an IncFI plasmid in the Albanian isolates (Tosini *et al.*, 1998; Carattoli *et al.*, 2001), and also in our two isolates the integrons were confirmed by plasmid isolation and hybridization to be located on plasmids. This is the second report confirmed by sequencing of *S. Typhimurium* isolates that carry a plasmid with both In-t1 and In-t2 where all isolates in both studies originated from infants with gastroenteritis. One isolate (1864/96) carried only a plasmid-borne In-t1 integron. This was a 28-year-old patient infected in India. The second IncL/M plasmid was absent in our isolates with the In-t1 and In-t2 integrons. This can possibly be ascribed to the higher conjugation frequency of the IncFI plasmid as compared to the IncL/M plasmid, giving it a higher potential to transfer between isolates. Isolate 3428/98 is additionally positive for the mercury resistance gene *merA* that is associated with

transposon Tn21 and some other known transposons, while isolate 900/98 is not. The In-t2 integron of 3428/98 is thus likely to be part of a larger transposon structure. The 200 bp amplicon was not consistently detected and was both present and absent in parallels of the same isolate, and after gel electrophoresis this 200 bp amplicon was always a fainter band than the amplicons of 1000 bp or longer. The 200 bp amplicon contained an ORF that was an internal segment of the *purG* gene encoding phosphoribosylformylglycinamide synthetase and has been described as a small integron in *S. Typhimurium* isolates from the Republic of Ireland amplified with the cassette primers (Daly *et al.*, 2000). We found that this 200 bp amplicon was a PCR artefact on closer examination of the sequence and after negative test PCRs with a primer set with one primer in the *intI1* gene and one in the *purG* fragment (IPUR-U/IPUR-L) (Table 4). The absence of the disinfectant gene *qacEΔ1* in one strain and the sulphonamide resistance gene in seven, indicates a possible increase in gene loss at the integron 3'-conserved segment region as noted by Hall *et al.* (1994). Isolate 8709/00 is a DT104 strain with an ACSSuT resistance profile, but the Pse-1 protein in this isolate is truncated as a consequence of a frameshift caused by a T insertion in the coding sequence just downstream for the ATG initiation codon. This truncated protein is believed to be non-functional. The 8709/00 isolate is still resistant to ampicillin which can only be explained by the presence of another functional β -lactamase, either chromosomal or plasmid-borne. The integron in strain 5180/00 was shown to contain the *dfrA17* and *aadA5* genes. The *dfrA17* gene confers resistance to trimethoprim and the recently described *aadA5* gene (Sandvang, 1999) confers resistance to spectinomycin, but not to streptomycin (White *et al.*, 2000). A sequence homology search in GenBank revealed three homologous integron sequences where two were from *E. coli* isolates and one was an unpublished sequence from a *Klebsiella pneumoniae* isolate. The first *E. coli* *dfrA17-aadA5* integron was from Australia and most likely had a chromosomal location (accession no. AF169041), the strain having been isolated from the urinary tract of a hospitalized patient (White *et al.*, 2000). The other *E. coli* *dfrA17-aadA5* integron was from a clinical urine specimen isolated in Taiwan; this integron (accession no. AF170088) was located on a 119 kb transferable plasmid (Chang *et al.*, 2000a). The third homologue to the 5180/00 *dfrA17-aadA5* integron was an unpublished sequence from a *K. pneumoniae* isolate (accession no. AF220757). The 5180/00 *S. Typhimurium* strain was isolated from a patient infected in the Mediterranean region and carries the *merA* gene, which suggests that this integron could be transposon-associated. The plasmid preparation from strain 5180/00 in our study was negative for the integron-associated *intI1*, *qacEΔ1* and *sulI* genes, making the integron most likely chromosomal in our isolate. This appears to be the first finding of this integron structure in *S. Typhimurium*, and the possible association with a *merA*-containing transposon will facilitate its further movement between strains.

When we only considered the non-DT104 isolates we

detected the *aadB-catB3* integron in three isolates, the *oxa1-aadA1* integron in five isolates, the *aadA2* integron in two isolates together with the *pse-1* integron, the *dfrA17-aadA5* integron in one isolate and the *dfrA12-orf-aadA2* and *dfrA1-aadA1* integrons also in one isolate each. The *pse-1* sequence in the 1200 bp cassette of strain 8709/00 displayed an insertion of an extra T nucleotide at nt 466 that would cause a frameshift and thus early termination at nt 510 in the 8709/00 *pse-1* gene as compared to the *pse-1* sequence from GenBank (accession no. AF313472).

S. Enteritidis integrons

In this report we additionally describe the presence of class I integrons among isolates of the important zoonotic pathogen *S. Enteritidis* from hospitals throughout Norway, isolated from 2000 to 2001, which makes them suited for describing the present situation concerning the frequency of integron-containing isolates of *S. Enteritidis* in Norway. The level of *S. Enteritidis* infections acquired domestically is very low in Norway. The majority of patients with illness caused by *S. Enteritidis* are infected abroad. Twenty of the investigated resistant isolates of *S. Enteritidis* contained integrons (22.2 %); in 2000 16.7 % (6/36) of the isolates contained integrons while in 2001 this number had risen to 25.9 % (14/54).

Gene cassette amplicons of 650, 1600 and 2000 bp were found. The 650 bp amplicon was shown to contain a fragment of the *sat1* gene, which in its full version gives resistance to streptothricin (Tschape *et al.*, 1984; Heim *et al.*, 1989). The 1600 bp amplicons were found to contain two integron gene cassettes, *dfrA1* and *aadA1*. The 2000 bp amplicons were found to carry an array of three cassettes, *dfrA12-orf-aadA2*. The *dfrA12* gene encodes a variant of the dihydrofolate reductase gene and the *aadA2* gene confers resistance to streptomycin. The *dfrA12* gene product confers a 10-times higher level of resistance to trimethoprim than the most common trimethoprim resistance gene *dfrA1* (Heikkilä *et al.*, 1993). The *dfrA12-orf-aadA2* gene cluster has previously been seen in several strains, including an *E. coli* O157 isolate (GenBank accession no. AF335108, unpublished), a *Serratia marcescens* isolate (GenBank accession no. AF284063, unpublished), a Spanish *S. Typhimurium* phage type U302 isolate (Guerra *et al.*, 2001; Echeita *et al.*, 2001), an *E. coli* Shiga-toxin producing O111:H8 isolate from the USA (Zhao *et al.*, 2001), *E. coli* isolates from Taiwan (Chang *et al.*, 2000b), *E. coli* and *Shigella* isolates from Finland (Heikkilä *et al.*, 1993), an epidemic *Vibrio cholerae* O1 strain from Guinea-Bissau (Dalsgaard *et al.*, 2000) and in an *S. Typhimurium* DT104a isolate originating from Australia (this report).

The results showed that integrons containing resistance to trimethoprim and streptomycin were frequently associated with multi-resistant *S. Enteritidis* isolates. Since the level of *S. Enteritidis* isolates containing resistance to two or more antibiotics is only about 4 % of the total isolates, the number of these mobile genetic elements brought into Norwegian

hospitals by *S. Enteritidis* is still modest. Our results, however, indicate a possible increase in the integron-containing isolates in Norwegian hospitals from 2000 to 2001. The frequency of integron carriers among the studied isolates rose from 17 % in 2000 to 25.9 % in 2001. In a study of antimicrobial resistance in *S. Enteritidis* isolated between 1990 and 1998 in Southern Italy it was reported that 44 of 1889 strains (2.3 %) were resistant to at least one antibiotic (Nastasi *et al.*, 2000). Of these 44 isolates, six were shown to carry integrons. This gives a frequency of integron carriers among resistant isolates of 13.6 % (6/44) in the strains isolated between 1990 and 1998 (Nastasi *et al.*, 2000). Our data indicate that this number might be increasing, and our frequency of about 22 % of resistant isolates carrying integrons is most likely a slight underestimate of the total frequency, since we have only looked at isolates containing resistance to two or more antibiotics. In a screening of all sulfamethoxazole-resistant *S. Enteritidis* isolates received by the Scottish Salmonella Reference Laboratory in 1997, integrons were detected in 19 % of the isolates (11/58) (Brown *et al.*, 2000), which is a frequency comparable to our data for 2000 of about 17 %. It will be interesting to see whether our increase in integron-carrying *S. Enteritidis* isolates from 2000 to 2001 will continue or if this was a fluctuation. Isolates from 2002 are being screened to address this in our laboratory. The only integron found in the 2000 isolates was the *dfrA1-aadA1* integron, while in 2001 we also detected the *aadA2* and *dfrA12* genes in addition to a fragment of the *sat1* gene in *S. Enteritidis*.

All *S. Enteritidis* integrons in this study were localized on plasmids, indicating that they contain the potential to spread further after introduction to a hospital environment.

Integron cassette promoters

The promoter regions in front of all the gene cassettes were PCR-amplified and sequenced. The promoters were homogeneous with all isolates showing both P_c - (also called P_{ant}) and P_2 -related sequences. Only one variation of the P_c promoter was found among all the integrons sequenced. The majority of samples had the P_c promoter sequence TGGACA-N₁₇-TAAGCT, irrespective of the cassette following this promoter (Table 5). Only the 5180/00 *S. Typhimurium* isolate had a variant P_c promoter with the sequence TGGACA-N₁₇-TAAACT in front of the *dfrA17-aadA5* gene cassettes (Table 5). In the P_2 -related sequences we found that all but seven isolates had the sequence TTGTTA-N₁₄-TACAGT, which is not a functional P_2 promoter. The *S. Typhimurium* isolates 1875/96, 1550/96, 1203/97, 2339/99, 1176/96 and 900/98 had all active P_2 promoters where the distances between the -35 and the -10 regions of the P_2 promoters were 17 nt (Table 5). All these isolates displayed active P_2 promoters in front of an *oxa1-aadA1* gene cassette (Table 5). We thus found that the majority of P_2 promoter signals in the In-t2 integrons (*oxa1-aadA1*) have a spacing between the -10 and -35 regions that is optimal for promoter function. These In-t2 isolates are thus believed to carry two active promoters (P_c and P_2). This is in accordance

Table 5. Integron cassette promoters

Nucleotides in *italic* are only P2-related sequences and are not part of an active promoter.

Isolate	Promoter sequences						Type
	P _C			P2			
	−35	N	−10	−35	N	−10	
Majority	TGGACA	17	TAAGCT	<i>TTGT</i> TA	14 [*]	<i>TACAGT</i>	Weak
5180/00	TGGACA	17	TAAACT	<i>TTGT</i> TA	14 [*]	<i>TACAGT</i>	Hybrid
3428/98	TGGACA	17	TAAGCT	<i>TTGT</i> TA	14 [*]	<i>TACAGT</i>	Weak
900/98	TGGACA	17	TAAGCT	TTGTTA	17	TACACA	Weak+mutated P2
1875/96	TGGACA	17	TAAGCT	TTGTTA	17	TACAGT	Weak+P2
1550/96	TGGACA	17	TAAGCT	TTGTTA	17	TACAGT	Weak+P2
1203/97	TGGACA	17	TAAGCT	TTGTTA	17	TACAGT	Weak+P2
2339/99	TGGACA	17	TAAGCT	TTGTTA	17	TACAGT	Weak+P2
1176/96	TGGACA	17	TAAGCT	TTGTTA	17	TACAGT	Weak+P2
4805/00	TGGACA	17	TAAGCT	<i>TTGT</i> TA	14 [*]	<i>T-CAGT</i>	Weak

* A spacing of 14 nucleotides between the -35 and -10 signal is outside the range of functional cassette promoters and these sequences do not constitute active promoters.

with the study by Tosini *et al.* (1998) (accession no. AJ009819) that also shows a spacing of 17 nt between the -10 and -35 regions in the In-t2 P2 promoter from plasmid *IncFI/97*, and a spacing of 14 nt between the P2 like -10 and -35 sequences in In-t1 (*aadB-catB3*) (accession no. AJ009818), similar to our results. Variations were noted, however: for example, the In-t2 integrons of isolate 3428/98 which had a spacing of 14 nt between the P2-like -10 and -35 signals, and isolate 900/98 which had a mutation in the P2 promoter sequence, TTGTTA-N₁₇-TACACA (the last 2 nt changed from GT) (Table 5). According to Carattoli *et al.* (2001) the In-t2 integron is likely to have been maintained unaltered in *IncFI* plasmids for nearly 30 years, while the plasmids with both In-t1 and In-t2 (*IncFI/97*) are considered to be new variants (Carattoli *et al.*, 2001). It thus appears as though the *IncFI/97*-associated In-t2 integron exists in two variant forms, one where both promoters are active and one where only P_C is active. The In-t2 integron with only a functional P_C promoter (strain 3428/98) is believed to exhibit weaker expression of the gene cassettes. The strength of the mutated P2 promoter in 900/98 is unknown and will be investigated further.

All of the *S. Enteritidis* promoters consisted of only the TGGACA-N₁₇-TAAGCT P_C promoter, which is identical to the most common *S. Typhimurium* P_C promoters, and did not have a functional P2 promoter.

ACKNOWLEDGEMENTS

Dr Truls Leergaard is gratefully acknowledged for providing data regarding antibiotic resistance of the *S. Typhimurium* isolates, and the staff of the National Salmonella Reference Laboratory is gratefully acknowledged for technical assistance and for providing data regarding antibiotic resistance of the *S. Enteritidis* isolates.

REFERENCES

- Baumler, A. J., Hargis, B. M. & Tsolis, R. M. (2000). Tracing the origins of *Salmonella* outbreaks. *Science* **287**, 50–52.
- Bito, A. & Susani, M. (1994). Revised analysis of *aadA2* gene of plasmid *pSa*. *Antimicrob Agents Chemother* **38**, 1172–1175.
- Briggs, C. E. & Fratamico, P. M. (1999). Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob Agents Chemother* **43**, 846–849.
- Brown, A. W., Rankin, S. C. & Platt, D. J. (2000). Detection and characterisation of integrons in *Salmonella enterica* serotype enteritidis. *FEMS Microbiol Lett* **191**, 145–149.
- Brown, H. J., Stokes, H. W. & Hall, R. M. (1996). The integrons In0, In2, and In5 are defective transposon derivatives. *J Bacteriol* **178**, 4429–4437.
- Bunny, K. L., Hall, R. M. & Stokes, H. W. (1995). New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene, *catB3*, in an integron in pBWH301. *Antimicrob Agents Chemother* **39**, 686–693.
- Carattoli, A., Villa, L., Pezzella, C., Bordi, E. & Visca, P. (2001). Expanding drug resistance through integron acquisition by *IncFI* plasmids of *Salmonella enterica* Typhimurium. *Emerg Infect Dis* **7**, 444–447.
- Chang, C. Y., Chang, L. L., Chang, Y. H., Lee, T. M., Li, Y. H. & Chang, S. F. (2000a). Two new gene cassettes, *dfr17* (for trimethoprim resistance) and *aadA4* (for spectinomycin/streptomycin resistance), inserted in an *Escherichia coli* class 1 integron. *J Antimicrob Chemother* **46**, 87–89.
- Chang, C. Y., Chang, L. L., Chang, Y. H., Lee, T. M. & Chang, S. F. (2000b). Characterisation of drug resistance gene cassettes associated with class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC. *J Med Microbiol* **49**, 1097–1102.
- Collis, C. M. & Hall, R. M. (1995). Expression of antibiotic resistance genes in the integrated cassettes of integrons. *Antimicrob Agents Chemother* **39**, 155–162.
- Dalsgaard, A., Forslund, A., Petersen, A. & 7 other authors (2000). Class 1 integron-borne, multiple-antibiotic resistance encoded by a 150-kilobase conjugative plasmid in epidemic *Vibrio cholerae* O1 strains isolated in Guinea-Bissau. *J Clin Microbiol* **38**, 3774–3779.

- Daly, M. & Fanning, S. (2000). Characterization and chromosomal mapping of antimicrobial resistance genes in *Salmonella enterica* serotype typhimurium. *Appl Environ Microbiol* **66**, 4842–4848.
- Daly, M., Buckley, J., Power, E., O'Hare, C., Cormican, M., Cryan, B. Wall, P. G. & Fanning, S. (2000). Molecular characterization of Irish *Salmonella enterica* serotype typhimurium: detection of class I integrons and assessment of genetic relationships by DNA amplification fingerprinting. *Appl Environ Microbiol* **66**, 614–619.
- Echeita, M. A., Herrera, S. & Usera, M. A. (2001). Atypical, *fljB*-negative *Salmonella enterica* subsp. *enterica* strain of serovar 4,5,12:i:- appears to be a monophasic variant of serovar Typhimurium. *J Clin Microbiol* **39**, 2981–2983.
- Guard-Petter, J. (2001). The chicken, the egg and *Salmonella enteritidis*. *Environ Microbiol* **3**, 421–430.
- Guerra, B., Soto, S. M., Arguelles, J. M. & Mendoza, M. C. (2001). Multidrug resistance is mediated by large plasmids carrying a class I integron in the emergent *Salmonella enterica* serotype [4,5,12:i:-]. *Antimicrob Agents Chemother* **45**, 1305–1308.
- Hall, R. M. & Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* **15**, 593–600.
- Hall, R. M. & Stokes, H. W. (1993). Integrons: novel DNA elements which capture genes by site-specific recombination. *Genetica* **90**, 115–132.
- Hall, R. M., Brown, H. J., Brookes, D. E. & Stokes, H. W. (1994). Integrons found in different locations have identical 5' ends but variable 3' ends. *J Bacteriol* **176**, 6286–6294.
- Heikkilä, E., Skurnik, M., Sundstrom, L. & Huovinen, P. (1993). A novel dihydrofolate reductase cassette inserted in an integron borne on a *Tn21*-like element. *Antimicrob Agents Chemother* **37**, 1297–1304.
- Heim, U., Tietze, E., Weschke, W., Tschape, H. & Wobus, U. (1989). Nucleotide sequence of a plasmid born streptothricin-acetyl-transferase gene (*sat-1*). *Nucleic Acids Res* **17**, 7103.
- Huovinen, P. & Jacoby, G. A. (1991). Sequence of the *PSE-1* beta-lactamase gene. *Antimicrob Agents Chemother* **35**, 2428–2430.
- Kapperud, G., Lassen, J., Dommarsnes, K., Kristiansen, B. E., Caugant, D. A., Ask, E. & Jahkola, M. (1989). Comparison of epidemiological marker methods for identification of *Salmonella typhimurium* isolates from an outbreak caused by contaminated chocolate. *J Clin Microbiol* **27**, 2019–2024.
- Kingsley, R. A. & Baumler, A. J. (2000). Host adaptation and the emergence of infectious disease: the *Salmonella* paradigm. *Mol Microbiol* **36**, 1006–1014.
- Lévesque, C., Brassard, S., Lapointe, J. & Roy, P. H. (1994). Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. *Gene* **142**, 49–54.
- Lévesque, C., Piche, L., Larose, C. & Roy, P. H. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother* **39**, 185–191.
- Mazel, D. & Davies, J. (1999). Antibiotic resistance in microbes. *Cell Mol Life Sci* **56**, 742–754.
- Mazel, D., Dychinco, B., Webb, V. A. & Davies, J. (2000). Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. *Antimicrob Agents Chemother* **44**, 1568–1574.
- Nastasi, A., Mammina, C. & Cannova, L. (2000). Antimicrobial resistance in *Salmonella enteritidis*, southern Italy, 1990–1998. *Emerg Infect Dis* **6**, 401–403.
- Nield, B. S., Holmes, A. J., Gillings, M. R., Recchia, G. D., Mabbutt, B. C., Nevalainen, K. M. & Stokes, H. W. (2001). Recovery of new integron classes from environmental DNA. *FEMS Microbiol Lett* **195**, 59–65.
- Partridge, S. R., Brown, H. J., Stokes, H. W. & Hall, R. M. (2001). Transposons *Tn1696* and *Tn21* and their integrons *In4* and *In2* have independent origins. *Antimicrob Agents Chemother* **45**, 1263–1270.
- Radstrom, P., Skold, O., Swedberg, G., Flensburg, J., Roy, P. H. & Sundstrom, L. (1994). Transposon *Tn5090* of plasmid *R751*, which carries an integron, is related to *Tn7*, *Mu*, and the retroelements. *J Bacteriol* **176**, 3257–3268.
- Rowe-Magnus, D. A., Guerout, A. M., Ploncard, P., Dychinco, B., Davies, J. & Mazel, D. (2001). The evolutionary history of chromosomal super-integrons provides an ancestry for multiresistant integrons. *Proc Natl Acad Sci U S A* **98**, 652–657.
- Sandvang, D. (1999). Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class I integron isolated from *Escherichia coli*. *Antimicrob Agents Chemother* **43**, 3036–3038.
- Sandvang, D., Aarestrup, F. M. & Jensen, L. B. (1997). Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett* **157**, 177–181.
- Shaw, K. J., Rather, P. N., Hare, R. S. & Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* **57**, 138–163.
- Stokes, H. W. & Hall, R. M. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* **3**, 1669–1683.
- Sunde, M. & Sørum, H. (1999). Characterization of integrons in *Escherichia coli* of the normal intestinal flora of swine. *Microb Drug Resist* **5**, 279–287.
- Tosini, F., Visca, P., Luzzi, I., Dionisi, A. M., Pezzella, C., Petrucca, A. & Carattoli, A. (1998). Class I integron-borne multiple-antibiotic resistance carried by *IncFI* and *IncLM* plasmids in *Salmonella enterica* serotype typhimurium. *Antimicrob Agents Chemother* **42**, 3053–3058.
- Tschape, H., Tietze, E., Prager, R., Voigt, W., Wolter, E. & Seltmann, G. (1984). Plasmid-borne streptothricin resistance in gram-negative bacteria. *Plasmid* **12**, 189–196.
- White, P. A., McIver, C. J., Deng, Y. & Rawlinson, W. D. (2000). Characterisation of two new gene cassettes, *aadA5* and *dfrA17*. *FEMS Microbiol Lett* **182**, 265–269.
- Zhao, S., White, D. G., Ge, B., Ayers, S., Friedman, S., English, L., Wagner, D., Gaines, S. & Meng, J. (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. *Appl Environ Microbiol* **67**, 1558–1564.