

# Comparison of Arbitrarily Primed PCR, Antibiotic Resistance and Plasmid Profiling for Differentiating *Salmonella enteritidis* Isolated from Fish

S. RADU<sup>1</sup>, A. M. SAHILAH<sup>1</sup>, G. RUSUL<sup>2</sup>, L. SAMUEL<sup>1</sup>, M. I. ZURAINI<sup>2</sup>  
and E. H. NASRELDIN<sup>1</sup>

<sup>1</sup>Department of Biotechnology  
Faculty of Food Science and Biotechnology  
University Putra Malaysia, 43400 UPM Serdang  
Selangor, Malaysia.

<sup>2</sup>Department of Food Science  
Faculty of Food Science and Biotechnology  
University Putra Malaysia, 43400 UPM Serdang  
Selangor, Malaysia.

## Abstract

A total of 28 isolates of *Salmonella enteritidis* isolated from *Tilapia* (*Tilapia mossambica*) were investigated through arbitrarily primed polymerase chain reaction (AP-PCR) fingerprinting using three primers, plasmid profiling and their antibiotic resistance patterns. All 28 isolates carried at least one plasmid ranging in size from 1.4 to 38 megadalton that enabled the *S. enteritidis* to be grouped into nine plasmid profiles. Isolates were resistant to nalidixic acid (21.1%), penicillin (39.3%) and streptomycin (71.4%), and were susceptible to carbenicillin, cephalothin, kanamycin, rifampicin and tetracycline. Seventeen isolates (60.7%) were resistant to a single antibiotic and eleven (39.3%) were resistant to two antibiotics; thus separating the isolates into six antibiotic resistance patterns. The AP-PCR results showed that the collection of isolates were genetically very heterogenous. AP-PCR enabled us to differentiate the isolates into 28 AP-PCR types. Analysis derived from these data in combination (AP-PCR, plasmid profiling and antibiotic resistance patterns) showed that all 28 isolates were distinct and were grouped into 28 individual groups. Our results demonstrate that AP-PCR fingerprinting method is more sensitive than plasmid profiling and antibiotic resistance patterns with respect to the individualization of the isolates used in this study.

## Introduction

*Salmonella enteritidis* is one of the most common causes of nontyphoidal salmonellosis after *S. typhimurium* and has now become the predominant serotype in many countries (Rodrigue et al. 1992). To determine the health risk associated with exposure to *S. enteritidis*, epidemiological

tracking is required. This may be achieved by using highly discriminatory molecular typing methodologies which allow rapid and sensitive differentiation between strains within a single serotype. Among these are plasmid analysis, ribotyping, IS200 profile, pulsed field gel electrophoresis and arbitrarily primed polymerase chain reaction (AP-PCR). These approaches are very helpful and have been applied by many researchers to investigate the evolutionary and epidemiological relationships of several *Salmonella* serotypes (Rodrigue et al. 1992; Olsen et al. 1994; Baquar et al. 1994; Fadl et al. 1995; Lin et al. 1996; Burr et al. 1998). In the present study, epidemiological typing was performed on 28 strains of *S. enteritidis* isolated from fish (*Tilapia mossambica*) using three different but complementary typing methods: arbitrarily primed PCR, plasmid typing and antibiotyping. The discriminatory powers of these typing methods for the same group of *S. enteritidis* isolates were compared.

## Materials and Methods

### *Fish*

Bacteria were isolated from the gills of *Tilapia* (*Tilapia mossambica*) re-tailed at several wet markets in Selangor, Malaysia.

### *Isolation and identification of bacterial strains*

Fish gills (20 g) were cut off using sterile scissors and put into a bottle containing alkaline peptone water and incubated overnight at 37°C. Then, 0.1 ml of the appropriate dilution was plated onto Salmonella-Shigella (SS) agar (Oxoid, Maryland). Plates were incubated for 18-24 hours at 37°C. Suspect colonies were picked and maintained on tryptone soy agar (Oxoid). Identification of isolated bacteria was carried out using Gram reaction and the API 20E test kit (BioMerieux, France).

### *Antibiotic susceptibility testing*

Susceptibility to antimicrobial agents was tested using the standard disc diffusion method (National Committee for Clinical Laboratory Standards 1993). Discs containing the following antibiotics were spotted with a 3 cm interval: carbenicillin at 100 µg, cephalothin at 30 µg, nalidixic acid at 30 µg, kanamycin at 30 µg, rifampicin at 5 µg, streptomycin at 10 µg and tetracycline at 30 µg (BBL Sensi-Disc, Becton Dickinson). The plates were incubated for 24 h at 37°C. The sensitivity or resistance of each tested isolate to these antibiotics was measured by the diameter of the inhibition zones around the antibiotic disc.

### *Bacterial growth and DNA preparation*

All isolates were grown in Luria-Bertani (LB) broth at 37°C with shaking at 200 rpm overnight. Prior to amplification by AP-PCR, total genomic

DNA of the *S. enteritidis* strains were extracted using the method described by Wilson et al. (1989). Small scale preparation of plasmid DNA from the *S. enteritidis* strains were obtained using the rapid alkaline extraction procedure described by Sambrook et al. (1989). The approximate molecular mass of each plasmid was determined by comparing it with a plasmid of known molecular mass of *E. coli* V517 (Macrina et al. 1978).

### **Primers**

A randomly designed 10-mer oligonucleotide set, designated as GEN1-50-01 to GEN1-50-10 was obtained from the Genosys Biotechnologies Inc. (TX, USA). The GEN1-50-01 (5'-GTGCAATGAG-3'), GEN1-50-08 (5'-GGAAGACAAC-3') and GEN1-50-09 (5'-AGAAGCGATG-3') were chosen for AP-PCR analysis because they yielded clear patterns.

### **AP-PCR fingerprinting**

PCR assays were routinely performed in a 25 µl reaction mixture containing 20-30 ng of template DNA, 2.5 ml 10x buffer, 1 unit *Taq* DNA polymerase, 0.2 mM primer, 2.5 mM MgCl<sub>2</sub> and 1 mM each of dCTP, dGTP, dATP and dTTP. AP-PCR was carried out using a thermal cycler (Perkin Elmer 2400). The cycling parameters were 1 min at 94°C, 1 min at 36°C and 2 min at 72°C for a total of 45 cycles, with a final cycle extending amplification conditions to 72°C for 5 min. The PCR amplification products were visualized by running 10 µl of the reaction on a 1.0% agarose gel and detected by staining with ethidium bromide. DNA ladder (Promega, USA) was used as DNA size marker.

## **Results and Discussion**

Fish (*Tilapia mossambica*) from several wet markets in Selangor, Malaysia were analysed in this study. During the five-month period, 28 *Salmonella enteritidis* strains were isolated from 10 of 23 samples. *S. enteritidis* has been associated with many outbreaks of human gastroenteritis and foodborne transmission has been implicated as one route of infection, especially by cross-contamination of precooked or raw foods (Hadfield et al. 1985; Threlfall et al. 1989; Rivera et al. 1991; Dorn et al. 1992). This underlines the need for consumer protection against infection by potential pathogenic strains of bacteria in fish, and as in other parts of East Asia, *tilapia* is popularly served in restaurants as well as at food stalls. The results of this study show that *tilapia* (*Tilapia mossambica*) is a potential vector for *S. enteritidis* in the study area.

All isolates were resistant to one or more of the antimicrobial agents tested. Their antibiotic resistance allow a clear distinction among the 28 isolates which made it possible to differentiate the *S. enteritidis* into six antibiotic resistance patterns on the basis of their susceptibility to nalidixic acid,

penicillin and streptomycin; and therefore found to be an acceptable marker for this set of isolates. The majority of isolates displayed resistance towards streptomycin (71.4%), penicillin (39.3%) and nalidixic acid (21.1%); but none is resistant to carbenicillin, cephalothin, kanamycin, rifampicin and tetracycline (Table 1). Our results confirm data reported by other authors indicating that the frequency of antibiotic resistance in *S. enteritidis* has been found to be low and stable (Cohen and Tauxe 1986; Lorian, 1986; Ward et al. 1990; Threlfall and Chart 1993). For epidemiological purposes, antibiotic resistance patterns provided only a limited degree of discrimination since 60.7% were resistant to a single antibiotic and 39.3% were resistant to two antibiotics. In addition, the limited degree of strain discrimination observed in antibiotyping could be due to the small number of antibiotics used in this study.

Determination of the plasmid profile using agarose gel electrophoresis reveal that all (100%) of the isolates carried at least one plasmid ranging in

Table 1. *Salmonella enteritidis* strains isolated from fish and their plasmid patterns, antimicrobial resistance and AP-PCR profiles.

Strains	Plasmid size (MDa) <sup>a</sup>	Antimicrobial resistance patterns <sup>b,c</sup>	AP-PCR profiles with primers:		
			GEN15001	GEN15008	GEN15009
FSE1	37 (1)	PSm (6)	A1	B1	C1
FSE2	37 (1)	PSm (6)	A2	UT	C1
FSE3	37 (1)	PSm (6)	A3	B2	C1
FSE4	37 (1)	PSm (6)	A3	B3	C1
FSE5	37 (1)	NaP (4)	A4	B4	C2
FSE6	37 (1)	Na (1)	A5	B4	C2
FSE7	37 (1)	P (2)	A6	B4	C2
FSE8	37 (1)	PSm (6)	A7	B5	C2
FSE9	37 (1)	PSm (6)	A8	B6	C3
FSE10	37, 1.4 (2)	PSm (6)	A9	B7	C4
FSE11	37, 1.7 (4)	P (2)	A10	B8	C5
FSE12	37, 3.0 (6)	Sm (3)	A11	B9	C6
FSE13	37, 3.0, 1.9 (9)	PSm (6)	A12	B10	C7
FSE14	37, 3.0, 1.9 (9)	Sm (3)	A12	B11	C7
FSE15	37, 3.0, 1.9 (9)	Sm (3)	A12	B12	C8
FSE16	37, 2.5, 1.5 (7)	Sm (3)	A13	B13	C9
FSE17	37 (1)	Na (1)	A14	B14	C10
FSE18	37 (1)	Na (1)	A15	B15	C11
FSE19	37, 2.0 (3)	Na (1)	A15	B16	C12
FSE20	37 (1)	Na (1)	A16	B16	C13
FSE21	37, 3.0, 1.7 (8)	Sm (3)	A17	B16	C14
FSE22	37, 1.4 (2)	NaSm (5)	A18	B17	C15
FSE23	37, 1.4 (2)	Sm (3)	A19	B18	C15
FSE24	37 (1)	PSm (6)	A19	B19	C16
FSE25	37 (1)	Sm (3)	A19	B20	C16
FSE26	37, 1.7 (4)	Sm (3)	A20	B21	C17
FSE27	37, 1.4 (2)	Sm (3)	A21	B22	C18
FSE28	37, 2.2 (5)	Sm (3)	A22	B23	C18

<sup>b</sup>Tested for carbenicillin, cephalothin, nalidixic acid (Na), kanamycin, penicillin (P), rifampicin, streptomycin (Sm) and tetracycline.

<sup>a,c</sup>Number in parenthesis indicates the plasmid pattern groups and antibiotic resistance groups, respectively.

UT – could not be classified according to type.

sizes from 1.4 to 38 megadalton (MDa). Generally, epidemiologically unrelated isolates expressed different plasmid profiles. In the present study, 14 (50%) of the plasmids containing *S. enteritidis* isolates were found to harbor a single plasmid of 38 MDa, nine and five isolates harbor two and three plasmid bands, respectively. The 38 MDa plasmid was present in all (100%) of the isolates, alone or in combination with other plasmids. The high prevalence of *S. enteritidis* isolates carrying the 38 MDa plasmid, singularly or in combination with other plasmids, has been reported previously (Helmuth et al. 1985; Nakamura et al. 1985; Lujan et al. 1990). Comparatively, the diversity of plasmid profiles was greater than antibiotic resistance patterns among the *S. enteritidis* isolated from fish. Using plasmid number and size as a basis, we could separate the isolates into nine groups (Table 1). Elsewhere, Threlfall and Chart (1993) reported that 78% of *S. enteritidis* isolates examined were characterized by a single plasmid of 38 MDa and that only nine plasmid profile types were observed. Though plasmid analysis could provide useful epidemiological information, the small number of plasmid carriage among the *S. enteritidis* studied compromise our ability to draw any conclusions.

Initially, ten primers were screened using a subsample of five isolates to detect polymorphism within *S. enteritidis*. However, only three oligonucleotides showed DNA polymorphism within the isolates tested. These three discriminating primers are GEN15001, GEN15008 and GEN15009. The G+C contents of these primers are 50% where *Salmonella* spp. have an average genome GC content of 50-52% (Marmur et al. 1963). They were chosen to analyse all of the 28 *S. enteritidis* isolates. The remaining primers (GEN15002, 03, 04, 05, 06, 07 and 10) gave bands with only some of the isolates or had poor reproducibility and were not examined further. Such diversity among primers has been previously reported by Oakey et al. (1995). Figures 1a and 1b, 2a and 2b, and 3a and 3b show the AP-PCR profiles of the *S. enteritidis* isolates obtained using primers GEN15001, GEN15008 and GEN15009, respectively. The possible number of AP-PCR patterns was estimated on the basis of changes in one or more clear bands or band sizes. Hence, the amplification of genomic DNAs from *S. enteritidis* using three oligonucleotides resulted in AP-PCR profiles consisting of 22, 23 and 18 distinct patterns of DNA fragments from primers GEN15001, GEN15008 and GEN15009, respectively, with molecular sizes ranging from 250 to 9000 base pairs (bp) (Table 1). All isolates could be classified according to types using primers GEN15001 and GEN15009 except Strain FSE2 using primer GEN15008. The combination of the results with the three primers showed 28 AP-PCR types among the 28 *S. enteritidis* isolates. Thus, the sensitivity of the AP-PCR was higher than antibiotic resistance pattern and plasmid profiling for fingerprinting, and that the high discriminating power of AP-PCR will undoubtedly be of great help in epidemiological studies.

A higher degree of discrimination was observed by combining the results of the three methods; the 28 *S. enteritidis* isolates could be grouped into 28 individual groups, confirming that all 28 isolates were highly heterogenous and genotypically distinct. This information helps to confirm that

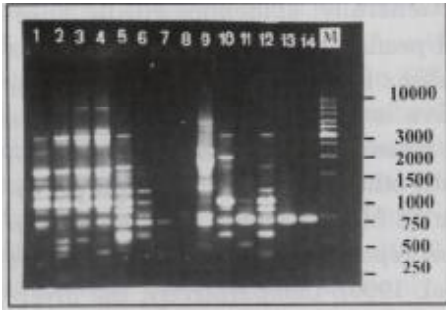


Fig. 1a. AP-PCR fingerprints of *Salmonella enteritidis* isolates FSE1 to FSE14 with primer GEN15001. Lane M contains molecular weight markers in base pairs (bp).

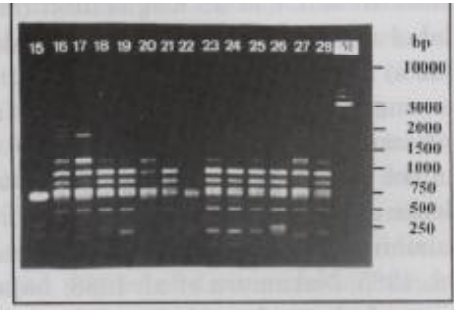


Fig. 1b. AP-PCR fingerprints of *Salmonella enteritidis* isolates FSE15 to FSE28 with primer GEN15001. Lane M contains molecular weight markers in base pairs (bp).

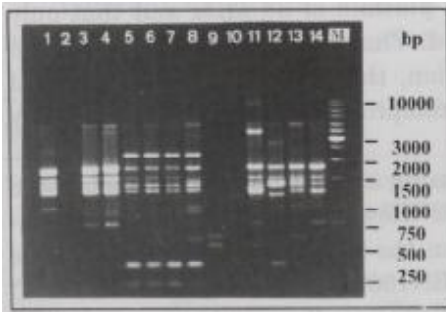


Fig. 2a. AP-PCR fingerprints of *Salmonella enteritidis* isolates FSE1 to FSE14 with primer GEN15008. Lane M contains molecular weight markers in base pairs (bp).

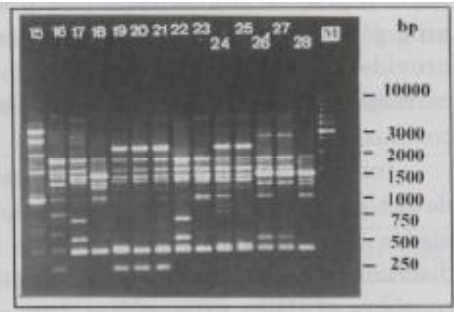


Fig. 2b. AP-PCR fingerprints of *Salmonella enteritidis* isolates FSE15 to FSE28 with primer GEN15008. Lane M contains molecular weight markers in base pairs (bp).

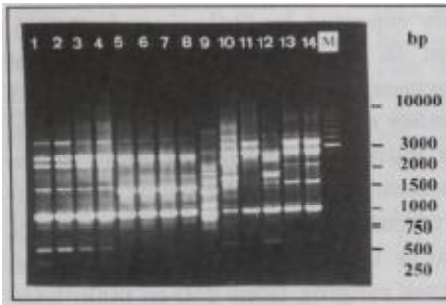


Fig. 3a. AP-PCR fingerprints of *Salmonella enteritidis* isolates FSE1 to FSE14 with primer GEN15009. Lane M contains molecular weight markers in base pairs (bp).

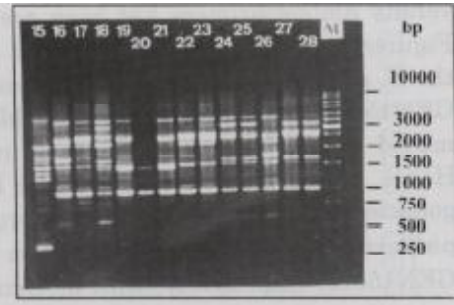


Fig. 3b. AP-PCR fingerprints of *Salmonella enteritidis* isolates FSE15 to FSE28 with primer GEN15009. Lane M contains molecular weight markers in base pairs (bp).

the isolates contained different genotypes which were genetically distinct, and demonstrates the value of using more than one typing technique in epidemiological investigations. As none of the isolates studied had been examined for disease potential using an animal challenge model, it is not possible to correlate genetic heterogeneity with virulence characteristic. However, it can be safely assumed that each of the *S. enteritidis* isolates can be patho-

genic as all of them carried the 38 MDa plasmid, which has been reported to be associated with virulence for mice (Jones et al. 1982; Terakado et al. 1983; Nakamura et al. 1985). In conclusion, AP-PCR in combination with plasmid profiles and antibiotic resistance patterns can provide substantial epidemiological data about *S. enteritidis* activity. As our data indicated, a variety of genetically diverse *S. enteritidis* strains were being spread among the fish population.

## Acknowledgments

The authors acknowledge Dora B. for her assistance in the preparation of this paper. This research was supported by the Malaysian Government through the Intensification of Research in Priority Areas (IRPA) grant mechanism.

## References

- Baquar, N., E. J. Threlfall, B. Rowe and J. Stanley. 1994. Phage type 193 of *Salmonella typhimurium* contains different chromosomal genotypes and multiple IS200 profiles. *FEMS Microbiology Letters* 115: 291-296.
- Burr, M. D., K. L. Josephson and I. L. Pepper. 1998. An evaluation of ERIC PCR and AP PCR fingerprinting for discriminating *Salmonella* serotypes. *Letters in Applied Microbiology* 27: 24-30.
- Cohen, M. L. and R. V. Tauxe. 1986. Drug-resistant *Salmonella* in the United States: an epidemiological perspective. *Science* 234: 964-969.
- Dorn, C. R., R. Silapanuntakul, E. J. Angrick and L. D. Shipman. 1992. Plasmid analysis and epidemiology of *Salmonella enteritidis* infection in three commercial layer flocks. *Avian Disease* 36: 844-851.
- Fadl, A. A., A. V. Nguyen and M. I. Khan. 1995. Analysis of *Salmonella enteritidis* isolates by arbitrarily primed PCR. *Journal of Clinical Microbiology* 58: 2180-2187.
- Hadfield, T. L., M. H. Monson and I. K. Wachsmuth. 1985. An outbreak of antibiotic-resistant *Salmonella enteritidis* in Liberia, West Africa. *Journal of Infectious Diseases* 151: 790-795.
- Helmuth, R., R. Stephen, C. Bunge, B. Hoog, A. Steinback and E. Bulling. 1985. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. *Infection and Immunity* 48: 175-182.
- Jones, G. W., D. K. Robert, D. M. Svinarich and H. J. Whitefield. 1982. Association of adhesive, invasive, and virulence phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. *Infection and Immunity* 38: 476-486.
- Lin, A. W., A. M. Usera, T. J. Barrett and R. A. Goldsby. 1996. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis*. *Journal of Clinical Microbiology* 34: 870-876.
- Lorian, V. 1986. *Salmonella* susceptibility patterns in hospitals from 1975 through 1984. *Journal of Clinical Microbiology* 23: 826-827.
- Lujan, R. A., A. Echeita, M. A. Usera, J. V. Martinez-Suarez, R. Alonso and J. A. Saez-Neito. 1990. Plasmid profile as an epidemiological marker for *Salmonella enterica* serotype *enteritidis* foodborne outbreaks. *Microbiologia SEM* 6: 45-50.
- Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers and S. M. McCowen. 1978. A multiple-plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* 1: 417-420.
- Marmur, J., S. Falkow and M. Mandel. 1963. New approaches to bacterial taxonomy. *Annual Review of Microbiology* 17: 329-372.
- Nakamura, M., S. Sato, T. Ohya, S. Suzuki and S. Ikeda. 1985. Possible relationship of a 36-megadalton *Salmonella enteritidis* plasmid to virulence in mice. *Infection and Immunity* 47: 831-833.

- National Committee for Clinical Laboratory Standards. 1993. Performance Standards for Antimicrobial Disc Susceptibility Tests: Tentative Standards, Vol. 13, No. 24. NCCLS document M2-A5. National Committee for Clinical Laboratory Standards, Villanova, PA.
- Oakey, H. J., J. T. Ellis and L. F. Gobson. 1995. Can RAPD-PCR be used to differentiate between *Aeromonas* genospecies? *Medical Microbiology Letters* 4: 373-381.
- Olsen, J. E., M. N. Skov, E. J. Threlfall and D. J. Brown. 1994. Cloning lines of *Salmonella* serotype Enteritidis documented by IS200-, ribo-, pulsed field gel electrophoresis and RFLP typing. *Journal of Medical Microbiology* 40: 15-22.
- Rivera, M. J., N. Rivera, J. Castillo, M. C. Rubio and R. Gomez-Lus. 1991. Molecular and epidemiological study of *Salmonella* clinical isolates. *Journal of Clinical Microbiology* 9: 927-932.
- Rodrigue, D. C., D. N. Cameron, F. W. Puh, F. W. Brenner, M. E. St. Louis, I. K. Wachsmuths and R. V. Tauxe. 1992. Comparison of plasmid profiles, phage types and antimicrobial resistance patterns of *Salmonella enteritidis* isolates in the United States. *Journal of Clinical Microbiology* 30: 854-857.
- Sambrook, J., E. F. Fritsch and M. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Terakado, N., T. Sekizaki, K. Hashimoto and S. Naitoh. 1983. Correlation between the presence of a fifty-megadalton plasmid in *Salmonella dublin* and virulence in mice. *Infection and Immunity* 41: 443-444.
- Threlfall, E. J. and H. Chart. 1993. Interrelationship between strains of *Salmonella enteritidis*. *Epidemiology and Infection* 111: 1-8.
- Threlfall, E. J., B. Bowe and L. R. Ward. 1989. Subdivision of *Salmonella enteritidis* types by plasmid profile typing. *Epidemiology and Infection* 102: 459-465.
- Ward, L. R., E. J. Threlfall and B. Rowe. 1990. Multiple drug resistance in salmonellae in England and Wales: a comparison between 1981 and 1988. *Journal of Clinical Pathology* 43: 563-566.
- Wilson, K. 1989. Preparation of genomic DNA from bacteria. In: *Current Protocols in Molecular Biology* (Ausubel, F. M., R. E. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl, Eds), pp. 2.4.1-2.4.5. John Wiley and Sons, New York.