BIOCHEMICAL AND CELL-SURFACE CHARACTERISTICS OF YERSINIA RUCKERI IN RELATION TO THE EPIZOOTIOLOGY AND PATHOGENESIS OF INFECTIONS IN FISH

A thesis submitted to the University of Stirling

for the degree of Doctor of Philosophy

by

Robert L. Davies

Institute of Aquaculture

University of Stirling

August 1989

Declaration

I declare that this thesis has been composed by myself and that it embodies the results of my own research carried out at the Institute of Aquaculture, University of Stirling, during the period 1985 to 1988.

Signature: R J Davies

Date: 27th August 1989

To Janet and

my Parents

Acknowledgements

This work may never have been completed without the support, comfort, perseverance, tolerance and genuine interest of my fiancee and I am sincerely indebted and grateful to her. Equally, the work may not have been completed without the additional support and assistance of my parents, and I am also extremely grateful to them. I am especially grateful for their assistance in the final preparation of the manuscript.

I would like to express my gratitude to the University of Stirling who provided me with the University Studentship to carry out the work described in this thesis. I am also extremely grateful to Professor R.J. Roberts within whose Institute the work was carried out. I am grateful also to Dr. G.N. Frerichs who took over the role of supervisor at a late stage of the work.

The work presented in this thesis would not have been possible without the cooperation of the people listed in Appendix 1 who kindly supplied me with isolates and often with additional information; I am sincerely indebted to these people.

I would like to express my thanks to the members of the Aquatic Vaccine Unit, Institute of Aquaculture, namely Dr. M. Tatner, Dr. R. Jones, Andrea Bundi and Niall Auchinachie, for helpful assistance and useful discussion at various stages of the work. I am also grateful to Dr. P. Brophy and Dr. M. North of the Department of Biological Sciences, University of Stirling, for helpful discussion relating to the SDS-PAGE work. In addition I am grateful to Mary Alexander and Stuart Miller, of the Institute of Aquaculture, for assistance with the biochemical characterisation of isolates.

I would also like to acknowledge my appreciation to Dr. S.P. Borriello of the Clinical Research Centre, Northwick Park Hospital, Middlesex, for reading Sections 1 to 3 of the manuscript and providing constructive criticism. I am also grateful to Dr. D. Alaandeen for reading and commenting on Section 7.

Special thanks must go to the photographer, Lewis Taylor, for making an excellent job of the photography, for much useful help, and for making the most of some poor-quality negatives.

Finally, special thanks must also go to the typist, Carole Campbell, and to Mike Still, for preparation of the manuscript.

Abstract

Isolates of Yersinia ruckeri were obtained from Europe, North America, Australia and South Africa. The biochemical and serological characteristics of the isolates were investigated. Biochemically the isolates were extremely uniform although motile, Tween positive isolates could be differentiated from non-motile, Tween negative isolates; these were designated biotypes 1 and 2 respectively. With the exception of two isolates, biotype 2 isolates were confined to the U.K. Five O-serotypes were recognised and an O-serotyping scheme is proposed; the relation of this scheme to previously described schemes is discussed. The geographic distribution of the different serotypes is also discussed. The lipopolysaccharide (LPS) and outer membrane protein (OMP) profiles of isolates were analysed by SDS-PAGE and Western-blotting using both rabbit and rainbow trout antisera. The relation of LPS-type to O-serotype, as well as variation within LPS-types, is discussed. Based on inter-strain variation in the molecular weight of a heat-modifiable protein and of peptidoglycan-associated (porin) proteins, an OMP-typing scheme was developed. Three major OMP-types comprised 95% of the isolates studied. Variation in biotype, serotype and OMP-type was used as an epizootiological tool, and six serotype O1 clonal groups were recognised which differed in their geographic distribution. The production of iron-regulated OMPs and siderophores was investigated. Four iron-regulated OMPs were produced in all of the isolates examined; siderophores appeared not to be produced by any of the isolates. Production of iron-regulated OMPs was not an important virulence determinant and appears to be a chromosomally-mediated factor. Resistance to the bactericidal effects of normal rainbow trout serum and virulence were also investigated. Serum-resistance was associated principally with two serotype O1 clonal groups and virulence was associated with the same two clonal groups. Other serotype O1 clonal groups and other serotypes were

generally serum-sensitive and avirulent. Thus, serum-resistance is an important virulence determinant in this organism. The role of outer membrane components in serum-resistance and virulence is discussed.

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Abbreviations

c.f.u.'s	colony forming units
ERM	enteric redmouth disease
HMP	heat-modifiable protein
LPS	lipopolysaccharide
MAC	membrane attack complex
MR	methyl red
OM	outer membrane
OMP	outer membrane protein
PAP	peptidoglycan-associated protein
PNRTS	pooled normal rainbow trout serum
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TSA	tryptone soya agar
TSB	tryptone soya broth
VP	Voges-Proskauer

General Introduction

The Epizootiology and Pathogenesis of Yersinia ruckeri Infections in Fish

The Epizootiology and Pathogenesis of Yersinia ruckeri Infections in Fish

1 THE ORGANISM

Yersinia ruckeri is a Gram-negative enteric bacterium which causes an acute to chronic bacteraemic infection of fish known as enteric redmouth (ERM) disease. Excellent reviews of Y. ruckeri and ERM have been given by Busch (1982) and Austin and Austin (1987) and the reader is referred to these for further information.

2 EPIZOOTIOLOGY

2.1 Geographic distribution of Y. ruckeri and its dissemination

Yersinia ruckeri was first isolated from hatchery-reared rainbow trout (Salmo gairdneri Richardson) in the Hagerman Valley of Idaho, U.S.A., in the early 1950s (Busch, 1982). Subsequently, Y. ruckeri was isolated in an increasing number of the states of the U.S.A. as well as from Canada (Ross et al., 1966; Rucker, 1966; McDaniel, 1971; Wobeser, 1973; Bullock et al., 1978; Busch, 1978). By 1982 Y. ruckeri was established as an enzootic infection in many of the major trout and salmon raising areas of North America although it was not considered to be ubiquitous in its occurrence (Busch, 1982).

The first isolation of Y. ruckeri in Europe was possibly from brown trout, Salmo trutta L., in England in 1977 (Scott, 1986) although Roberts (1983) described this identification as "presumptive". Busch (1978) stated that ERM had been reported in Italy prior to 1978 but the source of this information was not cited. However, Ghittino (1972) described epidemics of "Red Mouth" in

Italy, the aetiological agent of which was considered to be Aeromonas liquefaciens. If this was the source of Busch's statement it confirms the confusion caused by the term "red mouth" (Busch, 1978) since the first outbreak of ERM in farmed rainbow trout in Italy did not occur until 1983 (Giorgetti et al., 1985). The first published report of the isolation of Y. ruckeri in Europe appears to be that of Lesel et al. (1983) who described the isolation of Y. ruckeri from rainbow trout in south-western France in March 1981.

Subsequent to the isolation of Y. ruckeri in France in 1981 (Lesel et al., 1983) the organism was isolated in England (1981) (Roberts, 1983), West Germany (1981) (Fuhrmann et al., 1983), Finland (1982) (Rintamaki et al., 1986), Italy (1983) (Giorgetti et al., 1985), Denmark (1983) (Dalsgaard et al., 1984), Bulgaria (1983) (Gelev et al., 1984), Scotland (1984) (Frerichs et al., 1985), Norway (1985) (Sparboe et al., 1986), Switzerland (1985) (Meier, 1986), Spain (1985) (De la Cruz et al., 1986) and Ireland (1985) (McArdle and Dooley-Martin, 1985). Further isolations in France were described by Michel et al., (1986) and Vuillaume et al., (1987).

After 1981 the occurrence of ERM in Europe increased dramatically. For example, in north-west Germany the incidence of ERM increased from a single case in 1981 to 40 outbreaks in 1984/85 (Schlotfeldt et al., 1985). The situation was similar in Denmark where approximately 70 farms were affected in 1984 (Schlotfeldt et al., 1985). In Italy, after the first outbreak in 1983, the disease spread rapidly throughout the country and by 1986 more than 90% of the farms with a steady production of trout were considered infected (Giorgetti, personal communication). The disease also spread rapidly throughout the fish producing regions of France (Roberts, 1985). In the U.K. a significant proportion of the farms in the south of England were affected by 1985 (Scott, 1986). By 1986 Y. ruckeri had been isolated from rainbow trout in Hampshire,

Wiltshire, Cornwall, Devon, Yorkshire and Cumbria, as well as from Wales and at least three different regions of Scotland.

Yersinia ruckeri was isolated for the first time in Australia in about 1960. Four bacterial cultures, isolated from rainbow trout during an epizootic, were submitted to the National Fish Health Research Laboratory, Leetown, U.S.A. for identification but were not identified as Y. ruckeri until about 1977 (Bullock et al., 1977; 1978). Llewellyn (1980) described a bacterium, the "salmonid blood spot bacterium", which was isolated from brook trout Salvelinus fontinalis and Atlantic salmon Salmo salar L., and which showed similarities to Y. ruckeri. This bacterium was later confirmed as being Y. ruckeri by Stevenson and Airdrie (1984a). In an investigation of eleven freshwater salmonid hatcheries in southern Australia it was concluded that although Y. ruckeri was present in Australian salmonids, ERM and the classical "Hagerman" strain were exotic to Australian salmonids (Humphrey et al., 1987).

Yersinia ruckeri was isolated for the first time in South Africa in 1985. The organism was isolated from rainbow trout in the Lydenburg area of the Transvaal (Bragg & Henton, 1986).

Evidence from North America has suggested that the movement of carrier salmonids is the principal cause for the dissemination of ERM (Rucker, 1966; Wobeser, 1973). For example, the first report of ERM in Canada was an outbreak of disease due to the importation of asymptomatic carrier fish from Idaho, U.S.A. (Wobeser, 1973). Work carried out by the Ministry of Agriculture, Fisheries and Food, in England, has supported the suggestion that the spread of ERM is primarily associated with fish movements (Scott, 1986).

Bullock et al. (1978), however, suggested that Y. ruckeri may have originally been more widely distributed than was first thought. These authors suggested that Y. ruckeri was present in the mid-western, eastern and south-eastern United States before fish were shipped to these regions, and that its presence was overlooked. They also cited evidence to suggest that Y. ruckeri was present in Australia before the first importation of rainbow trout in 1894, although no mention was made of the fact that ornamental fish have been responsible for introducing several other serious diseases into Australia (Ashburner, 1976).

There is some evidence to suggest that Y. ruckeri was introduced into Europe by the importation of asymptomatic carrier fish. For example, Michel et al. (1986) isolated Y. ruckeri from two groups of minnows, Pimephales promelas, from Belgium and France respectively, which had been imported from Missouri and Arkansas, U.S.A. The fish had been imported into Europe for use as bait-fish and the first shipment of P. promelas into Western Europe could be dated from 1979. Yersinia ruckeri was also isolated from the gut of an apparently healthy goldfish believed to have originated in Singapore (McArdle and Dooley-Martin, 1985). The time lapse between the first outbreaks of ERM in North America and those in Europe also suggests that Y. ruckeri was imported into Europe from North America.

In the case of South Africa, no imports of free-swimming trout have been permitted into the country for at least 50 years although there is much international traffic of trout ova. Ova have been imported from the U.S.A., Italy, the U.K. and Denmark, all countries known to have ERM. There is also substantial international traffic in ornamental fish. The site at which Y. ruckeri was isolated, however, is in a reasonably inaccessible mountainous area and the origin of the organism is not known (Bragg, personal communication).

Finally, it should be noted that the movement of Y. ruckeri exactly mimics the classical disease movement of infectious pancreatic necrosis (IPN) which was first identified in the eastern states of the U.S.A. (1958), then the western states (1963), and then in Europe, where after being first reported in France (1965), it was found in Denmark (1969), Italy (1970) and Scotland (1971); it was also found in Japan in 1970 (Ashburner, 1976).

2.2 Host Range

Although ERM is considered a disease affecting both trout and salmon, rainbow trout, S. gairdneri, appears to be the most common natural host. Yersinia ruckeri has, however, also been isolated from the salmonids Salmo clarki (cutthroat trout), S. trutta (brown trout), Salmo aguabonita, S. fontinalis (brook trout), Salvelinus malma (Dolly Varden trout), Oncorhynchus kisutch (coho salmon), Oncorhynchus nerka (sockeye salmon), Oncorhynchus tshawytscha (chinook salmon) and S. salar (Atlantic salmon) as well as the non-salmonids Notropis atherinoides (emerald shiners), Cisco artedii (lake herring), Morone americana, Carassius auratus (goldfish), Lota lota (burbot), P. promelas (minnows), Coregonus peled and Coregonus muksun (whitefish), Cyprinus carpio (carp), Acipenser baeri (sturgeon), Thymallus thymallus (grayling), Esox lucius (pike), Gobio gobio (gudgeon), and Anguilla anguilla (eel).

Yersinia ruckeri has also been isolated from Ondatra zibethica (muskrat) and birds of prey as well as from man (Farmer et al., 1985).

2.3 Reservoirs of infection and transmission

Asymptomatic carrier fish in the environment that regularly shed large numbers of bacteria into the water through faeces probably serve as the

primary reservoir for disease (Busch, 1982). Indigenous fish species in rivers in southern England were shown to carry the bacterium in the gut. It was assumed that these fish were continually shedding the bacterium into the water and that this accounted for the recurrence of the disease 21 days after the cessation of antibiotic treatment (Roberts, 1985). Yersinia ruckeri was considered well adapted as a normal aquatic saprophyte (McDaniel, 1971) and may survive for up to two months in mud (Klontz and Huddleston, 1976). The organism may also occur in aquatic invertebrates, birds and mammals, all of which may serve as reservoirs of infection. For example, Y. ruckeri has been isolated from a musk rat (Stevenson and Daly, 1982) and from captive birds of prey (Bangert et al., 1988).

It has been demonstrated by various workers that transmission is probably of a direct horizontal nature through the water (Rucker, 1966; Busch, 1973; Hunter et al., 1980). An asymptomatic carrier state infection occurs in the lower intestine of fish surviving an epizootic infection and regular intestinal shedding of the bacterium occurs on a regular cyclic basis which can cause recurrent infection and mortality (Busch and Lingg, 1975). Enteric redmouth disease is a stress-related syndrome and heat-stress can cause the bacterium to be transmitted from infected carrier fish to uninfected recipient fish (Hunter et al., 1980). Vertical transmission through infected eggs has not been demonstrated (Busch, 1982).

2.4 Effect of stress

Enteric redmouth disease is a stress-related syndrome and consequently adverse environmental factors may lead to and influence the severity of an infection. Outbreaks of ERM are commonly associated with high water temperatures; as water temperature decreases to 10^{o}C or lower, the disease becomes less severe. Other stressful factors which may precipitate disease

outbreaks include handling, overcrowding and poor water quality. Weighing or moving apparently healthy fish is known to be succeeded by an epizootic (Rucker, 1966). Organic pollution may be very important in the epidemiology of ERM in Italy (Giorgetti et al., 1985). Overly fat or debilitated fish may be more susceptible to severe epizootics (Rucker, 1966).

2.5 Mortality and morbidity

The severity of infections of ERM is related to the virulence of the infecting strain, the resistance of the host and variation of environmental factors. Mortalities in rainbow trout usually range from 25-75% during the course of an untreated epizootic; brook trout and brown trout are more resistant with mortalities ranging from 5-10% (Busch, 1978). Pathogenesis of infection varies from peracute to chronic, depending on temperature, stress, species, age and so on. During the spring and early summer, when water temperatures are rising, peracute to acute infections usually occur with mortalities ranging from 50-70%. During the autumn and early winter, when water temperatures are falling, acute to subacute infections usually occur with mortalities ranging from 10-50%. Chronic infection often occurs in large mature fish and results in very low levels of mortality of the order of 10%.

3 PATHOGENESIS

Very little is known about the pathogenesis of Y. ruckeri infections. There is evidence to suggest, based on the virulence of serotype 1 isolates, that serotype antigens may be important in virulence but the precise role of these components in the disease process is not known. Little is known about the route of entry of the bacterium into the host, including any adhesion and invasive processes; little is known also about evasion of the host immune response. An ability to circumvent stimulation of the microbiocidal,

chemiluminescence-generating metabolism of fish phagocytes might be a feature of virulent strains and this property may be plasmid encoded (Stave et al., 1987). Although the histopathology of Y. ruckeri infections has been described by numerous workers, including Rucker (1966), Wobeser (1973) and Busch (1973), little is known about the mechanisms of tissue damage.

4 THE AIMS OF THE STUDY

From the above information it is apparent that there is a need to examine the population structure of Y. ruckeri, in terms of its distribution in North America, Europe, Australia and South Africa, to further our understanding of the epizootiology of this important fish pathogen. In addition it can be seen that there is a need to learn more about the virulence determinants and pathogenesis of this organism. The major objectives of this study were, therefore, as follows:

(1) To examine and compare isolates from these wide geographic locations using standard biochemical and serological techniques, as well as more refined methods of analysis of cell-surface components, including lipopolysaccharide (LPS) and outer membrane protein (OMP) analysis, and to identify clonal groups of epizootiological significance.

(2) To relate differences in biochemical and cell-surface characteristics to differences in virulence, and hence to identify virulent and avirulent clonal groups which can be recognised by biochemical and/or cell-surface markers.

(3) To examine the immune response of rabbits and rainbow trout to cell-surface components in order to identify important immunogenic antigens (antibodies against which could have an important protective function) and also to identify cross-reacting antigens common to isolates of different serotypes and which could provide cross-protection.

(4) To examine the effect of iron-limited growth on the expression of iron-regulated OMPs and on the production of siderophores (if siderophores are

produced by this organism) and to evaluate the importance of growth under iron-limiting conditions to virulence.

(5) To examine the role of cell-surface components in the resistance of this organism to the bactericidal effect of rainbow trout serum and to evaluate the importance of serum resistance to virulence.

PART I

EPIZOOTIOLOGY

Section 1

Morphological and Biochemical Characteristics of Yersinia ruckeri

Morphological and Biochemical Characteristics

of Yersinia ruckeri

1.1 INTRODUCTION

Variation in the cell morphology of Y. ruckeri has been described by Bullock et al.(1978) and by Austin et al.(1982). Busch (1982) stated that cell morphology may be affected by the age of the culture in that older stationary phase cultures tend to produce long filamentous or vegetative types of cells. Austin et al.(1982), however, showed that variation in cell morphology was not due to the age of the cultures or to the growth medium.

The biochemical characteristics of North American isolates of Y. ruckeri have been shown to be extremely uniform (Ross et al., 1966; Busch, 1973; O'Leary, 1977; Bullock et al., 1978; Ewing et al., 1978; Busch, 1982; Stevenson and Daly, 1982; Pyle et al., 1987). Some variation between isolates in certain biochemical reactions, notably the methyl red (MR) test, the Voges-Proskauer (VP) reaction, gelatin hydrolysis, Tween 80 hydrolysis and sorbitol fermentation, have been described by some authors (Ewing et al.,1978; Busch, 1982; Stevenson and Daly, 1982; Pyle et al., 1987) but these differences, with the exception of sorbitol fermentation, are not significant and only a single biotype is recognised (Busch, 1982).

Variation in sorbitol fermentation was first described by O'Leary (1977) who reported that sorbitol fermentation was associated with "a different serological type". It was later established that sorbitol non-fermenters comprised serotype I isolates only whereas sorbitol fermenters comprised a variety of serotypes (Stevenson and Airdrie, 1984a; Daly et al., 1986; Pyle et al., 1987). Stevenson

and Airdrie (1984a) identified a serotype I isolate which fermented sorbitol and concluded that sorbitol fermentation was not an accurate predictor of serotype (as had previously been thought). Other serotype I sorbitol-fermenting isolates were later identified by Michel et al.(1986), Rintamaki et al.(1986) and Pyle et al.(1987).

The biochemical characteristics of Y. ruckeri isolates responsible for disease outbreaks in various European countries (Fuhrmann et al., 1983; Lesel et al., 1983; Roberts, 1983; Frerichs et al., 1985; Giorgetti et al., 1985; De la Cruz et al., 1986; Meier, 1986; Rintamaki et al., 1986; Sparboe et al., 1986; Vuillaume et al., 1987) as well as South Africa (Bragg and Henton, 1986) and Australia (Llewellyn, 1980) have been described independently. These descriptions have, however, been restricted to only one or, at most, a few isolates responsible for a disease outbreak and have not included organisms obtained from routine surveillance of healthy fish.

Whereas the majority of Y. ruckeri isolates described have been motile, a small number of non-motile isolates have also been described (Ewing et al., 1978; Busch, 1982; Stevenson and Daly, 1982; Sparboe et al., 1986; Pyle et al., 1987). No correlations have been made, however, between motility and any of the biochemical characteristics.

The objective of this part of the study was to examine and compare the morphological and biochemical characteristics of European, North American, Australian and South African isolates of Y. ruckeri in an attempt to identify variable characteristics which could be of epizootiological significance.

1.2 MATERIALS AND METHODS

1.2.1 Bacterial isolates and growth conditions

The morphological and biochemical characteristics of 148 isolates of Y. ruckeri, including 143 field isolates, four reference strains and a vaccine production strain, were examined. The isolates examined are listed in Table 2 and further details of these isolates are given in Appendix 1.

Upon arrival in the laboratory bacteria were subcultured on tryptone soya agar (TSA) (Oxoid) at 22^{o}C for two or three days. As a purity check, single colonies were selected and subcultured a second time on TSA. In some cases, two or three colonies were individually subcultured and these subcultures were treated as individual isolates and subjected to the full range of biochemical tests. The purpose of this was to check for intra-strain variation in biochemical characteristics. These second subcultures were used for all morphological and biochemical tests. Twenty four hour growth was used for the morphological studies and three day growth was used for the biochemical tests.

1.2.2 Storage of isolates

All isolates were freeze-dried (for long term storage) and also stored on TSA slopes in universals (for short term storage or for as long as the bacteria remained viable). For freeze-drying, bacteria were grown overnight at 22^{o}C in tryptone soya broth (TSB) (Oxoid) containing 5% meso-inositol as cryo-protectant. Freeze-dried cultures and TSA slopes were stored at room temperature.

1.2.3 Morphology

Bacterial morphology was determined from Gram-stained preparations of 24 h TSA cultures (Cowan, 1974). Bacterial size was estimated with a calibrated eyepiece graticule and was based on the average size of ten organisms.

1.2.4. Biochemical characterisation

1.2.4.1 Biochemical tests used. Biochemical characterisation was carried out using the API 20E rapid identification system (API System S.A.) supplemented with additional tests. The following tests were available with the API 20E system: possession of β-galactosidase, ornithine and lysine decarboxylase, arginine dihydrolase, tryptophan deaminase and urease, production of hydrogen sulphide and indole, utilization of citrate, the VP reaction, hydrolysis of gelatin, possession of cytochrome oxidase, the reduction of nitrate to nitrite, and the production of acid from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose.

As studies by other workers had indicated that the VP test, gelatin hydrolysis and sorbitol fermentation were likely to be the most variable biochemical reactions, tests for these characteristics were also performed using an alternative method to the API 20E system. Utilization of citrate was also repeated using an alternative method to avoid the possibility of false negative results; this was also another reason for duplicating the gelatin hydrolysis test. Gelatin hydrolysis was determined using charcoal gelatin discs (Oxoid) on agar slopes, and the VP, Simmon's citrate and sorbitol fermentation tests were carried out conventionally as described by Cowan (1974). Reduction of nitrate was also determined by a conventional method (Cowan, 1974) because the API 20E test was found to be unreliable. Other tests carried out conventionally included the oxidation-fermentation, catalase, MR, casein hydrolysis and the

Tween 20 and Tween 80 hydrolysis tests, as well as growth on MacConkey agar, production of gas from glucose, and production of acid from fructose, galactose, lactose, maltose, mannose and trehalose (Cowan, 1974). The complete list of tests is given in Table 1.

1.2.4.2. Inoculation of tests. A light suspension of each isolate was prepared in 0.85% (w/v) saline from a TSA culture. This was used to inoculate the API 20E tests (as described in the API 20E Instruction Sheet), all of the liquid media tests and the Simmons citrate test. All other tests were inoculated directly from the TSA plate.

1.2.4.3 Incubation of tests and recording of results. All tests were incubated at 22°C. The results of the API 20E tests were recorded after 48 h if both the citrate and gelatinase tests were positive and after 72 h if either of these tests were negative after 48 h. All other tests were recorded after 48 h with the exception of the MR and VP tests which were recorded after 5 days of incubation. Negative tests were examined every day for up to two weeks. Results were recorded as positive or negative irrespective of the time taken for a positive reaction to occur. "Strong" and "weak" reactions were not differentiated on the grounds that different growth rates and different inocula sizes could account for these differences.

1.2.5 Motility

Motility was determined by the "hanging-drop" method using overnight growth in TSB (Cowan, 1974). Absence of flagella in non-motile isolates was confirmed by electron microscopical examination of negatively-stained preparations as described below. Motile isolates were examined as controls.

1.2.6 Electron microscopy

Bacteria were grown overnight on TSA at 22°C and were gently washed off with distilled water. The bacteria were diluted to give a light suspension (about 10^5 - 10^6 cells per ml) and were negatively stained by adding phosphotungstic acid (pH 6.8) to a final concentration of 1% (w/v). A drop of the negatively-stained bacterial suspension was placed on a carbon-coated formvar support grid which had previously been treated with 0.01% (w/v) bacitracin to reduce hydrophobicity and allow even spreading of the drop. The preparation was air-dried and examined in a JEOL JEM 100 transmission electron microscope.

1.3 RESULTS

1.3.1 Morphology

The majority of Y. ruckeri isolates (141 of 148) could be divided into two very similar morphological groups, i.e. very short, coccobacillary organisms 0.5 x 0.5 - 1.0 μm in size and slightly larger rods 0.5 x 1.0 - 2.0 μm in size. The remaining seven isolates, three of which were reference strains, were quite different in that the bacterial cells were longer, 0.5 x 1.0 - 3.0 μm in size, and chain formation was frequent.

1.3.2 Biochemical characteristics

The biochemical reactions of the 148 isolates of Y.ruckeri examined are summarized in Table 1. For the majority of the biochemical tests the reactions were extremely uniform with 99-100% of isolates giving the same positive or negative reaction. The results of the MR, VP, citrate utilization, gelatin and casein hydrolysis, Tween 20 and Tween 80 hydrolysis, production of acid from sorbitol and production of gas from glucose tests were less consistent. No

Table 1. Biochemical characteristics of isolates of Y. ruckeri.

Biochemical tests	Reference strains No +ve/4 strains tested	Field[a] isolates No +ve/144 isolates tested	Percentage +ve of all isolates tested
Motility	3	119	82
Fermentation (O/F test)	4	144	100
Cytochrome oxidase	0	0	0
Catalase	4	144	100
β-Galactosidase[b]	4	144	100
Ornithine decarboxylase[b]	4	144	100
Lysine decarboxylase[b]	4	144	100
Arginine dihydrolase[b]	0	0	0
Tryptophan deaminase[b]	0	0	0
Urease[b]	0	0	0
Hydrogen sulphide[b]	0	0	0
Indole[b]	0	0	0
Citrate utilization	4	143	99
Citrate utilization[b]	4	106	74
Methyl red test	4	132	92
Voges-Proskauer reaction[b]	1	137	93
Voges-Proskauer reaction	0	115	78
Gelatin hydrolysis	1	114	78
Gelatin hydrolysis[b]	2	103	71
Casein hydrolysis	1	110	75
Tween 20 hydrolysis	2	119	82
Tween 80 hydrolysis	2	119	82
Growth on MacConkey agar	4	143	99
Nitrate reduction	4	143	99
Gas from glucose	0	12	8
Acid from:			
Amygdalin[b]	0	0	0
Arabinose[b]	0	0	0
Fructose	4	144	100
Galactose	4	143	99
Glucose[b]	4	144	100
Inositol[b]	0	0	0
Lactose	0	0	0
Maltose	4	143	99
Mannitol[b]	4	143	100
Mannose	4	144	100
Melibiose[b]	0	0	0
Rhaminose[b]	0	0	0
Sorbitol[b]	0	29	19
Sorbitol	0	29	19
Sucrose[b]	0	0	0
Trehalose	4	144	100

[a] = including vaccine strain.

[b] = tests carried out with the API 20E system.

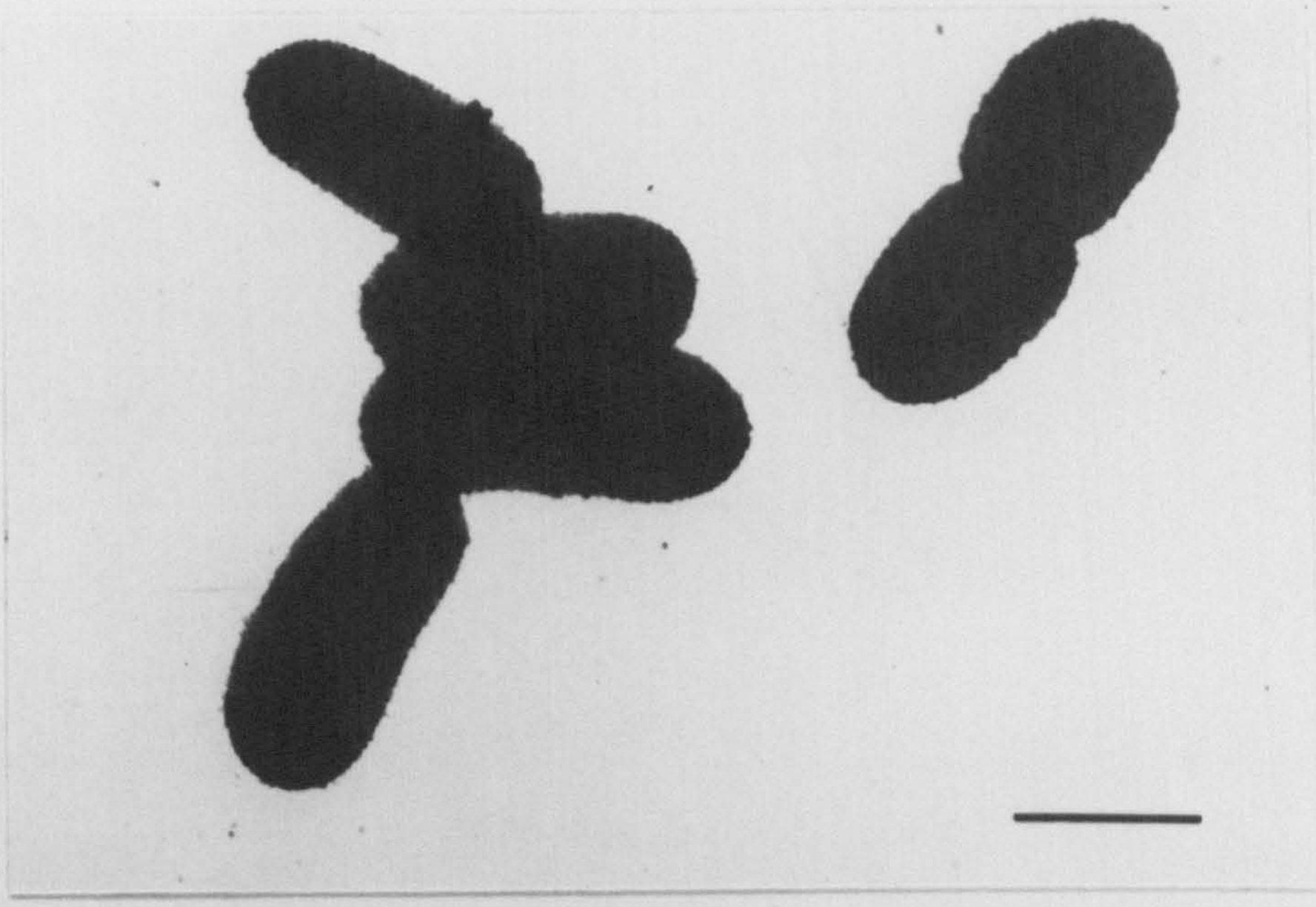
intra-strain variation was detected in the biochemical characteristics of the small number of isolates examined.

Ninety two percent of isolates were positive for the MR test. The API 20E system gave a higher incidence of positive reactions for the VP test (93% of isolates positive) than did the conventional test (78% of isolates positive). The majority of the VP positive isolates were also positive for the MR test indicating the presence of a mixed-acid fermentation metabolic pathway with the additional formation of acetoin and 2,3-butanediol (results not shown). Citrate utilization appeared to be a variable characteristic only when determined by the API 20E system. Thus, 99% of isolates utilized citrate according to the conventional method but only 74% of isolates utilized citrate according to the API 20E system. In the case of gelatin hydrolysis 71% of isolates gave a positive reaction according to the API 20E system but 78% were positive according to the conventional method. Seventy five per cent of the isolates hydrolysed casein and 82% of the isolates hydrolysed Tween 20 and Tween 80. Nineteen percent of the isolates produced acid from sorbitol (assumed to be due to fermentation) and 8% of the isolates produced gas from glucose.

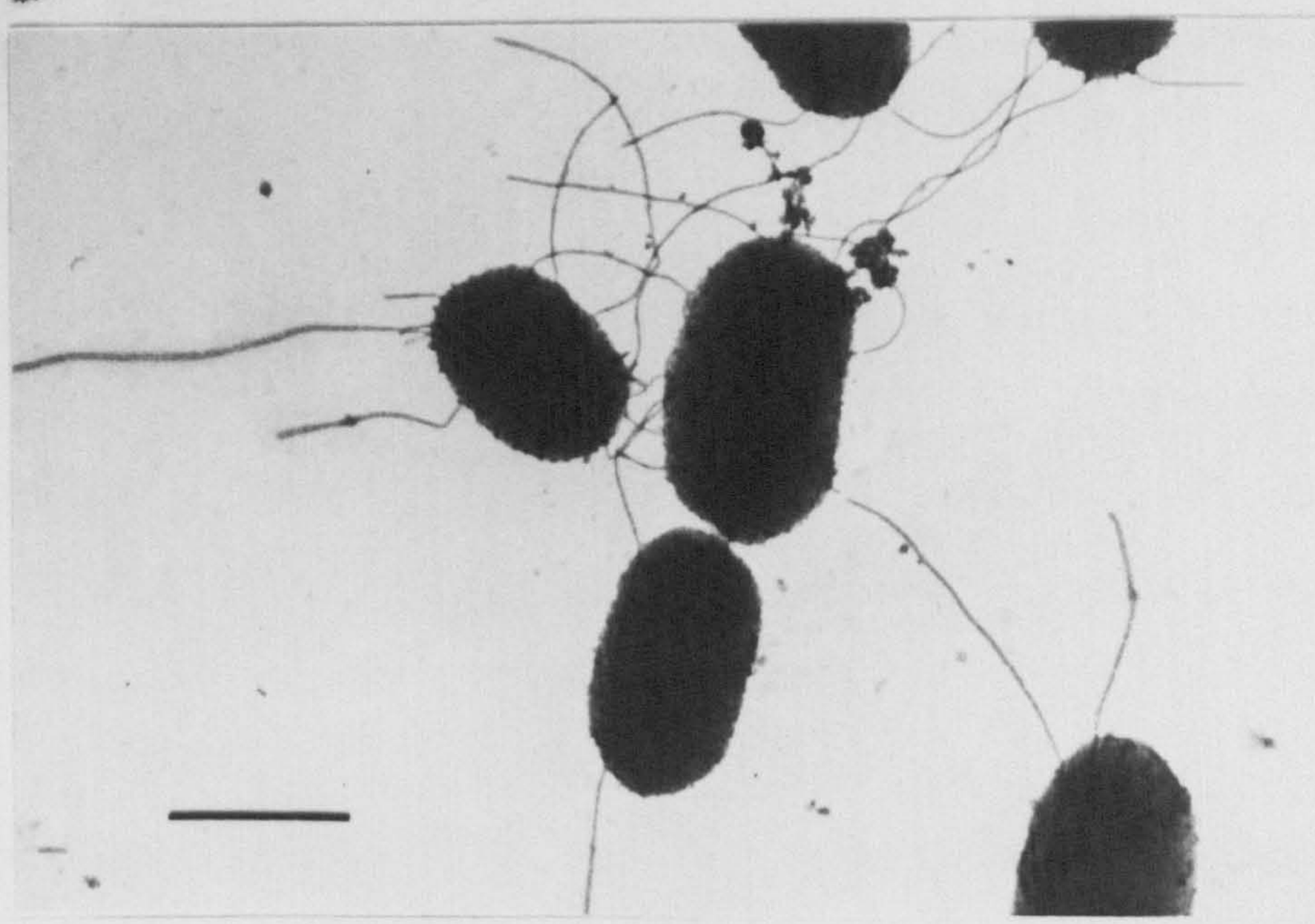
1.3.3 Motility

Twenty six isolates were shown to be non-motile. Of these, 23 were obtained from the U.K., one from Norway and one from Canada; one of the reference strains was also found to be non-motile. Electron microscopy confirmed that all non-motile isolates lacked flagella whereas motile isolates possessed peritrichous flagella (Figure 1).

A



B



1.3.4 Association of Tween hydrolysis with motility

With the exception of a single motile reference strain all Tween 20 and Tween 80 negative (Tween negative) isolates (26 isolates) were non-motile, and, conversely, all Tween positive isolates (121 isolates) were motile. It was significant, however, that of the Tween negative, non-motile isolates, 23 were from the U.K.; of the others, one was from Canada, one from Norway, and one was a reference strain.

1.4 DISCUSSION

The variation in cell morphology observed in isolates of Y. ruckeri was similar to that described by Austin et al. (1982). However, whereas Austin et al. (1982) differentiated between long, thin rods as well as filamentous cells, in this study these two cell types were not distinguished. Seven isolates were identified whose cells were much longer than the other 141 isolates and which frequently formed chains. These isolates were presumably analogous to the long, thin rods described by Austin et al. (1982). The remainder of the isolates were either very short, coccobacillary organisms or slightly larger rods although it was often difficult to differentiate between these two cell types and the difference may have been apparent rather than real. These morphological types were not associated with any of the other characteristics studied such as biochemical characteristics, serotype (Section 2), LPS-type (Section 3) and OMP-type (Section 4) and probably have no epizootiological significance and no relation to virulence. Variation in cell morphology, due to age of culture, was not studied.

With the exceptions discussed below the results confirmed previous findings, based on work carried out on North American isolates, that the biochemical characteristics of Y. ruckeri are extremely uniform. Variation in gelatin and

casein hydrolysis, the MR test and VP reaction, and in the utilization of citrate was not significant. Furthermore, with the exception of certain isolates from the U.K., Norway and Canada, it was shown that isolates from Europe, North America, Australia and South Africa were indistinguishable on the basis of their biochemical reactions.

The most notable finding of the study was the relationship between motility and hydrolysis of Tween 20 and Tween 80 (Tween hydrolysis), i.e. motile isolates hydrolysed Tween 20 and Tween 80 whereas non-motile isolates did not. This finding was totally unexpected. In a comparison of the biochemical reactions of North American isolates of Y. ruckeri Busch (1982) noted that 18% of isolates were non-motile and 14% did not hydrolyse Tween 80; a relationship between motility and Tween hydrolysis, however, was not observed. Busch (1982) also reported that 100% of isolates hydrolysed Tween 20; in this study there was no difference in the hydrolysis of Tween 20 and Tween 80. Waltman and Shotts (1984) found that 7/60 biochemically "atypical" isolates did not hydrolyse Tween 80 and Hastings and Bruno (1985) described three U.K. isolates which were similarly Tween 80 negative but neither group of workers identified any relationship between Tween hydrolysis and motility.

Previously, the biochemical reactions of Y. ruckeri have been considered sufficiently uniform for only a single biotype to be recognized (Busch, 1982). In view of the demonstrated association between motility and Tween hydrolysis, it is proposed that motile, Tween positive isolates of Y. ruckeri be designated biotype 1 and non-motile, Tween negative isolates be designated biotype 2.

With the exception of a single biotype 2 isolate from Norway and Canada respectively, as well as a single reference strain, all of the biotype 2 isolates originated from the U.K. The apparent geographic restriction of these isolates mainly to the U.K. suggested that they formed an epizootiological distinct

group of isolates. It will be shown later that the Canadian and Norwegian biotype 2 isolates differ from the U.K. isolates in terms of serotype and OMP-type respectively (Sections 2 and 4).

The distribution of biotype 2 isolates may be more widespread in North America than the results suggest, since relatively few North American isolates were tested, and these had been subject to selection. Nevertheless, the U.K. biotype 2 isolates were highly virulent (Section 9) and, if present in North America, it is surprising that they have not already been described. The identification of a geographically isolated and biochemically distinct strain of Y. ruckeri in Europe raises obvious questions about its origin and epizootiology. The biotypes and geographic origins of the isolates examined are shown in Table 2.

As expected, sorbitol fermentation was related to the serotype (Section 2) in that sorbitol non-fermenting isolates were of serotype O1 and sorbitol-fermenting isolates, with the exception of four serotype O1 isolates, were of serotypes O2, O5, O6 and O7. It will be shown later (Section 4) that the sorbitol-fermenting serotype O1 isolates form a distinct clonal group; sorbitol fermentation is, therefore, a marker of this clonal group. Sorbitol fermentation is useful as an initial indicator of serotype but has limited epizootiological significance. The sorbitol fermentation reactions and geographic origins of the isolates examined are shown in Table 2.

Citrate utilization, gelatin hydrolysis, the VP reaction and the nitrate reduction test of the API 20E system showed some variation from the conventional tests. In particular, a large number of negative citrate tests were obtained with the API 20E system. Extended incubation periods, for up to three days, were sometimes necessary to avoid false negative citrate utilization and gelatin hydrolysis tests. The nitrate reduction test of the API 20E system also

Table 2. Geographic origins, biotypes and sorbitol reactions of isolates of Y. ruckeri

Geographic origin	Biotype	Sorbitol fermentation	No. of isolates	Isolates
Australia	1	-	6	RD44,RD46,RD156,RD158,RD184,RD186
Bulgaria	1	-	13	RD296,RD298,RD300,RD302,RD304,RD306, RD308,RD310,RD312,RD314,RD316,RD318, RD320
Canada	1	-	2	RD178,RD182
	1	+	8	RD52,RD54,RD166,RD168,RD176,RD190, RD192,RD194
	2	+	1	RD174
Denmark	1	-	7	RD112,RD114,RD116,RD118,RD120,RD122, RD124
	1	+	1	RD150
Finland	1	-	4	RD20,RD24,RD26,RD160
	1	+	2	RD22,RD162
France	1	-	6	RD136,RD138,RD140,RD142,RD144,RD278
	1	+	3	RD280,RD282,RD284
Italy	1	-	41	RD74,RD76,RD78,RD80,RD82,RD84, RD86,RD88,RD90,RD92,RD94,RD96, RD98,RD100,RD102,RD104,RD106,RD108, RD110,RD146,RD196,RD198,RD200,RD202, RD204,RD206,RD208,RD210,RD212,RD214, RD216,RD218,RD220,RD222,RD224,RD226, RD228,RD230,RD232,RD234,RD236
Norway	1	-	1	RD292
	1	+	1	RD294
	2	-	1	RD154
South Africa	1	-	2	RD286,RD288
U.K.	1	+	3	RD28,RD34,RD290
	2	-	23	RD6,RD10,RD16,RD30,RD128,RD130, RD132,RD148,RD152,RD246,RD248,RD250, RD252,RD254,RD256,RD258,RD260,RD264, RD266,RD268,RD270,RD272,RD274
U.S.A	1	-	5	RD38,RD40,RD42,RD170,RD172
	1	+	7	RD36,RD48,RD50,RD56,RD58,RD60, RD188
West Germany	1	-	3	RD62,RD70,RD72
	1	+	3	RD64,RD66,RD68
Reference	1	-	3	RD4,RD32,RD180
Strains	2	-	1	RD2
Vaccine Strain	1	-	1	RD126

sometimes gave false negative results. A higher number of VP positive results were recorded with the API 20E system than with the conventional test method. It is suggested, therefore, that the citrate utilization, gelatin hydrolysis and nitrate reduction tests, in particular, be duplicated by conventional methods in studies on Y. ruckeri when the API 20E system is used.

The variation observed in the MR and VP tests (VP+/MR+, VP+/MR-, VP-/MR+ strains were all identified) suggests that an analysis of fermentation end-products may be useful in taxonomic and epizootiological studies of Y. ruckeri. The potential value of such a procedure has been demonstrated by Tracey et al. (1986) who used gas-liquid chromatography to analyse the metabolites of 55 strains of bacteria (principally Hafnia alvei and Hafnia protea) including Y. ruckeri ATCC 29473.

Section 2

Serological Characterisation of Yersinia ruckeri

Serological Characterisation of Yersinia ruckeri

2.1 INTRODUCTION

Initial serological studies showed that Y. ruckeri consisted of only a single serotype (Busch, 1973; Ross et al., 1966). A second serotype was described by O'Leary (1977) who designated the new serotype as serotype 1 and the original serotype as serotype 2. A third serotype originating from Australia was described by Bullock et al. (1978).

Due to the confusing numerical type designations which occurred in the literature, primarily due to O'Leary's designation of the original serotype as serotype 2, Anderson and Dixon (1980) proposed specific serotype designations "that reflected the historical precedence, comparative incidence, economic importance and relative virulence of the three recognized serotypes" (Busch, 1982). Thus, the original "Hagerman" strain was designated serotype 1 and the "Big Creek" strain identified by O'Leary (1977) was designated serotype 2. The Australian serotype was designated serotype 3.

Although McCarthy and Johnson (1982) demonstrated only a single serotype (serotype 1) among North American isolates of Y. ruckeri, Stevenson and Daly (1982) described cross-reactivity in sorbitol-fermenting isolates and concluded that variation in the serological properties of Y. ruckeri was greater than previously thought. Stevenson and Airdrie (1984a) identified two further serotypes which were designated serotypes IV and V. It was later shown, however, that the serotype IV isolate was not in fact Y. ruckeri (De Grandis et al., 1984; 1988). In addition, Stevenson and Airdrie (1984a) identified a serotype I sorbitol-fermenting isolate and concluded that sorbitol fermentation was an inaccurate predictor of serological reactions. A further serological

investigation by Daly et al. (1986) revealed a serological variety which was designated serotype VI as well as an isolate resembling the serotype III Australian isolate (Bullock et al., 1978). Unlike the Australian isolate, however, this isolate fermented sorbitol.

The serotyping described by Stevenson and Airdrie (1984a) and Daly et al. (1986) was based on whole-cell antigens using unabsorbed and cross-absorbed antisera. Pyle and Schill (1985), however, described at least four O-serotypes among sorbitol-fermenting isolates of Y. ruckeri. These authors showed that lipopolysaccharide band patterns in silver-stained polyacrylamide gels could be used to predict O-serologic specificity as determined by Western-blotting and microplate agglutination assays using somatic O-antigens. The four O-serotypes were designated 2, 4, 5 and 6. These designations did not, however, correspond to those of Stevenson and Airdrie (1984a) and Daly et al. (1986).

In a serological study of 79 isolates of Y. ruckeri originating principally from North America, but also including some Australian isolates, Pyle et al. (1987) distinguished at least six serotypes which were designated 1 to 6. Inexplicably, with the exception of serotypes 1 and 2, these designations did not correspond either with those of Stevenson and Airdrie (1984a) and Daly et al. (1986), or with those of Pyle and Schill (1985). Furthermore, the technique used (immunofluorescence assay) was not able to differentiate between the serotypes of 10 isolates, some of which had previously been shown to be different (Pyle and Schill, 1985).

In Europe, the serotypes of relatively few isolates of Y. ruckeri have been described in the literature (Giorgetti et al., 1985; McArdle and Dooley- Martin, 1985; Meier, 1986; Michel et al,. 1986; Vuillaume et al., 1987). This serotyping has been relatively crude and the isolates have all been identified as serotype

1. A detailed serological investigation of European isolates of Y. ruckeri has not been carried out.

The aim of this part of the study was two-fold. First, it was necessary to produce a serotyping scheme which was based on defined antigens and which could be related to the three schemes described above. The second objective was to compare and contrast the serological characteristics of European, North American, Australian and South African isolates of Y. ruckeri. Serological analysis of these isolates would, in particular, show the relatedness of European and North American strains of Y. ruckeri and hence would be of importance to the study of the epizootiology of Y. ruckeri in Europe. The analysis would also provide information relevant to the virulence and pathogenesis aspects of the study.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial isolates

The serological characteristics of 132 isolates of Y. ruckeri were examined, including 127 field isolates, four reference strains and a vaccine production strain. Details of these isolates are given in Table 8 and in Appendix 1.

2.2.2 Preparation of antigens

The antigens used in serological tests were formalin-killed whole-cells and O-antigens. Isolates were grown on TSA at 22^{o}C for two or three days. For preparation of formalin-killed whole-cells, 50 ml TSB was inoculated from a TSA culture and the cells were grown at 22^{o}C overnight. Formalin was added to a final concentration of 0.5% (v/v) and the cells were left for a further 24 h. The cells were harvested by centrifugation at 1000 x g for 15 min, washed, and

resuspended in 0.85% saline to an optical density of 2.0 at 610 nm. Somatic O-antigens were prepared from the cells of a 48 h bacterial lawn, which was prepared by spreading 0.1 ml of a light saline suspension, made from the TSA culture, of each isolate on a dry TSA plate. The bacteria were harvested in 10 ml 0.85% saline and heated at 100^{o}C in a boiling water bath for 2 h. The cells were collected by centrifugation at 1000 x g for 15 min, washed, and resuspended in 0.85% saline. For microplate agglutination assays the cells were resuspended to an optical density of 2.0 at 610 nm; for slide agglutination tests the cells were resuspended in 0.5 ml 0.85% saline to give a dense suspension.

2.2.3 Preparation of hyperimmune rabbit antisera

Formalin-killed cells were washed and resuspended in 0.85% saline to an optical density of 1.0 at 610 nm. Increasing volumes were injected intravenously into female New Zealand white rabbits as follows: day 1, 0.25 ml; day 4, 0.5 ml; day 8, 1.0 ml; day 11, 2.0 ml. Washed live cells, adjusted to the same optical density, were then injected as follows: day 15, 0.5 ml; day 18, 1.0 ml; day 22, 2.0 ml. The rabbits were bled from the marginal ear vein on days 29 and 36. The blood was allowed to clot at room temperature for 1 h and was left at 4^{o}C overnight. After centrifugation at 1000 x g for 5 min the serum was separated, filter sterilized through a 0.45 μm Millipore filter, and stored at -70^{o}C. For short term use antisera was preserved with 0.01% methiolate and stored at 4^{o}C.

2.2.4 Cross-absorption of antisera

Bacteria from a 48 h lawn were harvested in 10 ml 0.85% saline, washed, and resuspended in 2.0 ml antiserum. The antiserum was incubated at 37^{o}C for 1 h with occasional mixing and the bacteria were removed by centrifugation. The

process was repeated twice more after which the antiserum was filter sterilized and stored at -70^{o}C.

2.2.5 Microplate agglutination assays

Microplate agglutination assays were carried out essentially as described by Stevenson and Daly (1982) using whole-cells or O-antigens and unabsorbed anti whole-cell antisera. Two-fold serial dilutions of antisera were made in 50 μl volumes of 0.85% saline incorporating 0.005% (w/v) safranin in 96-well round-bottomed microtitre plates. The last well of each row was used as a control and contained only saline. Thus, the antisera dilutions were from 1 in the first well of each row (i.e. undiluted) to 1/1024 in the eleventh well. In the case of the whole-cell antigen assays the antisera were diluted 1/10 before serial dilution; in this case, therefore, the antisera dilutions were from 1/10 in the first well to 1/10,240 in the eleventh well. Additional controls incorporated preimmune serum instead of hyperimmune serum. Fifty microlitres of bacterial suspension were added to each well and the plates were incubated at 37^{o}C for 2 h and overnight at 4^{o}C. Agglutination titres were recorded against a black background and were expressed as the reciprocal of the highest dilution showing a macroscopic reaction.

2.2.6 Slide agglutination tests

Ten microlitres of the bacterial O-antigen preparation were reacted, on a glass slide, with the same volume of undiluted antiserum. The result was recorded against a black background after 30-60 s.

2.3 RESULTS

The serotype designations employed in this study were O1, O2, O5, O6 and O7. These were based on the agglutination reactions of O-antigens with whole-cell antisera. The corresponding whole-cell antisera were designated types 1, 2, 5, 6 and 7. Serotypes O1, O2, O5 and O6 were equivalent to the serotypes I, II, V and VI, respectively, of Stevenson and Airdrie (1984a) and Daly et al. (1986); serotype O7 was not described by these authors. These designations will be discussed more fully later.

Antisera were first raised against isolates RD170 and RD168, the serotype O1 "Hagerman" strain and the serotype O2 "Big Creek" strain, respectively. All isolates were initially "screened" by microplate agglutination assay using whole-cell antigens and types 1 and 2 antisera. Based on the results obtained, antisera were raised against isolate RD28 (which gave an identical reaction to known serotype V isolates, i.e. RD188 and RD192 [Stevenson and Airdrie, 1984a]), isolate RD194 (known to be a serotype VI isolate [Daly et al., 1986]) and isolate RD150 (of unknown serotype).

Twelve serotype O1 isolates and all of the non-serotype O1 isolates were examined further, using these additional antisera, by microplate agglutination assay of whole-cell antigens. The O-antigens of these isolates were then tested against all five antisera by microplate agglutination assay and finally, the O-antigens of all 132 isolates were tested, by slide agglutination tests, against the five antisera.

2.3.1 Microplate agglutination assays

2.3.1.1 Whole-cell antigens. The ranges of reactions obtained with whole-cell antigens in microplate agglutination assays are shown in Table 3. Although it

Table 3. Results of microplate agglutination assays with whole-cell antigens against unabsorbed antisera.

Serotypes of isolates	No. of isolates	Antisera[a]				
		Type 1	Type 2	Type 5	Type 6	Type 7
01	12	160-320	0-40	0-40	0	0
02	11	0-80	110-1280	0	0	0
05	5	20-40	0	640-1280	0	0
06	4	40	20	0	320-1280	0
07	5	20-40	0	0	40	80
UT	1	0	0	0	0	0

[a] = ranges of agglutination reactions expressed as the reciprocal of the highest dilution showing a macroscopic reaction

UT = untypable

Table 4. Results of microplate agglutination assays with O-antigens against unabsorbed antisera.

Serotypes of isolates	No. of isolates	Antisera[a]				
		Type-1	Type-2	Type-5	Type-6	Type-7
01	12	128-256	0	16-32	0	0
02	11	0	128-256	0	0	0
05	5	0-8	0	64-128	0	0
06	4	0	0	0	128-256	0
07	5	0	0	0	16-32	32-64
UT	1	0	0	0	0	0

[a] = ranges of agglutination reactions expressed as the reciprocal of the highest dilution showing a macroscopic reaction.

UT = untypable

was possible to serotype the majority of isolates by this method the results were not entirely satisfactory. Some isolates could not be typed due to auto-agglutination and end-points were often difficult to determine due to fine agglutination after the major reaction. Furthermore, cross-agglutination occurred which also made interpretation of the results difficult. All isolates, however, gave stronger reactions with the homologous antiserum, than with the heterologous antisera.

2.3.1.2 O-antigens. Problems associated with auto-agglutination, determination of end-points, and cross-agglutination, were eliminated when O-antigens were used in microplate agglutination assays (Table 4). All isolates, with one exception, gave strong unambiguous reactions with the homologous antiserum although minor cross-agglutination occurred between isolates of serotypes O1, O5 and O7 and types 5, 1 and 6 antisera respectively. The exception was the Australian isolate RD46 which did not react with any of the antisera.

2.3.2 Slide agglutination tests

Slide agglutination tests with O-antigens were in agreement with the results of the O-antigen microplate agglutination assays. All isolates gave a strong, rapid, granular agglutination with the homologous antiserum but isolates of serotypes O1 and O7 gave weaker, fine agglutination with types 5 and 6 antisera respectively (Table 5). This cross-agglutination was removed when the antisera were cross-absorbed with the heterologous serotype (Table 6).

2.3.3 Comparison of serotyping schemes

Three different serotyping schemes have been described for Y. ruckeri

Table 5. Results of slide agglutination tests with O-antigens against unabsorbed antisera.

Serotypes of isolates	No. of isolates	Antisera Type-1	Type-2	Type-5	Type-6	Type-7
01	106	+	-	+	-	-
02	11	-	+	-	-	-
05	5	-	-	+	-	-
06	4	-	-	-	+	-
07	5	-	-	-	+	+
UT	1	-	-	-	-	-

UT = untypable

Table 6. Results of slide agglutination tests with O-antigens against cross-absorbed antisera.

Serotypes of isolates	No. of isolates	Antisera Type-1	Type-2	Type-5[a]	Type-6[b]	Type-7
01	106	+	-	-	-	-
02	11	-	+	-	-	-
05	5	-	-	+	-	-
06	4	-	-	-	+	-
07	5	-	-	-	-	+
UT	1	-	-	-	-	-

[a] = type 5 antiserum cross-absorbed with serotype 01 whole-cells.

[b] = type 6 antiserum cross-absorbed with serotype 07 whole-cells.

UT = untypable

(Stevenson and Airdrie, 1984a and Daly et al., 1986; Pyle and Schill, 1985; Pyle et al., 1987). The work described here included representative isolates of the various serotypes described in all three of these schemes and so a direct comparison could be made. The numerical designations used in this study were based on those of Stevenson and Airdrie (1984a) and Daly et al. (1986). Unlike the scheme described by these authors, however, the scheme described in this study was based on defined antigens, i.e. O-antigens. Pyle and Schill (1985) also proposed a serotyping scheme based on O-antigens, but their numerical designations did not correspond to those of Stevenson and Airdrie (1984a) and Daly et al. (1986). A third scheme was proposed by Pyle et al. (1987) which differed again from the previous two schemes. For reasons discussed below serotypes O3 and O4 were not recognised in the scheme proposed in this study. The three published serotyping schemes, as well as the one proposed in this study, are compared in Table 7.

2.3.4 Serotypes of Y. ruckeri isolates

Using O-antigens in conjunction with microplate agglutination assays or slide agglutination tests it was possible, with the exception of a single untypable isolate (RD46), to serotype all of the isolates examined (132 isolates) into one of five O-serotypes. Thus, 106 isolates were serotype O1, 11 isolates were serotype O2, five isolates were serotype O5, four isolates were serotype O6, five isolates were serotype O7 and one isolate was untypable. The serotypes and geographic origins of the isolates examined are shown in Table 8.

Table 7. Relationship between serotyping schemes of Stevenson and Airdrie (1984) and Daly et al. (1986), Pyle and Schill (1985), Pyle et al. (1987) and the scheme proposed in this study.

Serotypes designated by Stevenson and Airdrie (1984) and Daly et al.(1986)	Serotypes designated by Pyle and and Schill (1985)	Serotypes designated by Pyle et al (1987)	Serotypes designated in this study
I	NT	1	01
II	2	2	02
III	NT	6	-[b]
IV[a]	-	-	-
V	6	5	05
VI	5	4	06
NT	4	3	07

[a] = no longer recognised as Y. ruckeri

[b] = not recognised as an O-serotype in this scheme

NT = not tested

Table 8. Geographic origins and serotypes of isolates of Y. ruckeri

Geographic origin	Serotype	No. of isolates	Isolates
Australia	01	5	RD44,RD156,RD158,RD184,RD186
Bulgaria	01	13	RD296,RD298,RD300,RD302,RD304,RD306,RD308,RD310, RD312,RD314,RD316,RD318,RD320
Canada	01	3	RD178,RD182,RD190
Denmark	01	7	RD112,RD114,RD116,RD118,RD120,RD122,RD124
Finland	01	5	RD20,RD22,RD24,RD26,RD160
France	01	8	RD136,RD138,RD140,RD142,RD144,RD278,RD280,RD282
Italy	01	20	RD74,RD76,RD78,RD80,RD82,RD84,RD86,RD88,RD90,RD92,RD94, RD96,RD98,RD100,RD102,RD104,RD106,RD108,RD110,RD146
Norway	01	2	RD154,RD292
South Africa	01	2	RD286,RD288
Switzerland	01	4	RD322,RD324,RD326,RD328
U.K.	01	24	RD6,RD10,RD14,RD16,RD30,RD128,RD130,RD132,RD148, RD152,RD246,RD248,RD250,RD252,RD254,RD256,RD258, RD260,RD264,RD266,RD268,RD270,RD272,RD274
U.S.A.	01	5	RD38,RD40,RD42,RD170,RD172
West Germany	01	3	RD62,RD70,RD72
Reference Strains	01	4	RD2,RD4,RD32,RD180
Vaccine Strain	01	1	RD126
Canada	02	3	RD54,RD174,RD176
France	02	1	RD284
Norway	02	1	RD294
U.K.	02	1	RD34
U.S.A	02	3	RD56,RD58,RD168
West Germany	02	2	RD64,RD68
Canada	05	1	RD192
U.K.	05	2	RD28,RD290
U.S.A.	05	2	RD50,RD188
Canada	06	1	RD194
Finland	06	1	RD162
U.S.A.	06	1	RD48
West Germany	06	1	RD66
Canada	07	2	RD52,RD166
Denmark	07	1	RD150
U.S.A.	07	2	RD36,RD60
Australia	UT	1	RD46

2.4 DISCUSSION

The use of whole-cell antigens in microplate agglutination assays was found to be unsatisfactory due to difficulties in determining end-points, auto-agglutination and cross-agglutination. Slide agglutination tests using whole-cell antigens were attempted and abandoned due to excessive cross-agglutination. These problems were eliminated when O-antigens were used, both in microplate agglutination assays and in slide agglutination tests. Toranzo et al. (1987) also described strong cross-agglutinations between type 1 and 2 Y. ruckeri isolates in slide agglutination tests and eliminated these cross-reactions by using somatic O-antigens. In addition, Pyle and Schill (1985) used somatic O-antigens in a serological study of Y. ruckeri without problems of cross-agglutination. The results described by these authors, together with the results described in this study, indicate that the use of O-antigens in the serotyping of Y. ruckeri is extremely effective and has advantages over the use of whole-cell antigens. It was also shown that the rapid slide agglutination technique is a reliable, quick and accurate method of determining O-serotype when the necessary precaution of cross-absorbing certain antisera (i.e. types 5 and 6 antisera) is taken. The cross-reactivity observed between certain O-serotypes was not unexpected since similar cross-reactivity occurs in E.coli O-groups and is also removed by cross-absorption (Edwards and Ewing, 1972; Orskov and Orskov, 1984).

The antisera used in this study were raised against whole-cell antigens because the sera were required for Western-blotting studies of OMPs (Section 6). The method could have been improved by using O-antisera. Slide agglutination tests could then have been performed with whole-cells taken direct from TSA plates, thus saving additional time necessary for the heat-treatment of cells. It is suggested, therefore, that serotyping of Y. ruckeri should be carried out with

O-antisera and, to avoid confusion, the numerical O-serotype designations proposed in this study should be used.

In the work described by Stevenson and Airdrie (1984a) and Daly et al. (1986) whole-cell antigens were used in conjunction with unabsorbed and cross-absorbed whole-cell antisera. This methodology had the disadvantages that defined antigens were not employed and the results needed careful interpretation. These authors identified three isolates (RD184, RD186 and RD190 in this study) as being serotype III (Stevenson and Airdrie, 1984a; Daly et al., 1986; De Grandis et al., 1988) whereas the work described in this study identified these isolates as serotype O1. Evidence is presented in Section 3 which shows that these isolates have an LPS structure similar to other serotype O1 isolates whereas the original Australian serotype III isolate (RD46) has a rough-type LPS. It is suggested, therefore, that the original serotype 3 Australian isolate described by Bullock et al. (1978) is a rough-type mutant and that serotype 3 (or O3) does not exist. Furthermore, it is suggested that the three isolates described as serotype III by the above authors have been wrongly serotyped and are, in fact, serotype O1. The serotype IV isolate described by Stevenson and Airdrie (1984a) has since been shown not to be Y. ruckeri (De Grandis et al., 1984; 1988). For these reasons, therefore, serotypes O3 and O4 were not included in the serotyping scheme proposed in this study.

The serological analysis of the European isolates was significant in that three of the serotypes, serotypes O5, O6 and O7, have not previously been described in Europe. Although serotype O2 isolates have been recognised in Europe their isolation has not been well documented in the literature. Serotype O2 isolates appeared to be more common and more widely distributed than isolates of serotypes O5, O6 and O7. Serotype O5 and O7 isolates were restricted to the U.K. and Denmark respectively, whereas serotype O6 isolates occurred in Finland and West Germany. Serotype O2 isolates were present in France,

Norway, the U.K. and West Germany. These distributions are probably not true representations because the sample size was very small. The recognition of additional serotypes of Y. ruckeri in Europe and the association of these with disease outbreaks will have important implications on the diagnosis of ERM and on the vaccination of fish against the disease.

The range of serotypes present in Europe was identical to the range present in North America. Serotypes O1, O2, O5, O6 and O7 were found to be present on both continents and there were no serotypes unique to either North America or Europe. In Australia and South Africa, only serotype O1 isolates were found to occur, with the exception of the untypable Australian isolate. These findings suggest that the North American and European populations of Y. ruckeri are not isolated and that there is movement from one continent to the other or in both directions. This tends to support evidence that suggests that Y. ruckeri was introduced (and is possibly still being introduced) into Europe from North America by the importation of asymptomatic carrier fish (McArdle and Dooley-Martin, 1985; Michel et al., 1986). Conversely, the findings suggest that Australia and South Africa, each with a much smaller range of serotypes, are to a large extent isolated from the spread of Y. ruckeri, although it should be recognised that only a small number of isolates were examined from these two countries.

Serotype O1 isolates comprised, by far, the majority of isolates from Europe (91%) and were the only isolates obtained from Australia and South Africa. The relatively small number of serotype O1 isolates obtained from North America was due to the selection of the isolates and was not a true representation of their distribution. The large number of serotype O1 isolates obtained from Europe can be explained by the virulent nature of the serotype O1 phenotype in comparison to the other serotypes. It will be shown later, however, that serotype O1 isolates can be divided into six clonal groups

(Section 5) and that, of these, two are highly virulent whereas four are avirulent (Section 9).

A relationship between sorbitol fermentation and serotype has been known since the work of O'Leary (1977). Generally, serotype O1 isolates do not ferment sorbitol, although a small number of serotype O1 isolates have been described that do ferment sorbitol (Stevenson and Airdrie, 1984a; Michel et al., 1986; Rintamaki et al., 1986; Pyle et al., 1987). It was shown in this study that of the 29 isolates that fermented sorbitol (Section 1) only four were of serotype O1; the others were of serotypes O2, O5, O6 and O7. These results were in agreement, therefore, with previous findings. Sorbitol fermentation appears to be a marker of a small, uncommon clonal group of serotype O1 isolates which can also be distinguished by their OMP profile (Section 4). Michel et al. (1986) isolated a sorbitol-fermenting serotype O1 isolate from imported baitfish in France (these fish had been imported from the U.S.A.). The recognition of a similar isolate in Finland gives rise to speculation about its origin also, since these isolates possibly have a common origin.

It should be noted that the relation of O-serotype to sorbitol-fermentation in Y. ruckeri is analogous to the situation found in E. coli, where the O157:H7 strain (which is responsible for hemorrhagic colitis) does not rapidly ferment D-sorbitol, in contrast to the 95% of other E. coli strains which do ferment D-sorbitol (Farmer and Davis, 1985).

Section 3

Lipopolysaccharide Analysis of Yersinia ruckeri

Lipopolysaccharide Analysis of Yersinia ruckeri

3.1 INTRODUCTION

The outer surfaces of bacteria are extremely important in that they provide a physical and functional barrier between the inside of the cell and the environment. In Gram-negative bacteria, with the exception of bacteria possessing a polysaccharide capsule (K antigen), it is the outer membrane (OM) which constitutes this barrier and a major component of the OM is LPS. Lipopolysaccharide confers on the OM properties which are important in providing the bacterial cell with protection against adverse environmental factors. Protection against the bile salts, fatty acids and glycerides present in the gut is vital for members of the Enterobacteriaceae and the LPS of these bacteria are particularly important in this respect. Lipopolysaccharides are also extremely important in pathogenic bacteria in which they provide protection against such factors as complement, antibodies, lysozyme and other peptidoglycan-degrading enzymes, and phagocytes. Lipopolysaccharides are, therefore, important virulence determinants. Other OM components, such as certain proteins, are also important in providing the bacterial cell with protection. Further information on the structure and function of the Gram-negative cell envelope will be found in reviews by Nikaido and Nakae (1979), Lugtenberg (1981) and Lugtenberg and Van Alphen (1983).

The LPS of members of the Enterobacteriaceae is composed of three well defined regions : (1) the O-specific antigen, also called O-antigen or somatic antigen, which is a long-chain polysaccharide consisting of repeating units containing one to seven monosaccharide sugars, (2) the oligosaccharide core and, (3) lipid A. Details of the structure of LPS will be found in reviews by Luderitz et al. (1982) and Hitchcock et al. (1986).

Lipopolysaccharide O-antigens form the basis of O-serotyping schemes of members of the Enterobacteriaceae (Edwards and Ewing, 1972; Orskov and Orskov, 1978) and have also been shown to form the basis of heat-stable serotyping systems in non-Enterobacteriaceae including Campylobacter fetus (Perez-Perez et al., 1986), Campylobacter jejuni and Campylobacter coli (Logan and Trust, 1984; Preston and Penner, 1987) and Legionella pneumophila (Nolte et al., 1986).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used for the analysis of the LPS of numerous bacterial species including fish pathogens (Chart et al., 1984; Chart and Trust, 1984; Dooley et al., 1985; Nomura and Aoki, 1985; Pyle and Schill, 1985). Jann et al. (1975) first used the technique to study bacterial LPS and demonstrated heterogeneity of the LPS of Escherichia coli, Salmonella typhimurium and Citrobacter sp. Independently, Goldman and Leive (1980) and Palva and Makela (1980) demonstrated that the O-antigenic side chains in the LPS of E.coli and S.typhimurium were heterogeneous in length. They concluded that the banding pattern in polyacrylamide gels was probably due to LPS molecules with increasing numbers of repeating units in the O-antigenic side chain.

Lipopolysaccharide analysis by SDS-PAGE was used to subtype strains of Haemophilus influenzae and the technique was reported to have epidemiological potential (Inzana, 1983; Inzana and Pichichero, 1984). It was subsequently shown, however, that the LPS profiles of H. influenzae are not stable and that the technique is not useful for the epidemiological analysis of this organism (Kimura and Hansen, 1986; Tolan et al., 1986). Lipopolysaccharide analysis by SDS- PAGE was able to identify four LPS types among 018 E.coli strains and it was concluded that other E.coli O-serogroups are probably chemically heterogeneous (Pluschke et al., 1986). An analysis of

the LPS patterns of sorbitol-fermenting isolates of Y. ruckeri demonstrated a correlation between LPS pattern and the O-serotype (Pyle and Schill, 1985).

Since the first use of Western-blotting in the study of bacterial LPS (Cousland and Poxton, 1983; Bradbury et al., 1984; Karch et al., 1984; Sturm et al., 1984) the technique has been used in numerous studies on the antigenicity and cross-reactivity of LPS. In particular the LPS of the fish pathogens Aeromonas salmonicida (Chart et al., 1984; Pyle and Cipriano, 1986), Aeromonas hydrophila (Dooley et al., 1985), Vibrio anguillarum and Vibrio ordalii (Chart and Trust, 1984) and Y. ruckeri (Pyle and Schill, 1985) have been analysed by this technique. Analysis of LPS by Western-blotting may be used to test the specificity of O-typing sera, to evaluate the extent of cross-reactivity among O-serogroups and to detect any modification in the antigenic structure of LPS (Karch et al., 1984).

An analysis of the LPS of Y. ruckeri isolates by SDS-PAGE and Western-blotting was carried out with the following objectives in mind : (1) to compare the LPS patterns of serotype O1 isolates, which have not previously been examined, with the patterns of serotype O2, O5, O6 and O7 isolates, and to examine the relationship between the LPS patterns and the O-serotypes; (2) to ascertain whether there was any geographic variation in the LPS patterns of individual serotypes and whether, by the identification of distinct LPS subtypes, such variation could have epizootiological significance; (3) to demonstrate, by Western-blotting, that LPS is the serospecific antigen responsible for the O-serotyping scheme and to test for cross-reactivity between LPS types; and (4) to identify any correlation between LPS patterns and virulence.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial isolates and growth conditions

The LPS of 56 isolates of Y. ruckeri, including 52 field isolates and four reference strains, were examined by SDS-PAGE and Western-blotting. The isolates used in the study are shown in Table 9.

Bacteria were grown on TSA at 22^{o}C for two or three days. Growth from five or six colonies was used to inoculate 5 ml TSB and 1 ml of the resulting suspension was used to inoculate 200 ml TSB. Cultures were grown overnight at 22^{o}C resulting in cells which were in the stationary phase of the growth cycle at the time of harvest.

3.2.2 Preparation of hyperimmune rabbit antisera

Preparation of hyperimmune rabbit anti-sera is described in Section 2.

3.2.3 Cross-absorption of antisera

Cross-absorption of antisera is described in Section 2.

3.2.4 Isolation of outer membranes

Outer membrane fractions were prepared essentially by the method described by Chart and Trust (1983). Cells from 200 ml stationary phase broth cultures were harvested by centrifugation at 5000 x g (MSE High Speed 18 Centrifuge) for 30 min, washed in 20 ml 20 mM Tris-hydrochloride (pH 7.2) buffer, and resuspended in 7 ml of this buffer containing 10 mM EDTA. The cells were disrupted, on ice, by sonication (MSE 150 watt ultrasonic disintegrator) using 4

x 60 s bursts with 30 s cooling periods. Unbroken cells were removed by centrifugation at 5000 x g at 4°C for 30 min and the total cell envelope fraction was pelleted by centrifugation at 50,000 x g (Beckman L8-55M Ultracentrifuge) at 4°C for 60 min. The total cell envelope fraction was resuspended in 0.5% (w/v) sodium N-lauroyl sarcosinate (Sarkosyl) for 20 min at room temperature to selectively solubilize the inner membrane (Filip et al., 1973) and the remaining OM fraction was pelleted by centrifugation at 50,000 x g at 4°C for 60 min. The OM fraction was washed by resuspending in 20 mM Tris-hydrochloride (pH 7.2) and centrifuging at 50,000 x g at 4°C for 60 min, and was finally solubilized in 2.0 ml sample buffer (Appendix 2). Undissolved OM material, as well as other particulate contamination, was removed by centrifuging the samples in Eppendorf tubes in a microcentrifuge (MSE Micro-Centaur Centrifuge) at 11,600 x g for 5 min. The supernatants were transferred to clean Eppendorf tubes and stored, in 1 ml aliquots, at -70°C.

3.2.5 Preparation of lipopolysaccharide

Lipopolysaccharide was obtained by proteinase K digestion of OM preparations essentially by the method of Hitchcock and Brown (1983) with the exception that OMs were treated rather than whole cells. Fifty microlitres of the OM preparation were diluted in 200 μl sample buffer and heated at 100°C for 5 min. After cooling, 25 μg proteinase K (Sigma) in 20 μl sample buffer were added, and the preparation was incubated at 60°C for 1 h. After cooling, the samples were either used immediately or stored at -70°C.

3.2.6 SDS-PAGE

Lipopolysaccharide profiles were analysed by SDS-PAGE using the SDS-discontinuous system of Laemmli (1970). A Protean I Dual Slab Cell (Bio-Rad, Richmond, CA94804) apparatus was used according to the Bio-Rad Instruction

Manual. Lipopolysaccharides were stacked in a 4% acrylamide stacking gel and separated in a 12% acrylamide resolving gel (Appendix 2). The buffer system used was the Tris-glycine system consisting of 25mM Tris-hydrochloride, 192mM glycine and 0.1% SDS (pH 8.3) (Appendix 2). Twenty five microlitres of each sample (original OM sample diluted 1:5 in sample buffer and digested with proteinase K) were applied to each lane and electrophoresis was carried out at a constant current of 20 mA per gel through the stacking gel (until the dye-front had entered into the resolving gel) and at a constant current of 30 mA per gel through the resolving gel until the dye-front was about 1 cm from the bottom of the gel.

3.2.7 Silver-staining

Lipopolysaccharides were visualized by silver-staining using the method described by Wray et al. (1981). When electrophoresis was complete the gels were soaked in 50% methanol for 2 h and stained with silver nitrate solution (Appendix 2) for 15 min. After washing the gels in distilled water for 5 min the silver-stain was developed by soaking the gels in developer (Appendix 2) until the bands appeared (about 10 min). Development was carried out in a water bath at 40^{o}C. The gels were washed with distilled water and placed in 50% methanol to stop development. Developed gels were photographed immediately because the stain deteriorated very quickly.

3.2.8 Western-blotting

Lipopolysaccharides were separated by SDS-PAGE and transferred from the slab gel to nitrocellulose membrane by the Tris-glycine-methanol method described by Towbin et al. (1979). Electrophoretic transfer was carried out in a Bio-Rad Trans-Blot Cell (Bio-Rad, Richmond, CA94804) following the Bio-Rad Operating Instructions. Transfer was carried out overnight at 30 V

followed by 3 h at 70 V in buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3 (Appendix 2).

Detection of LPS was carried out with a Bio-Rad Immun-Blot Goat Anti-Rabbit Horseradish Peroxidase assay kit following the Bio-Rad Instruction Manual. After completion of electrophoretic transfer the nitrocellulose membrane was washed in 20 mM Tris, 500 mM sodium chloride buffer, pH 7.5 (TBS) (Appendix 2) for ten minutes. Non-specific binding was blocked by incubation for 1 h in 3% (w/v) gelatin in TBS and the membrane was washed (two 5 min washes) in 0.05% Tween 20 in TBS (TTBS). The membrane was incubated for 2 h in primary rabbit antiserum diluted 1:500 in 1% (w/v) gelatin in TTBS, and was washed (two 5 min washes) in TTBS. This was followed by incubation for 2 h in horseradish peroxidase labelled goat anti-rabbit IgG diluted 1:3000 in 1% (w/v) gelatin in TTBS. The membrane was washed in TTBS (two 5 min washes) and in TBS (one 5 min wash) and developed in a substrate solution containing 0.015% hydrogen peroxide and 4-chloro-1-naphthol in TBS (Appendix 2). Development was stopped by immersing the membrane in distilled water for 10 min. The membrane was dried on filter paper and photographed.

3.3 RESULTS

3.3.1 Lipopolysaccharide profiles

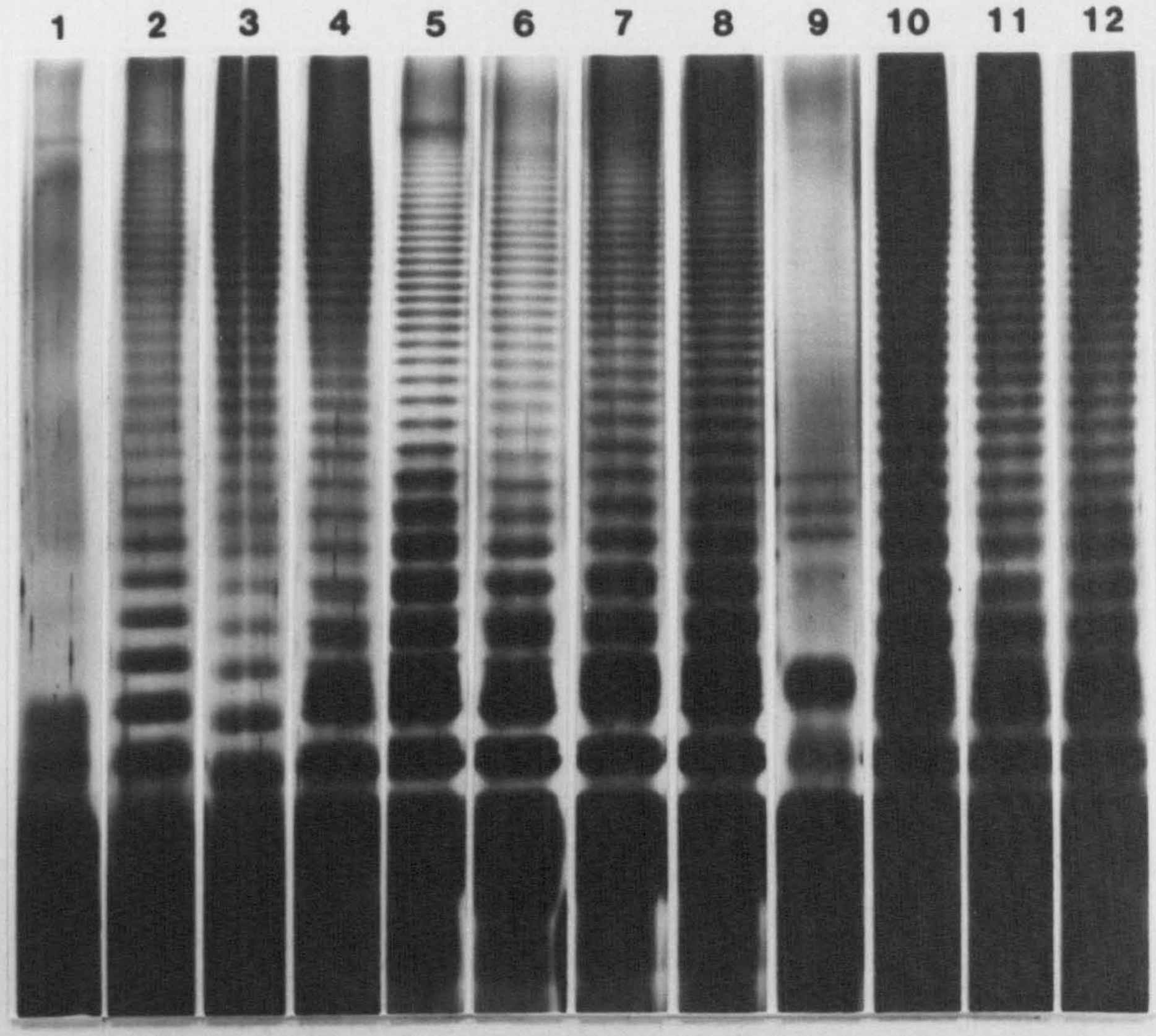
The LPS profiles of the 56 isolates of Y. ruckeri examined are shown in Figure 2. With the exception of a single isolate all isolates had LPS having the ladder-like banding pattern characteristic of smooth-type LPS found in the Enterobacteriaceae. The exception was the untypable Australian isolate, RD46, which appeared to have a rough-type LPS.

Figure 2. Lipopolysaccharide patterns of *Y. ruckeri* serotypes in silver-stained polyacrylamide gels.

(A). Lanes 1 - 12 represent isolates RD2, RD4, RD32, RD180, RD44, RD156, RD158, RD184, RD186, RD6, RD128, RD152 (serotype 01 isolates).

(B). Lanes 1 - 11 represent isolates RD124, RD82, RD84, RD86, RD88, RD62, RD70, RD136, RD138, RD140, RD142 (serotype 01 isolates).

A



B

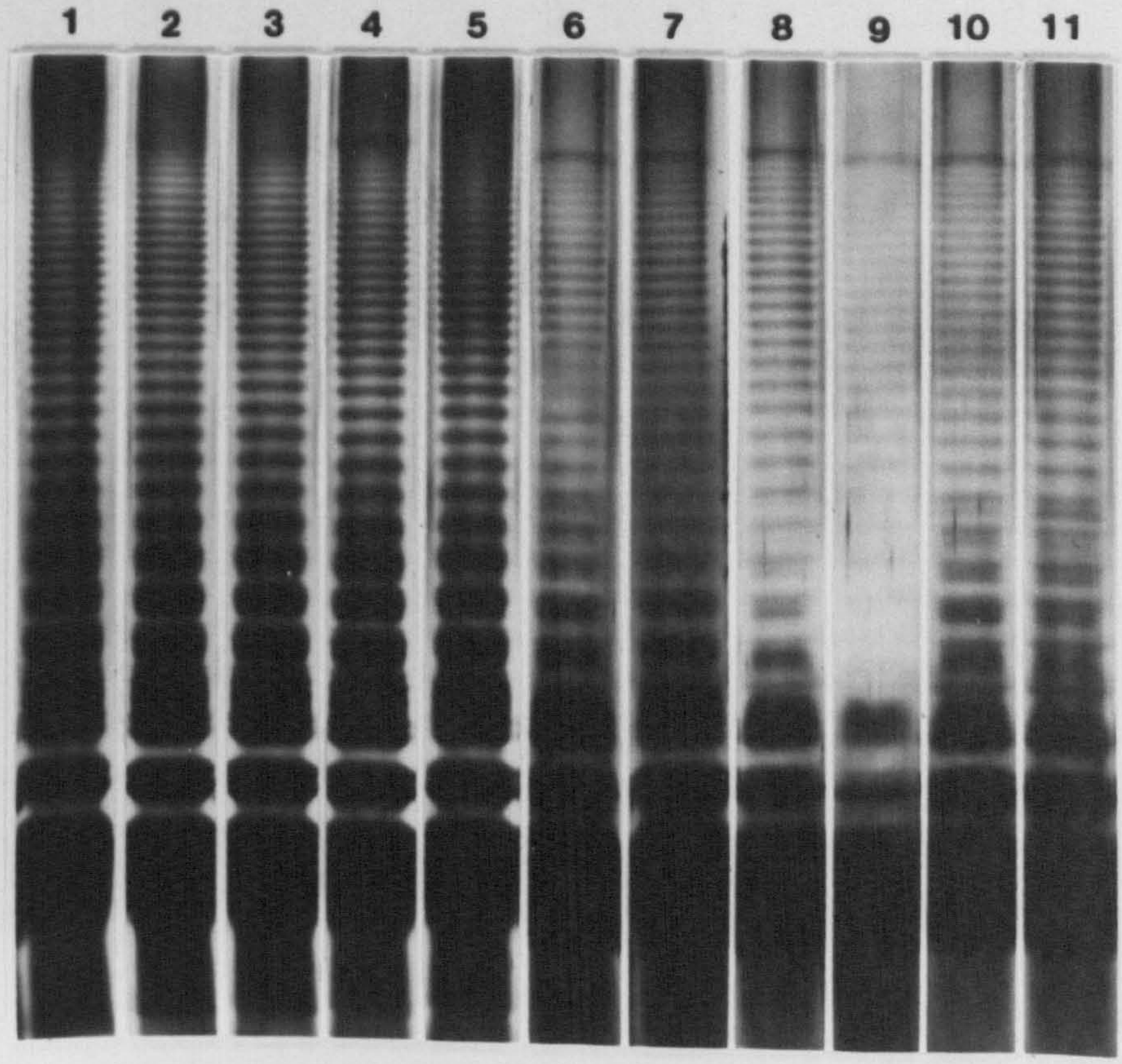
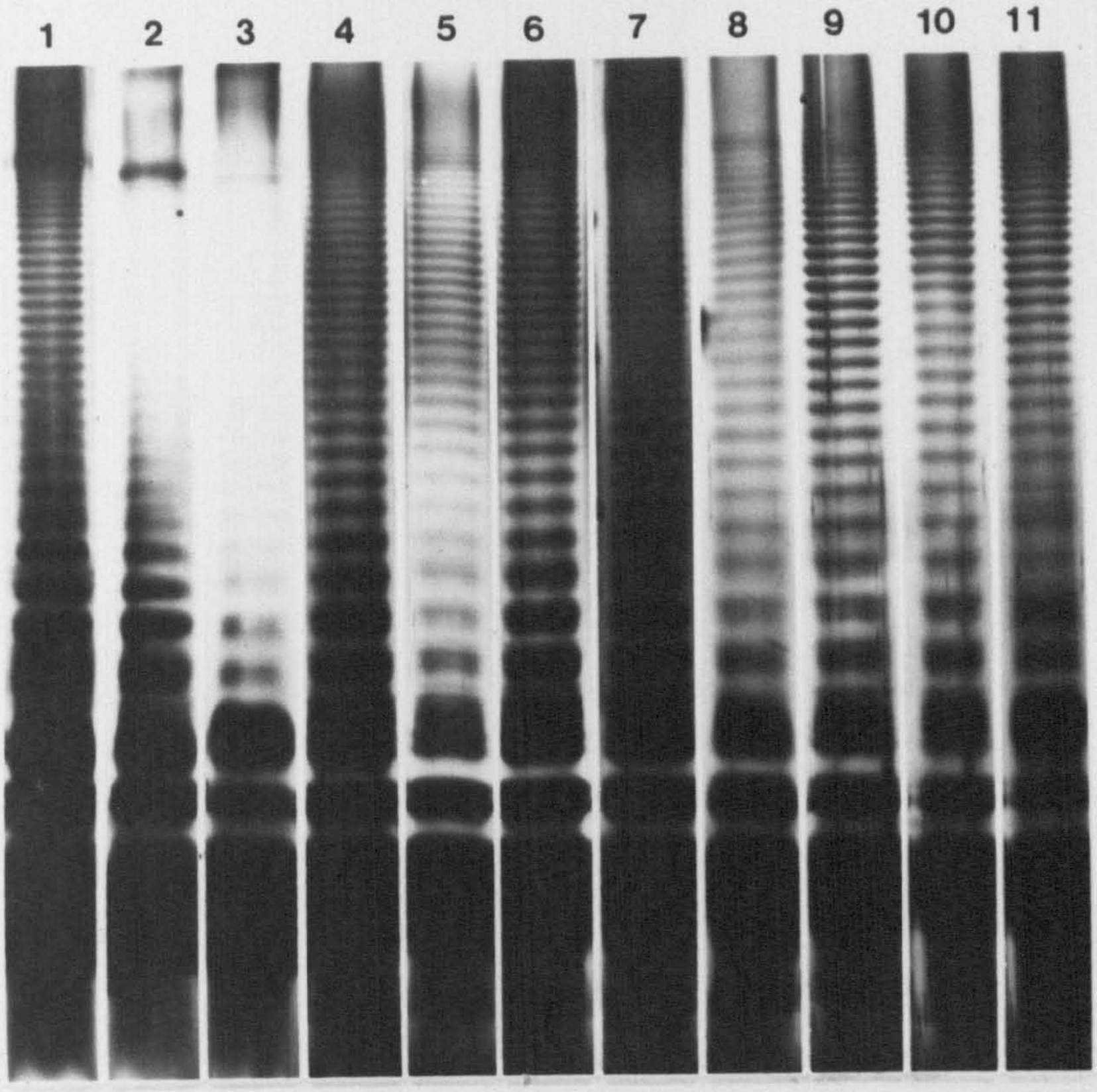


Figure 2 (continued). Lipopolysaccharide patterns of *Y. ruckeri* serotypes in silver-stained polyacrylamide gels.

(C). Lanes 1 - 11 represent isolates RD154, RD20, RD22, RD38, RD40, RD42, RD170, RD172, RD178, RD182, RD190 (serotype 01 isolates).

(D). Lanes 1 - 12 represent isolates RD34, RD64, RD56, RD58, RD168, RD174, RD54, RD176 (serotype 02 isolates), RD28, RD50, RD188, RD192 (serotype 05 isolates).

C



D

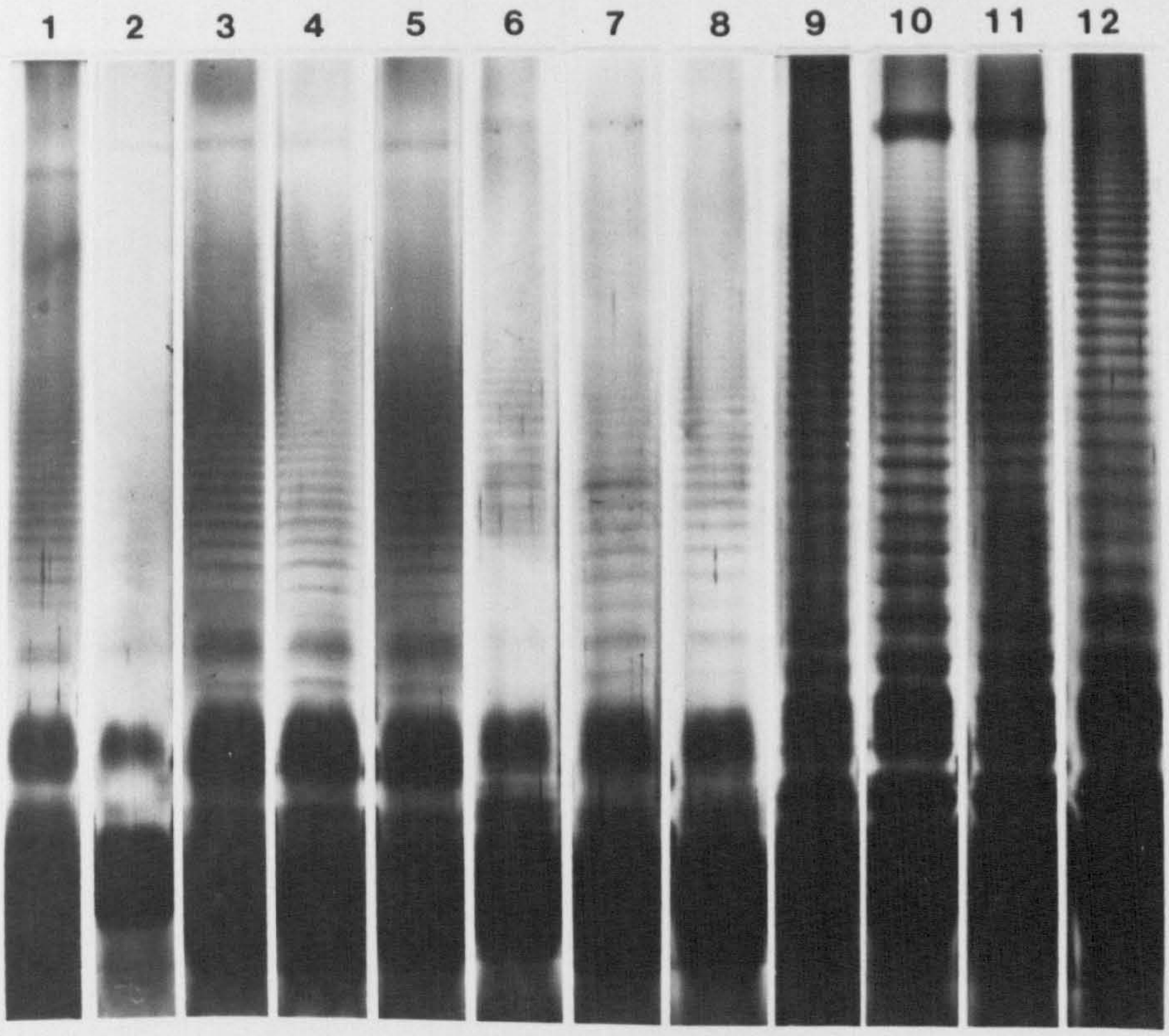
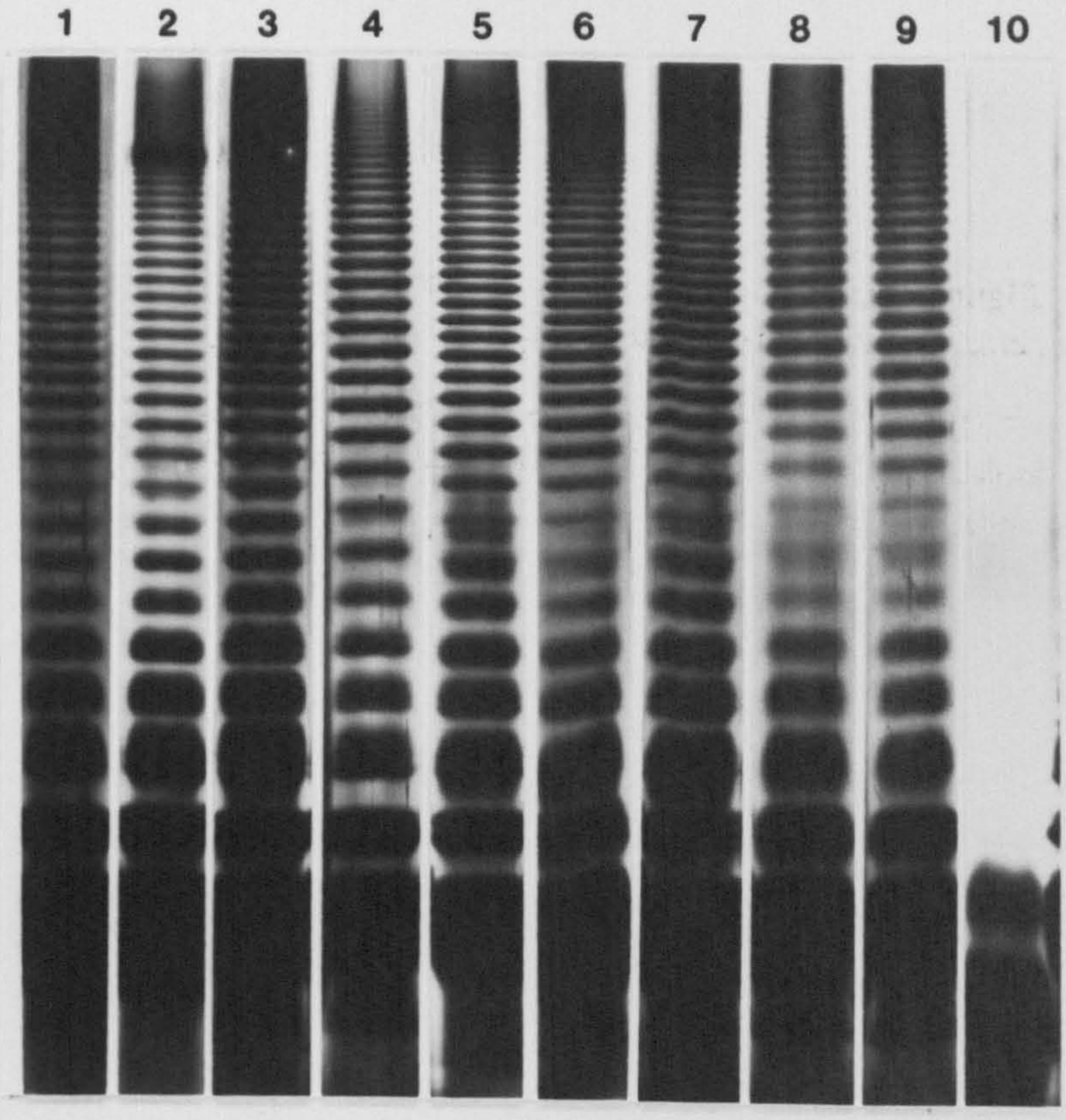


Figure 2 (continued). Lipopolysaccharide patterns of *Y. ruckeri* serotypes in silver-stained polyacrylamide gels.

(E). Lanes 1 - 10 represent isolates RD66, RD162, RD48, RD194 (serotype 06 isolates), RD150, RD36, RD60, RD52, RD166 (serotype 07 isolates), RD46 (untyped).

E



The LPS profiles of serotype O1, O6 and O7 isolates were very similar and it was difficult to differentiate between these with confidence (Figures 2A, B, C and E). However, the presence of a doublet band (see below) in the LPS profiles of serotype O7 isolates to some extent enabled these isolates to be differentiated from serotype O1 and O6 isolates. The LPS profiles of the serotype O2 and O5 isolates, however, were quite distinct and could easily be differentiated both from each other and from the LPS profiles of the other serotypes (Figure 2D). The LPS profiles of isolates of serotypes O1 to O7 were designated LPS-types 1 to 7 respectively (Table 9).

The LPS profiles of the serotype O1 isolates showed some degree of heterogeneity (Figures 2A, B and C) although isolates could not be subtyped on the basis of these variations. An important difference was that a number of isolates had LPS profiles containing doublet bands. Doublet bands were present, for example, in the LPS profiles of isolates RD180, RD62, RD136, RD140, RD142, RD154, RD20 and RD190. The LPS profile of isolate RD186 differed from those of the other serotype O1 isolates in that the ladder pattern, with the exception of three prominent bands, was very weak. Weak ladder patterns were also observed in isolates RD138, RD20 and RD22. In the case of isolate RD2 a distinct ladder pattern was not present although the LPS did not appear to be of the rough type (compare with isolate RD46, Figure 2E). The LPS profiles of isolates RD4 and RD32 differed from those of other serotype O1 isolates in the low-molecular-weight regions.

The LPS profiles of the serotype O2, O5, O6 and O7 isolates did not show the same degree of heterogeneity as was shown by the serotype O1 isolates, although the number of isolates examined was smaller. Nevertheless, there was slight variation in some of the profiles within each of these serotypes. For example, the profiles of isolates RD34 and RD64 (serotype O2),RD188 and RD192 (serotype O5), RD162 and RD48 (serotype O6), and RD60 and RD52

Table 9. Relation between O-serotypes, LPS profiles and LPS-immunotypes in isolates of Y. ruckeri.

Isolates	O-serotype	LPS type	LPS immunotype
RD2	01	1[a]	1A
RD4	01	1[a]	1A
RD32	01	1[a]	1A
RD180	01	1[a]	1A
RD44	01	1	1A
RD156	01	1	1A
RD158	01	1	1A
RD184	01	1	1A
RD186	01	1[a]	1A
RD6	01	1	1A
RD128	01	1	1A
RD152	01	1	1A
RD124	01	1	1A
RD82	01	1	1A
RD84	01	1	1A
RD86	01	1	1A
RD88	01	1	1A
RD62	01	1[a]	1A
RD70	01	1	1A
RD136	01	1[a]	1A
RD138	01	1[a]	1A
RD140	01	1[a]	1A
RD142	01	1[a]	1A
RD154	01	1[a]	1B
RD20	01	1[a]	1A
RD22	01	1[a]	1A
RD38	01	1	1A
RD40	01	1	1A
RD42	01	1	1A
RD170	01	1	1A
RD178	01	1	1A
RD182	01	1	1A
RD190	01	1[a]	1A
RD34	02	2	2
RD64	02	2	2
RD56	02	2	2
RD58	02	2	2
RD168	02	2	2
RD174	02	2	2
RD54	02	2	2
RD176	02	2	2
RD28	05	5	5
RD50	05	5	5
RD188	05	5	5
RD192	05	5	5
RD66	06	6	6
RD162	06	6	6
RD48	06	6	6
RD194	06	6	6

RD150	07	7[b]	7
RD36	07	7[b]	7
RD60	07	7[b]	7
RD52	07	7[c]	7
RD166	07	7[c]	7
RD46	UT	R	-

[a] = 01 LPS types having slightly different profiles to other 01 LPS types, e.g. by possessing doublet bands (see text).

[b] = 07 LPS types having identical profiles.

[c] = 07 LPS types having identical profiles.

UT = untypable

R = rough-type LPS

(serotype O7) showed slight differences. It should be noted that these pairs of isolates also differed in OMP profiles (Section 4).

In addition to certain O1 LPS types, doublet bands were also present in the LPS profiles of serotype O5, O6 and O7 isolates and possibly also in serotype O2 isolates. In the serotype O7 isolates the position of the doublet band was the same in isolates RD150, RD36 and RD60, but slightly different in isolates RD52 and RD166 (Figure 2E). These slight differences in the position of the doublet band enabled these two groups of serotype O7 isolates to be differentiated from one another. It was also found that the two groups of isolates could be differentiated on the basis of their OMP profiles (Section 4). It should be noted that isolates RD36 and RD60, and RD52 and RD166, were in fact the same isolates derived from different sources (Appendix 1). The position of doublet bands in isolates RD66 and RD162 (serotype O6 isolates) were also identical. It should be noted that both of these isolates originated from Europe (Appendix 1) and also had similar OMP profiles (Section 4).

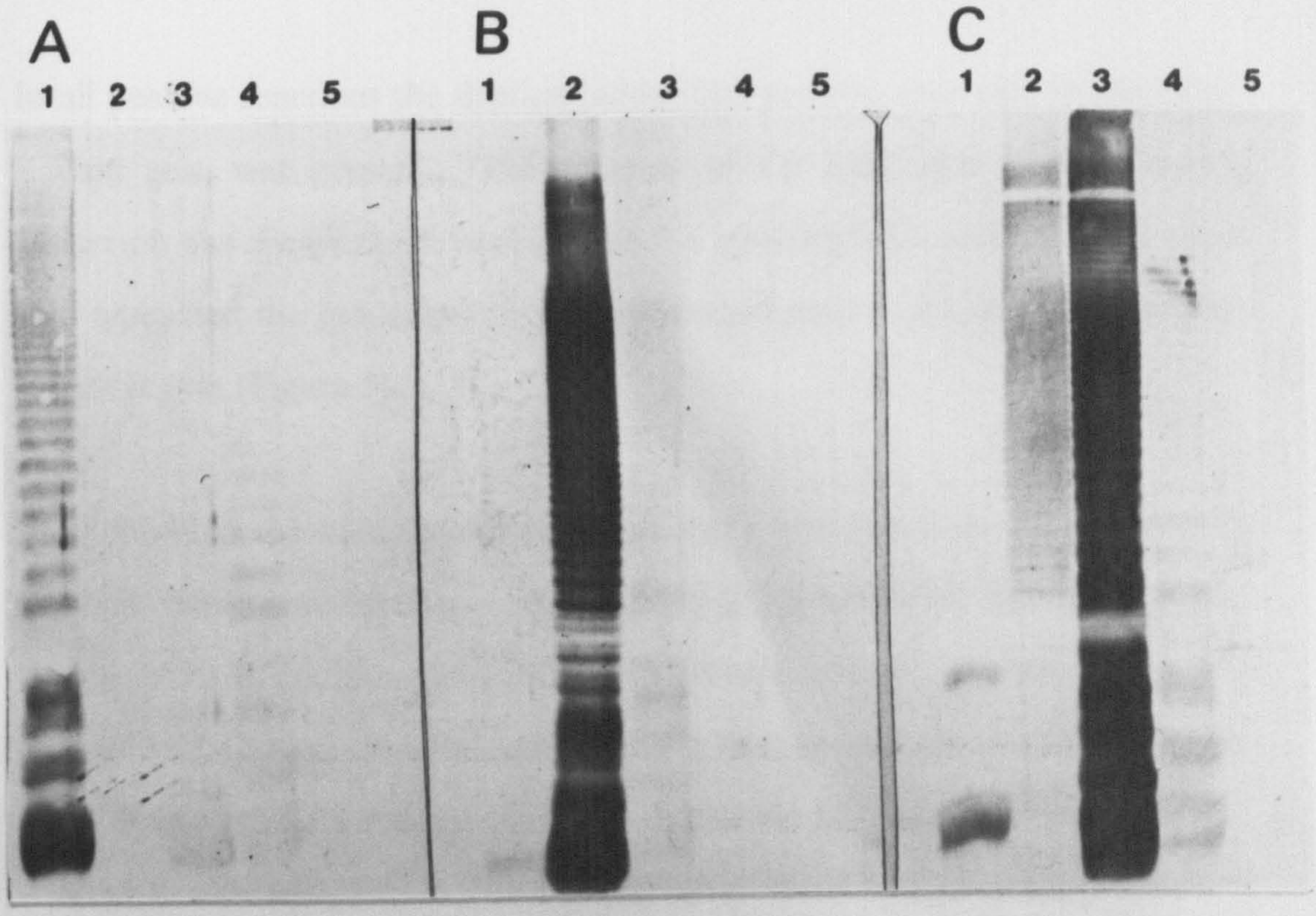
3.3.2 Western-blotting

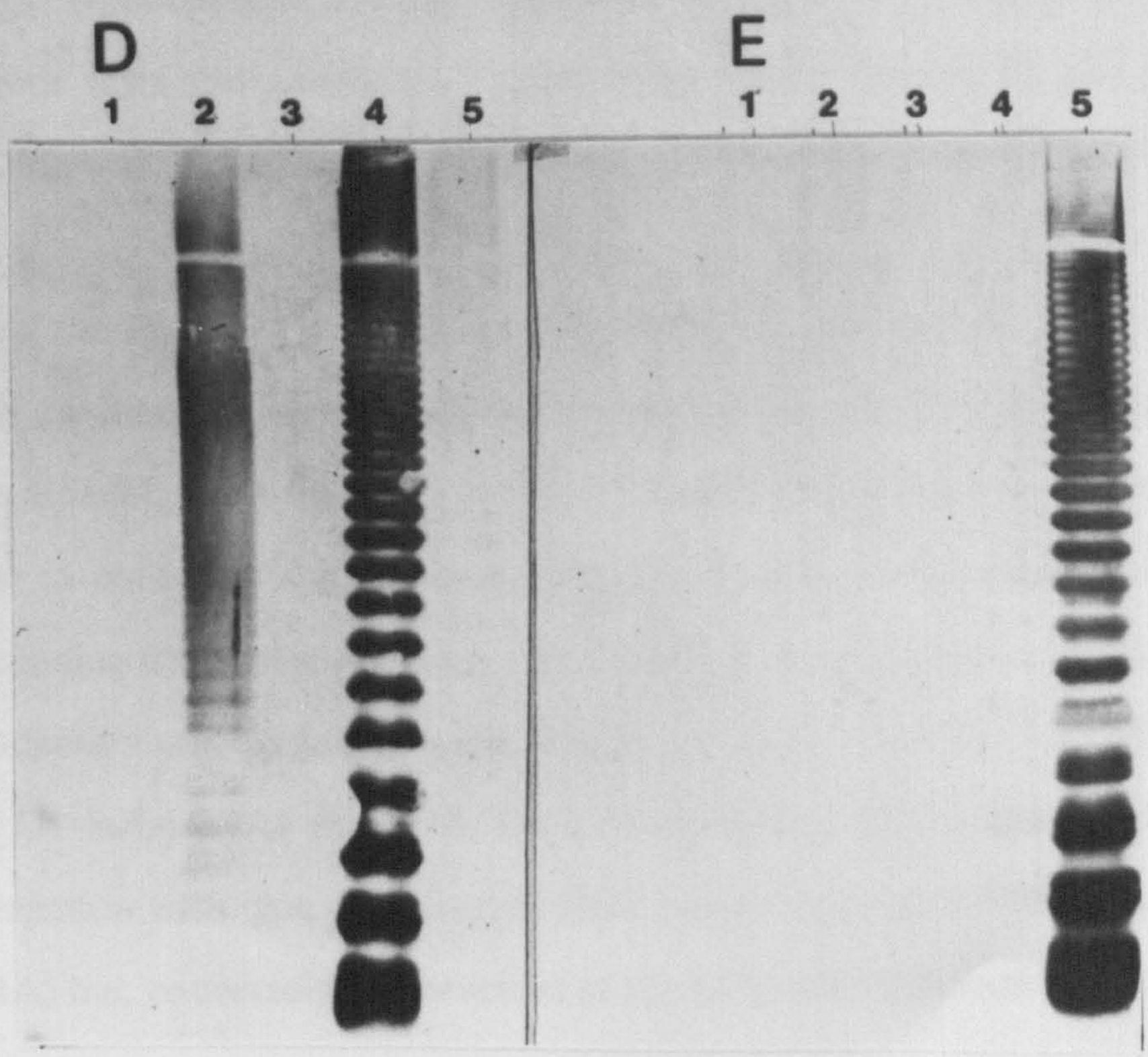
All LPS types were initially reacted with type 1 and type 2 antisera. Isolates not reacting with either of these antisera were reacted with type 5 antiserum and negatively-reacting isolates were reacted with type 6 and type 7 antisera. Representative isolates of each serotype were reacted with the homologous and heterologous antisera to test for cross-reactivity (Figure 3). A strong reaction was demonstrated between each LPS type and its homologous antiserum (as determined in the agglutination studies - Section 2) (Figure 3), with the exception of the LPS of isolate RD46 which did not react with any of the antisera, thus confirming that LPS was the determinant of the O-serotype. Lipopolysaccharide immunotypes were assigned to the isolates based on the reactions of LPS with the

Figure 3. Western-blots showing reactions of 01, 02, 05, 06 and 07 lipopolysaccharides with types 1, 2, 5, 6 and 7 rabbit antisera.

(A) - (E) represent reactions with types 1, 2, 5, 6 and 7 antisera, respectively.

Lanes 1 - 5 represent lipopolysaccharides of serotype 01 (isolate RD170), serotype 02 (isolate RD168), serotype 05 (isolate RD28), serotype 06 (isolate RD194) and serotype 07 (isolate RD150), respectively.


A
1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5


D
1 2 3 4 5
E
1 2 3 4 5

homologous antiserum. These results are summarized in Table 9.

In all positive reactions the distinct ladder-like pattern, seen also in the silver-stained gels, was present. The reaction of O1 LPS with its homologous antiserum was always much weaker than the homologous reactions of the other LPS types and the ladder-pattern was often incomplete in the low-molecular-weight region (Figure 3).

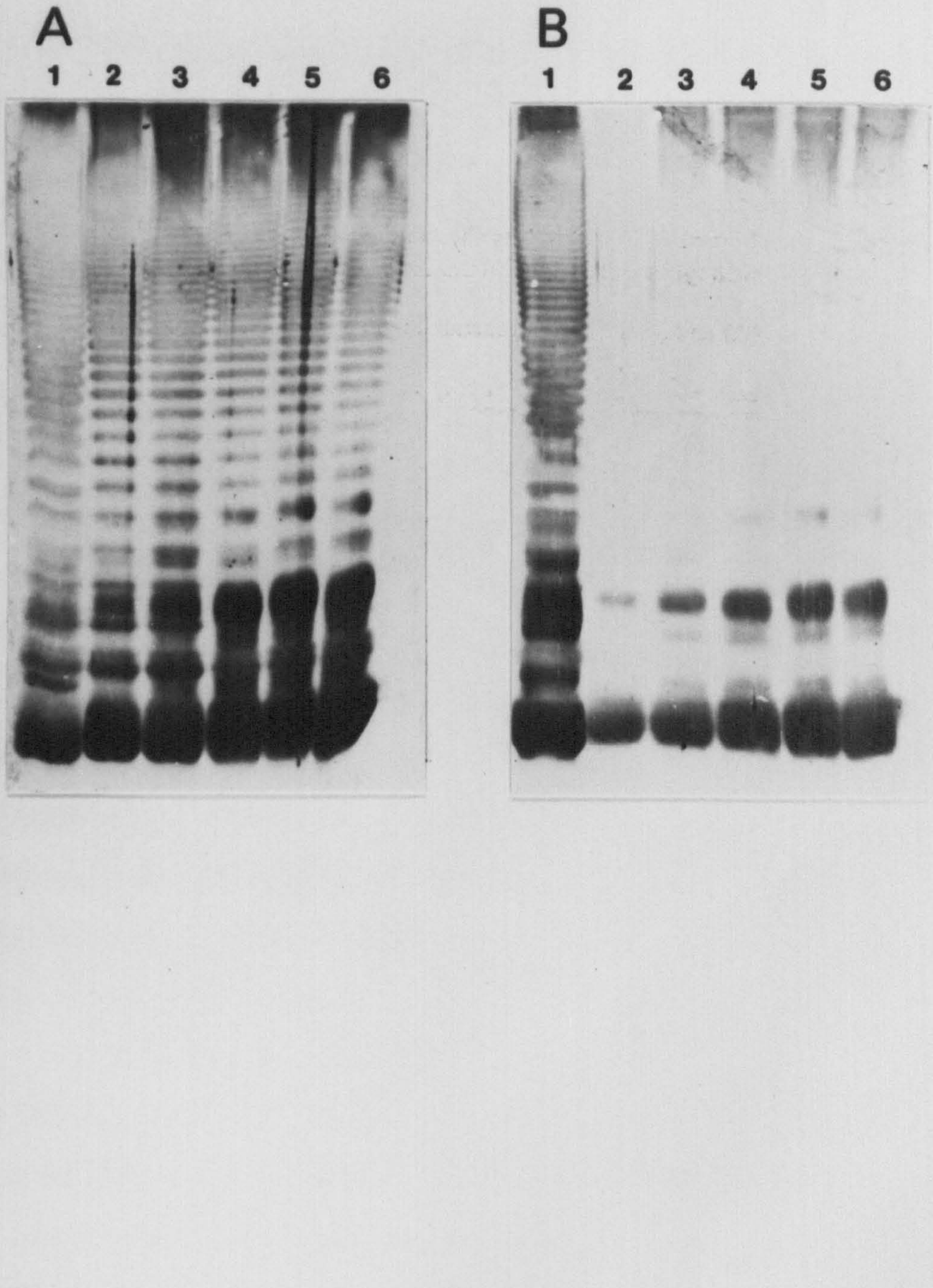
In addition to the homologous reactions there was also some cross-reactivity present. Most noticeable was the reaction of O2 LPS with type 6 antiserum (Figure 3D). In addition, O5 and O6 LPS reacted weakly with type 1 antiserum (Figure 3A), O1 and O5 LPS reacted weakly with type 2 antiserum (Figure 3B), O1, O2 and O6 LPS reacted with type 5 antiserum (Figure 3C) and O7 LPS reacted weakly with type 6 antiserum (Figure 3D). Cross-reactivity between O7 LPS and type 6 antiserum was removed by cross-absorbing the antiserum with a serotype O7 isolate, RD150 (results not shown). It should also be noted that type 6 antiserum cross-agglutinated serotype O7 isolates in slide agglutination tests and microplate agglutination assays (Section 2); this cross-agglutination was also eliminated when cross-absorbed antiserum was used.

In view of the fact that serotype O1 isolates cross-agglutinated with type 5 antiserum (Section 2), the LPS of selected serotype O1 isolates, including that of isolate RD154, were reacted with type 5 antiserum, as well as with type 1 antiserum as control. Isolate RD154 was specifically chosen because in the initial screening it was observed that the reaction of the LPS of this isolate with type 1 antiserum was noticeably weaker than the reactions of the LPS of other serotype O1 isolates. As expected, the LPS of isolate RD154 gave a slightly weaker reaction with type 1 antiserum than did the LPS of the other isolates (Figure 4A) but, conversely, the reaction of the LPS of RD154 with type 5

Figure 4. Western-blots showing reactions of 01 lipopolysaccharides with types 1 and 5 rabbit antisera.

(A) and (B) represent reactions with types 1 and 5 antisera, respectively.

Lanes 1 - 6 represent lipopolysaccharides of isolates RD154, RD178, RD184, RD190, RD280 and RD282, respectively.


A
1 2 3 4 5 6
B
1 2 3 4 5 6

antiserum was much stronger than the reactions of the LPS of the other isolates which reacted only weakly with type 5 antiserum (Figure 4B). In addition, it should be noted that the LPS profile of isolate RD154 in silver-stained gels was slightly different from the LPS profiles of other serotype O1 isolates. These results indicated, therefore, that the LPS of isolate RD154 was in some way different from the LPS of other serotype O1 isolates and that this LPS could constitute a subtype of O1 LPS. Based on these results, the LPS of isolate RD154 was designated immunotype 1B whereas the LPS of all other serotype O1 isolates was designated immunotype 1A (Table 9).

3.4 DISCUSSION

The analysis of LPS by SDS-PAGE showed that Y. ruckeri possesses an LPS structure similar to other members of the Enterobacteriaceae. That is, the LPS molecule has a lipid A-oligosaccharide core subunit and an O-antigenic sidechain composed of repeating units which gives rise to the ladder-like pattern observed in silver-stained gels and Western-blots (Goldman and Leive, 1980; Palva and Makela, 1980). The O-polysaccharide chains of Y. ruckeri consisted of at least 30 repeat units although it was not possible to count these accurately and compare the five LPS types. This figure compares with more than 40 repeat units described in E.coli (Goldman and Leive, 1980) and more than 30 in S.typhimurium (Munford et al., 1980). However, molecules with long O-antigenic side chains constitute a minority of outer membrane LPS in S.typhimurium (Munford et al., 1980) and the same may or may not be the case for Y. ruckeri.

Whereas the profiles of O2 and O5 LPS types were easily distinguishable and could be used to predict the O-serotype, the profiles of O1, O6 and O7 LPS types were more alike. Nevertheless, serotype O7 isolates could be differentiated from serotype O1 and O6 isolates due to the presence of a

doublet band in the O7 LPS profiles. These observations therefore confirmed those of Pyle and Schill (1985) who suggested that the LPS morphology of sorbitol-fermenting isolates could be used to predict O-serologic specificity. The O1 LPS profiles were more heterogeneous than the other LPS types but some of these profiles were very similar to the O6 LPS profiles. Thus, it would be difficult to predict the O-serotype of serotype O1 and O6 isolates based on their LPS profiles. Pyle and Schill (1985) did not examine serotype O1 isolates and would not have made this observation. Thus, analysis of LPS profiles by SDS-PAGE can be used to predict the O-serotype of O2, O5 and O7 isolates, but it is more difficult to differentiate between O1 and O6 isolates using this technique.

With the exception of serotype O6 and O7 isolates, LPS profiles did not show any variation that could be correlated with geographic origin or with OMP profiles (Section 4). In particular, the LPS of isolates belonging to the six serotype O1 clonal groups (Section 5) could not be differentiated. Lipopolysaccharide analysis therefore has limited epizootiological potential for Y. ruckeri (other than being able to differentiate between serotypes) although it may be useful for demonstrating relatedness of isolates of serotypes O6 and O7. Although the stability of LPS profiles was not examined, the fact that the profiles of isolates RD36 and RD60, and RD52 and RD166, (these pairs of isolates were actually the same isolate obtained from different sources - Appendix 1), were identical suggested that the LPS profiles of these isolates were stable after in vitro passage. By comparison, the LPS profiles of H.influenzae have been shown to be unstable after in vitro and in vivo passage (Kimura and Hansen, 1986; Tolan et al., 1986).

The presence of doublet bands was observed in the LPS profiles of a number of isolates of serotypes O1, O5, O6 and O7 as well as in some serotype O2 isolates (although they were not clearly visible in the latter case). Such doublet bands

have been described in E.coli, S.typhimurium, Salmonella minnesota, Serratia marcescens and Yersinia enterocolitica (Palva and Makela, 1980; Goldman and Leive, 1980; Hitchcock and Brown, 1983; Hitchcock et al., 1986). The precise reason for the doublet banding is unknown although it has been postulated that it might be due to variation in phosphorylation (Hitchcock et al., 1986). Goldman and Leive (1980), however, suggested that the upper band is produced from the lower band after storage at -20°C or -80°C. The presence of some doublet bands (particularly those in the low-molecular-weight regions) may be related to the amount of LPS loaded on the gels. At high LPS densities doublet bands may be hidden by excessive staining. These observations require further analysis.

In view of the protective-role of LPS against such factors as bile-salts it was interesting to note that the LPS profile of isolate RD178 (Figure 2C), which was the only isolate unable to grow on MacConkey agar (Section 1 and Table 1), did not differ from the profiles of other serotype O1 isolates.

Western-blot analysis demonstrated that LPS was the sero-specific antigen responsible for the O-agglutination reactions and that variation in LPS formed the basis of the O-serotyping scheme. This confirmed the observations of Pyle and Schill (1985) that LPS is responsible for O -serologic specificity. Unlike the work of Pyle and Schill (1985), however, cross-reactivity was demonstrated between LPS and heterologous antisera. This cross-reactivity was able to account for the cross-agglutinations observed in the O-agglutination reactions (Section 2). Some cross-reactions identified by Western-blotting were not, however, detected in agglutination reactions (Section 2). In particular, the reaction of O2 LPS with type 6 antiserum was not detected in agglutination reactions. Similar findings were described by Gaston et al. (1988) for S.marcescens. These authors suggested that LPS antigens detected by Western-blotting were not available on the surface of boiled cells to mediate

agglutination reactions. When these workers used autoclaved antigens rather than boiled antigens the immunoblotting results were corroborated in agglutination reactions. Unfortunately autoclaved antigens were not tested in this study.

Western-blotting demonstrated that at least two subtypes of O1 LPS possibly occur. All but one of the serotype O1 isolates appeared to have the same O1 subtype but one isolate, RD154, appeared to have a slightly different LPS type. This finding supported the observation that the LPS profile of RD154 in silver-stained gels was slightly different from other O1 LPS profiles. Other differences in the profiles of O1 LPS, as demonstrated in silver-stained gels, were not confirmed by Western-blotting. The existence of a unique O1 LPS subtype would need to be confirmed by raising antisera against isolate RD154 and by carrying out further cross-absorption experiments.

Hitchcock and Brown (1983) demonstrated that differences in LPS profiles in polyacrylamide gels are related to biochemical differences in LPS. Differences in the antigenic structure of LPS may be due to differences in the composition or structure of the LPS molecule, particularly of the O-antigenic side chains. For example, different serotypes of H. influenzae were shown to have quantitative differences in the chemical composition of their LPS which correlated with the antigenic specificity of their homologous antisera and with their mobility in polyacrylamide gels (Zamze and Moxon, 1987). Chemical analysis of the different LPS types of Y. ruckeri could be very useful in attempting to explain the differences described above and could also account for differences observed in the susceptibility to the bactericidal effect of rainbow trout serum and in the virulence of isolates of different LPS types (Sections 8 and 9). Such analyses would have particular relevance for the differentiation of different LPS subtypes among serotype O1 isolates.

Section 4

Analysis of the Outer Membrane Protein Profiles

of Yersinia ruckeri

Analysis of the Outer Membrane Protein Profiles

of Yersinia ruckeri

4.1 INTRODUCTION

The OM of Gram-negative bacteria is composed of protein, LPS and phospholipid. Characteristically the number of protein species in the OM is relatively small and they are usually classified into major and minor proteins according to their relative abundance in SDS-PAGE gels. Growth conditions, however, greatly influence the relative abundance of certain proteins (Lugtenberg et al., 1976; Brown and Williams, 1985) and this nomenclature can be mis-leading. The classification, structure and function of Gram-negative OMPs is discussed in detail in reviews by Nikaido and Nakae (1979), Osborn and Wu (1980) and Lugtenburg and Van Alphen (1983).

For the purpose of the work described in this Section reference is made to two classes of OMPs, namely, porin proteins and heat-modifiable proteins (HMPs). Porin proteins form pores through the OM which allow for the diffusion of small, hydrophilic solutes through the otherwise impermeable membrane (Nikaido and Nakae, 1979; Hancock, 1987). In E.coli K12 two major porins exist, namely, the Omp C and Omp F proteins, which have molecular weights of 36 and 37 KDa respectively. An important and characteristic feature of the porin proteins is that they remain tightly associated with peptidoglycan in 2% sodium dodecyl sulphate at 60^{o}C (and are hence referred to as peptidoglycan-associated proteins [PAPs]) but are dissociated upon boiling (Rosenbusch, 1974). In the peptidoglycan-associated form porin proteins will not pass into SDS-PAGE gels. Escherichia coli K12 possesses a HMP known as the Omp A protein. This protein migrates in SDS-PAGE gels with an apparent molecular

weight of about 28 KDa when heated below 56°C but when heated at 100°C the protein migrates with an apparent molecular weight of about 35 KDa (Beher et al., 1980). Unlike the porin proteins the Omp A protein is not tightly associated with peptidoglycan and migrates into the gel without prior heat-treatment. Other E.coli strains have been shown to possess proteins related to the Omp A, Omp C and Omp F proteins of E.coli K12 (Overbeeke and Lugtenberg, 1980a). Heat-modifiable proteins have also been described in other species of Enterobacteriaceae as well as in non-Enterobacteriaceae species (Beher et al., 1980). Similarly, PAPs (or porin proteins) have also been described in other bacterial species (Lugtenberg et al., 1977; Hancock, 1987).

Outer membrane protein analysis has been used to study strain variation and relatedness in numerous bacterial species, including many pathogenic bacteria. It is a powerful epidemiological tool and can also be useful in the identification of virulence determinants. Outer membrane protein analysis has led to the development of typing schemes based on OMP patterns in SDS-PAGE gels in a number of bacterial species including C. jejuni (Blaser et al., 1983), Haemophilus ducreyi (Odumeru et al., 1983), H.influenzae (Barenkamp et al., 1981; Murphy et al., 1983), Haemophilus pleuropnuemoniae (Rapp et al., 1986) and Neisseria meningitidis (Mocca and Frasch, 1982). The technique has also been used for comparing other fish pathogenic bacteria from different geographic origins, including V. anguillarum (Buckley et al., 1981) and A. hydrophila, A. salmonicida and Edwardsiella tarda (Aoki and Holland, 1985).

An analysis of the OMP profiles of Y. ruckeri isolates was carried out with the following objectives in mind: (1) to use the technique to investigate the relatedness of isolates from widely different geographic locations and to determine whether OMP analysis could be used as an epizootiological tool; and, (2) to identify those OMP types associated with virulent, disease-causing isolates and those OMP types associated with avirulent, non-disease-causing

isolates, and to identify differences in the OMP profiles of virulent and avirulent isolates which could perhaps account for the differences in virulence.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial isolates and growth conditions

The OMP profiles of 137 isolates of Y. ruckeri were examined including 132 field isolates, four reference strains and a vaccine production strain. A complete list of the isolates examined is given in Table 11 and further details of the isolates are given in Appendix 1.

The conditions of growth for the preparation of OMPs were identical to the conditions for the preparation of LPS and have been described in Section 3. In fact, the same OM preparations were used for both LPS and OMP analysis.

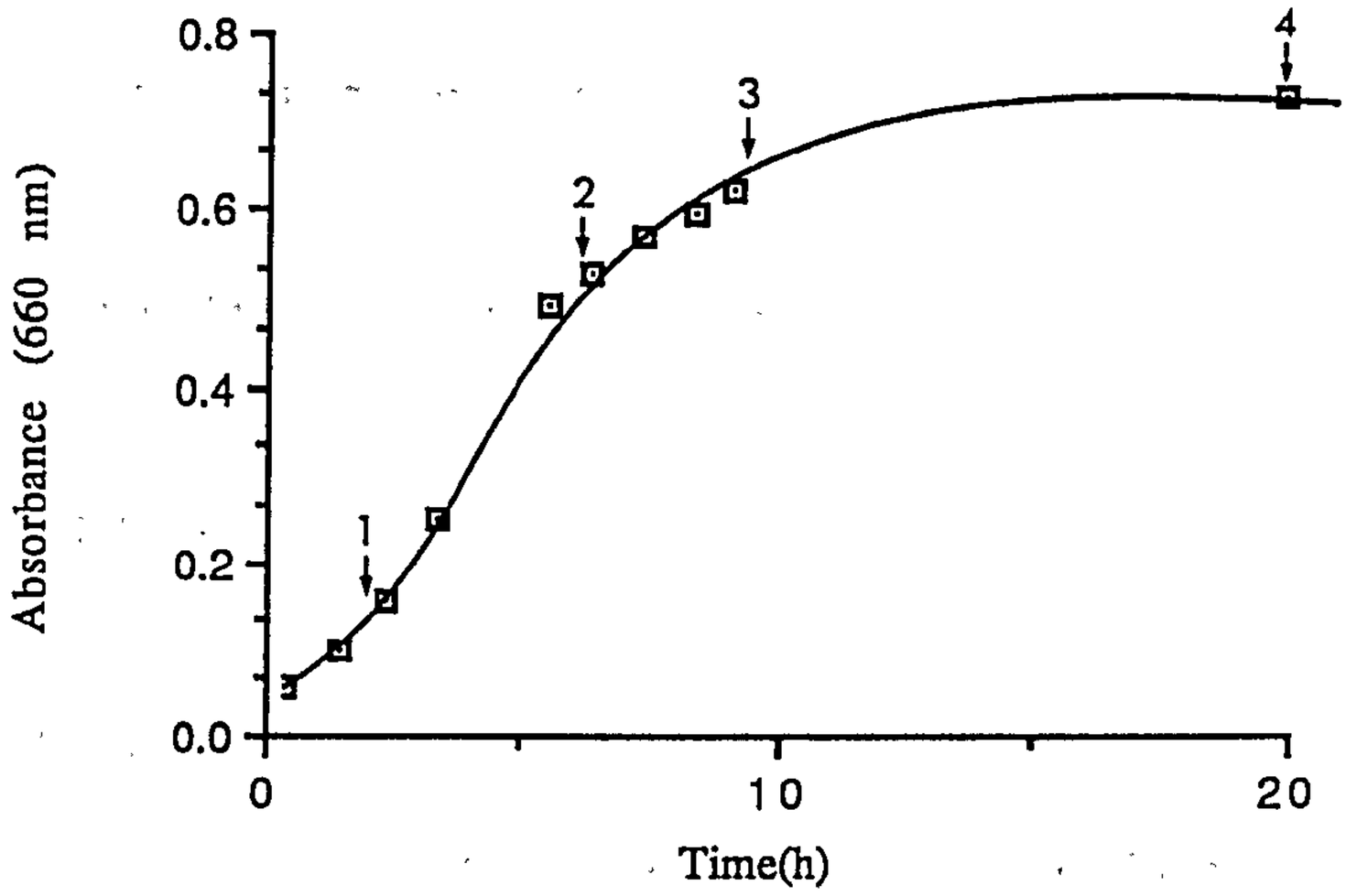
4.2.2 Evaluation of the stability of outer membrane protein profiles

To evaluate the effect of in vitro passage isolates RD6 and RD124 were each subcultured 20 times on TSA (at 22^{o}C for two or three days) and OM fractions were prepared after 5, 10, 15 and 20 subcultures. In each case, 200 ml TSB were inoculated as described in Section 3 and incubated overnight at 22^{o}C.

To evaluate the effect of colony selection on OMP profiles, eight single colonies of isolate RD124 were each used to inoculate 200 ml TSB as described in Section 3; the cultures were incubated overnight at 22^{o}C.

To evaluate the effect of phase of the growth cycle on OMP profiles, OM fractions of isolates RD6 and RD124 were prepared from TSB cultures harvested at various points of the growth cycle (Figure 5). In this case, 800 ml

(a)



(b)

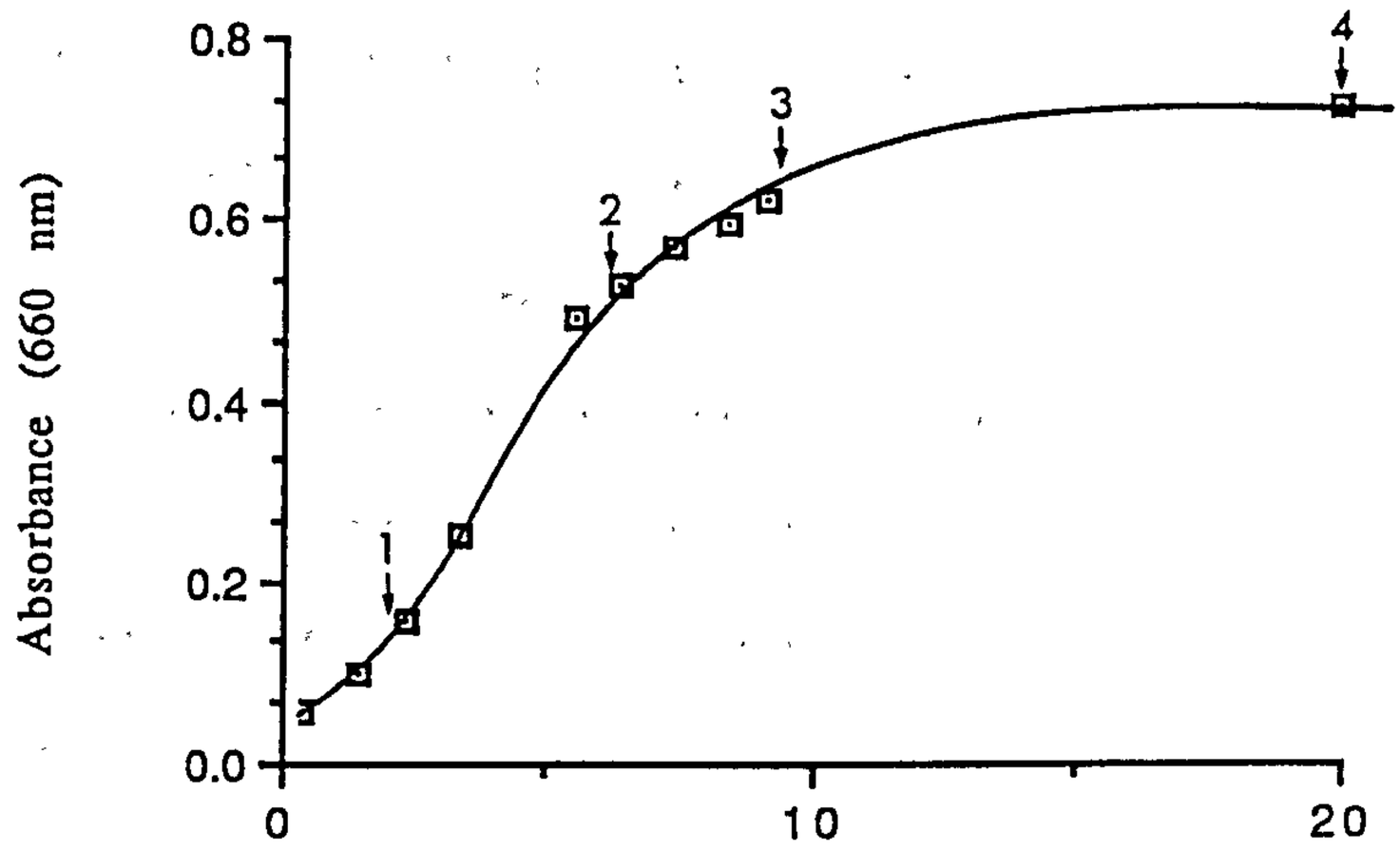


Figure 5. Growth curves of isolates RD6 (a) and RD124 (b) showing points of the growth cycle at which cells were harvested for isolation of outer membrane proteins.

TSB were inoculated as described above and incubated at 22°C, and 200 ml were removed after 2, 6, 9 and 20 h. Growth was monitored spectrophotometrically at 660 nm.

4.2.3 Isolation of outer membranes

The isolation of OMs is described in Section 3.

4.2.4 SDS-PAGE

SDS-PAGE of OMPs was carried out as described in Section 3. Proteins were stacked in 4% acrylamide stacking gels and, for the majority of the work, were separated in 12% acrylamide resolving gels; for peptide mapping 15% resolving gels were used. To improve the resolution of certain major OMPs, proteins of selected isolates were also separated in 8-20% linear gradient resolving gels (Hames, 1981a). The composition of the mixtures for all gels are given in Appendix 2. Aliquots of the original OM preparations were diluted 1:5 in sample buffer (50 µl OM sample were added to 200 µl sample buffer). This dilution was found to give optimal resolution of the protein bands when 10-30 µl were loaded. After heat-treatment of the samples, as described below, 10 or 30 µl of each sample were applied to each lane of the gel. Standard molecular weight markers (Appendix 2) were applied (approximately 25 µg per lane) where appropriate and 10 or 30 µl sample buffer were applied to empty wells. The buffer system used and the conditions of electrophoresis were as described in Section 3. After completion of electrophoresis proteins were visualized by staining overnight with 0.2% Coomassie blue solution (Appendix 2) and by destaining with "destaining solution" (Appendix 2). The apparent molecular weights of the OMPs were estimated from a calibration curve prepared with the molecular weight standards. The gels were photographed and dried with a slab gel dryer.

4.2.5 Evaluation of the effect of solubilization temperature on the migration of outer membrane proteins

To evaluate the effect of solubilization temperature on the migration of the HMPs and PAPs in particular, and on the other OMPs in general, samples of OM preparations of selected isolates (RD6, RD20, RD28, RD30, RD34, RD38, RD42, RD44, RD52, RD54, RD168, RD170, RD176, RD178, RD200 and RD210) were heated at 37, 50, 70, 80, 90 and 100^{o}C for 5 min and examined by SDS-PAGE. These isolates were selected because they represented a variety of OMP profiles after heat-treatment at 100^{o}C for 5 min.

4.2.6 Demonstration that heat-modification of the 31 KDa HMP results in a change of molecular weight to 38 or 36.5 KDa and that other major proteins in the molecular weight range 36 to 40.5 KDa are peptidoglycan-associated

Samples (1:5 dilutions) of OM preparations of isolates RD6, RD20, RD28, RD34, RD38, RD42, RD52, RD54, RD168 and RD170 were heated at 37^{o}C for 5 min and 40 μl of each sample were applied to each of two wells of a 12% slab gel. Electrophoresis was carried out as described above and the gels were stained with Coomassie blue for 5 min and destained for 30 min. The gels were rinsed with distilled water, placed on a light box, and for each isolate, the 31 KDa protein bands (HMPs) and the PAPs (which had been unable to pass into the stacking gel and were located at the bottom of the wells) were excised from the gels. The gel slices were equilibrated with 125 mM Tris-hydrochloride (pH 6.8), 0.1% SDS for 30 min. The proteins were eluted from the gel slices by macerating the combined gel slices (i.e. two slices for each protein for each isolate) in 600 μl sample buffer in Eppendorf tubes and incubating at 37^{o}C for 2 h. The PAP samples were heated at 100^{o}C for 5 min before incubation at 37^{o}C. The macerated gel was removed by centrifugation at 11,600 x g for 5 min

in a microcentrifuge, and the supernatants were transferred to clean Eppendorf tubes. The HMP samples were divided into two equal aliquots, one of which was heated at 100°C for 5 min. One hundred microlitres of each of the samples (i.e. unheated HMP, heated HMP and heated PAPs) for each of the isolates were applied to the wells of two 12% slab gels and electrophoresis was carried out as described above. The gels were stained overnight and destained as described above.

4.2.7 Peptide mapping

Peptide mapping of the 38 and 36.5 KDa HMPs and of the 38 and 36.5 KDa PAPs was carried out essentially by the method of Cleveland et al. (1977) as described by Hames (1980b). Fifteen microlitres of undiluted OM samples of isolates RD26 and RD74, previously heated at 100°C for 5 min, were applied to each of 4 lanes of a 12% slab gel. Electrophoresis was carried out as described above. The gel was stained with Coomassie blue for 5 min, destained for 30 min and rinsed with distilled water. For each isolate the HMP (36.5 KDa for RD26; 38 KDa for RD74) and variable PAP (38 KDa for RD26; 36.5 KDa for RD74) were excised from the gel and the gel slices were equilibrated with 125 mM Tris-hydrochloride (pH 6.8), 0.1% SDS for 30 min.

A second slab gel was prepared with a 15% acrylamide resolving gel (Appendix 2) and an exceptionally tall (about 4.5 cm high) 4% stacking gel. The sample wells were filled with 125 mM Tris-hydrochloride (pH 6.8), 0.1% SDS and the gel slices, trimmed so that they fitted easily into the sample wells, were positioned to lie horizontally at the bottom of each well. The HMP and the PAP slices were set up in different gels. The gel slices were overlayered with 10μl of 125 mM Tris-hydrochloride (pH 6.8), 0.1% SDS, containing 20% glycerol. Finally, 25 μl of 125 mM Tris-hydrochloride (pH 6.8), 0.1% SDS containing 10% glycerol, 0.001% bromophenol blue and 2.5 μg protease (100

μg/ml) was overlayered into each well. The proteases used were chymotrypsin, papain and V8 protease (Sigma), and optimal concentrations were determined in a pilot experiment. Controls consisted of proteins alone and proteases alone.

Electrophoresis was begun at a constant current of 20 mA per gel until the tracking dye had traversed about two thirds of the stacking gel; the power was then turned off for 45 min. After proteolysis, electrophoresis was continued at 20 mA per gel until the dye front had passed into the resolving gel at which point the current was increased to 30 mA per gel. Electrophoresis was continued until the dye front reached the bottom of the resolving gel. The gels were stained overnight and destained as described above.

4.2.8 Analysis of the outer membrane protein profiles of Y. ruckeri isolates

As a result of the experiment to examine the effect of solubilization temperature on the migration of OMPs it was found necessary to examine the OMP profiles of all isolates after solubilization at both 80 and 100^{o}C. Only by examining the OMP profiles after solubilization at these two temperatures could the 38 and 36.5 KDa HMPs and the 38 and 36.5 KDa PAPs be distinguished (see Results). The OMP profiles of selected isolates were also examined in gradient gels in order to obtain better resolution of the proteins (and of the major proteins in particular).

4.3 RESULTS

4.3.1 Stability of outer membrane protein profiles

To assess the effect of culture passage on the OMP profiles of Y. ruckeri, isolates RD6 and RD124 were passaged 20 times on TSA. The OMP profiles

were examined after 5, 10, 15 and 20 passages and were shown to be identical, for both isolates, after each series of passages (Figure 6A).

To evaluate the effect of colony selection on OMP profile eight individual colonies of isolate RD124 were selected and cultured in TSB. The OMP profiles of the eight clones were shown to be identical indicating that there was no variation in OMP profile due to colony selection (Figure 6B).

To assess the effect of phase of growth on the OMP profiles OM fractions of isolates RD6 and RD124 were prepared at four different stages of the growth cycle (Figure 5). With the exception of the 39.5 KDa PAP (see below) the OMP profiles of both isolates were identical at all phases of the growth cycle. In both isolates, however, the 39.5 KDa PAP was expressed in greater abundance during the later stages of growth, i.e. during stationary phase. Thus, the protein was not expressed during log phase but was expressed slightly during early stationary phase. The protein was expressed even more as stationary phase progressed and maximum expression occurred when the cells were well into stationary phase (Figures 5 and 6C).

4.3.2 Effect of solubilization temperature on the outer membrane protein profiles

The effect of solubilization temperature on the OMP profiles of selected Y. ruckeri isolates was examined by heating the samples at 37, 50, 70, 80, 90 and 100^{o}C prior to electrophoresis. Two important observations were made. First, a protein of molecular weight 31 KDa was the only major protein present at temperatures of 37^{o}C and 50^{o}C. This protein was also present at 70^{o}C and 80^{o}C but decreased substantially in abundance at 90^{o}C and at 100^{o}C appeared like a minor protein. As the 31 KDa protein decreased in abundance at 90^{o}C and 100^{o}C, a protein of higher molecular weight appeared. This protein first

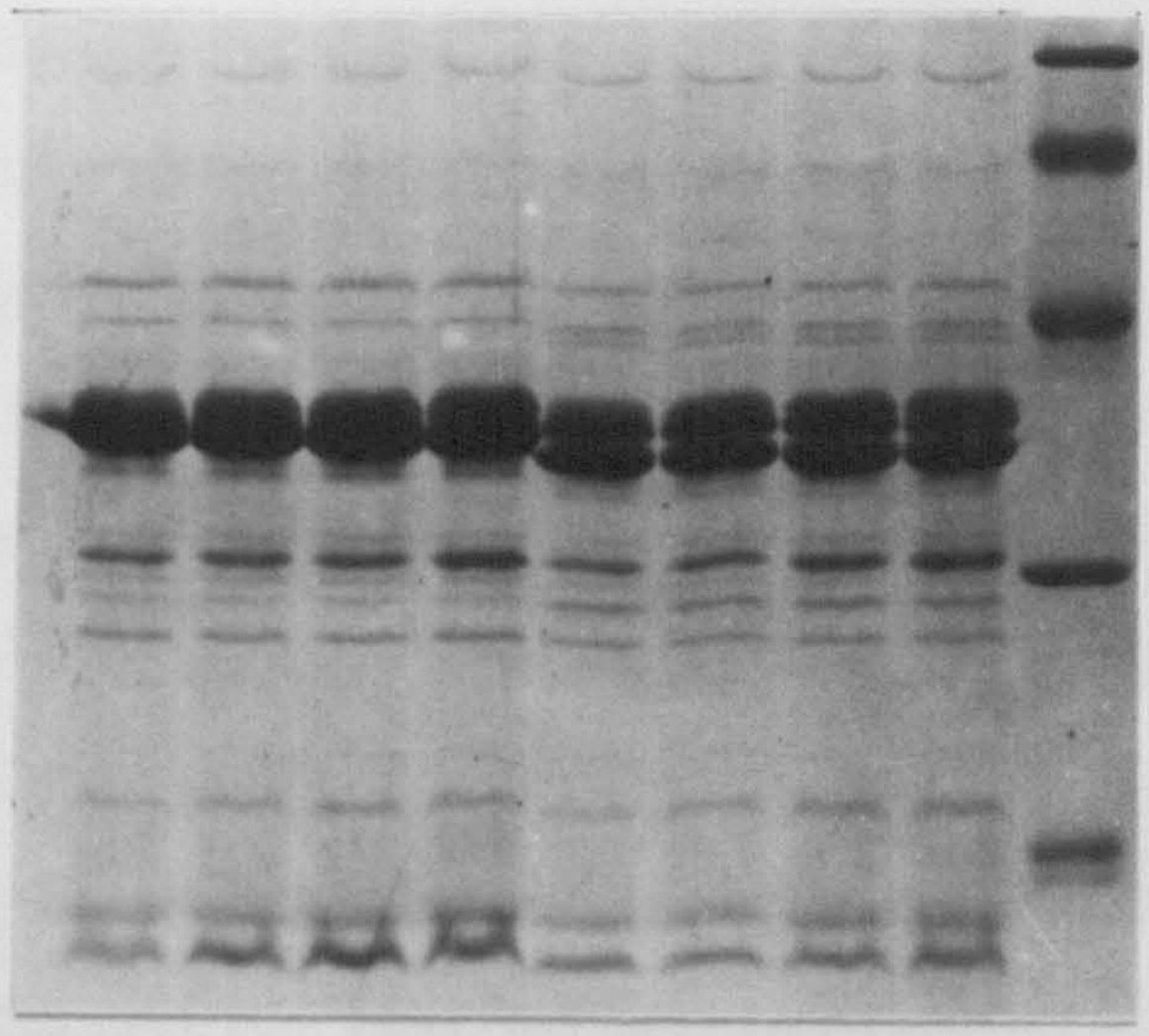
Figure 6. Stability of outer membrane protein profiles of *Y. ruckeri*.

(A). OMPs of isolates RD6 (lanes 1 - 4) and RD124 (lanes 5 - 8) after 5 (lanes 1 and 5), 10 (lanes 2 and 6), 15 (lanes 3 and 7) and 20 (lanes 4 and 8) subcultures on TSA. Molecular weight standards (KDa) are shown in lane 9.

(B). OMPs of isolate RD124 derived from eight individual single colonies (lanes 1 - 8). Molecular weight standards (KDa) are shown in lane 9.

(C). OMPs of isolates RD6 (lanes 1 - 4) and RD124 (lanes 5 - 8) obtained at different stages of the growth cycle (see Figure 5). Lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8 represent OMPs obtained from cells harvested at points 1, 2, 3 and 4, respectively, on the growth curves shown in Figure 5. Molecular weight standards (KDa) are shown in lane 9.

A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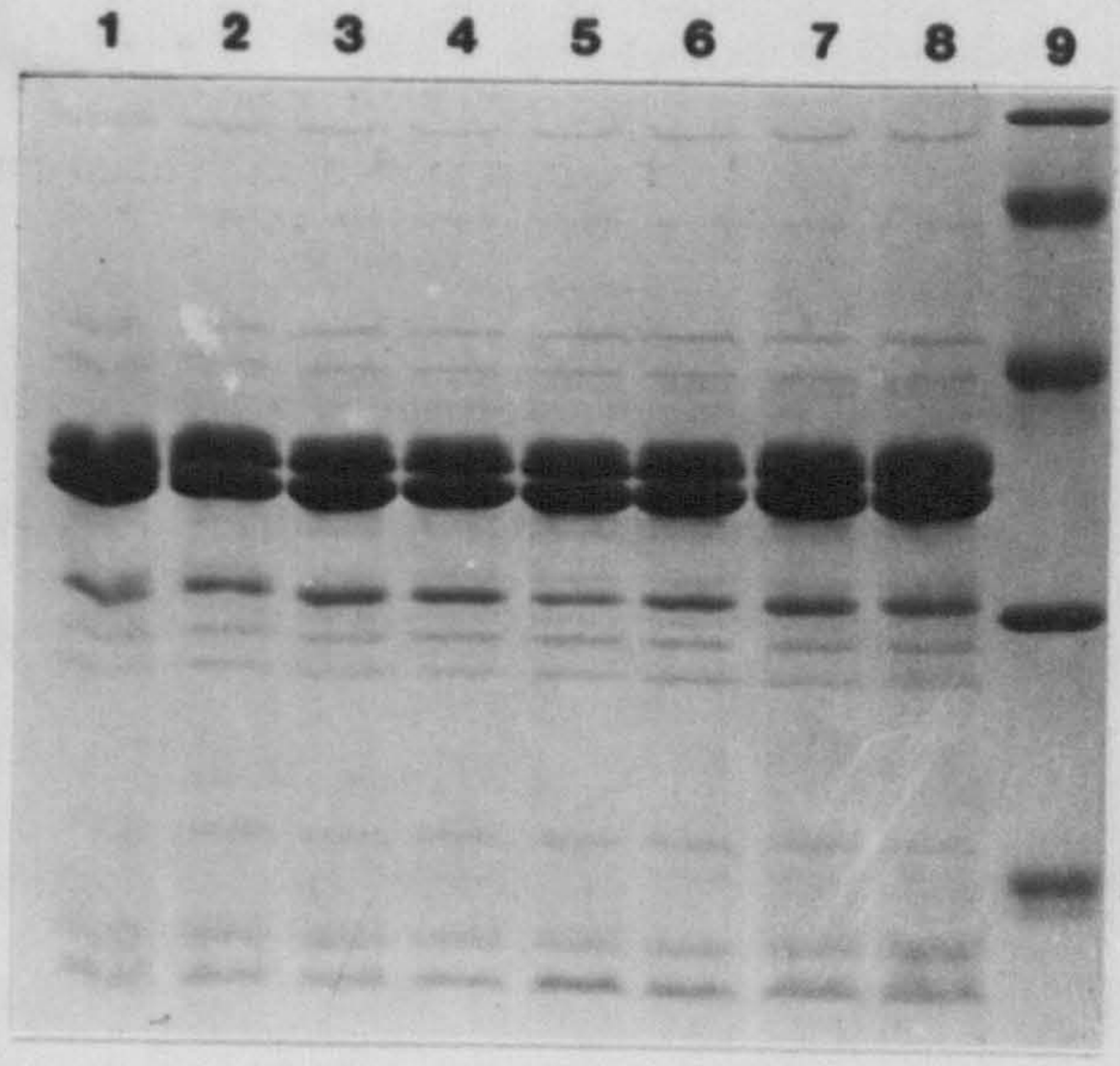
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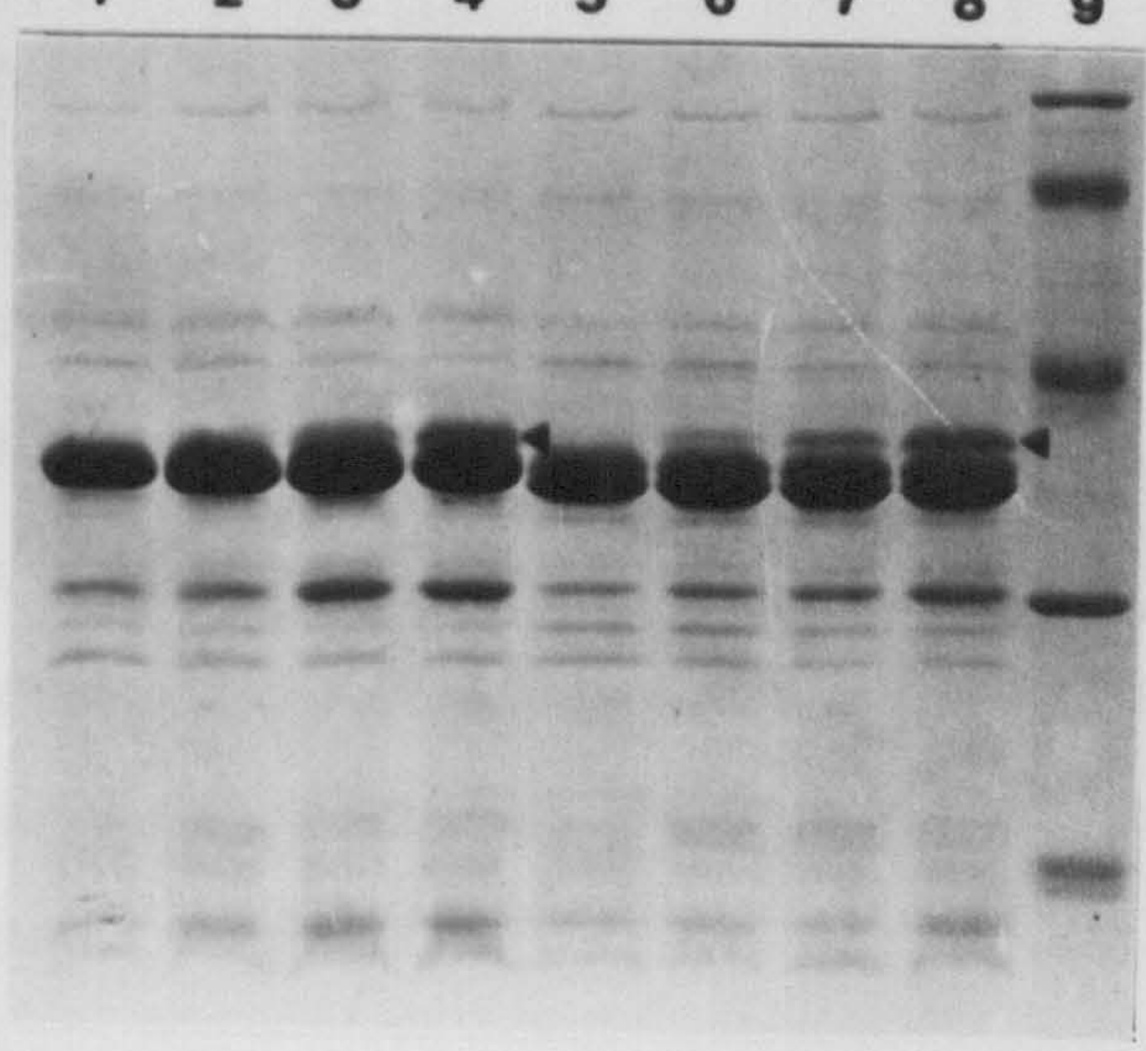
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appeared at 80°C but at 90°C and 100°C it was present in maximum abundance. The decrease in abundance of the 31 KDa protein at 90°C and 100°C and the simultaneous increase in the abundance of the higher molecular weight protein suggested that the two proteins were the same, but were present in two different heat-modifiable forms. This protein was probably the equivalent of the heat-modifiable Omp A protein of E.coli K12 which has also been described in other members of the Enterobacteriaceae (Beher et al., 1980). Although the molecular weight of the unheated form of the HMP was identical in all of the isolates examined, this was not the case for the heat-modified form of the protein. Thus, the heat-modified HMP had a molecular weight of 38 KDa in isolates RD6, RD30, RD34, RD42, RD44, RD54, RD170, RD176, RD178, RD200 and RD210, and had a molecular weight of 36.5 KDa in isolates RD20, RD28, RD38, RD52 and RD168 (Figures 7 and 8).

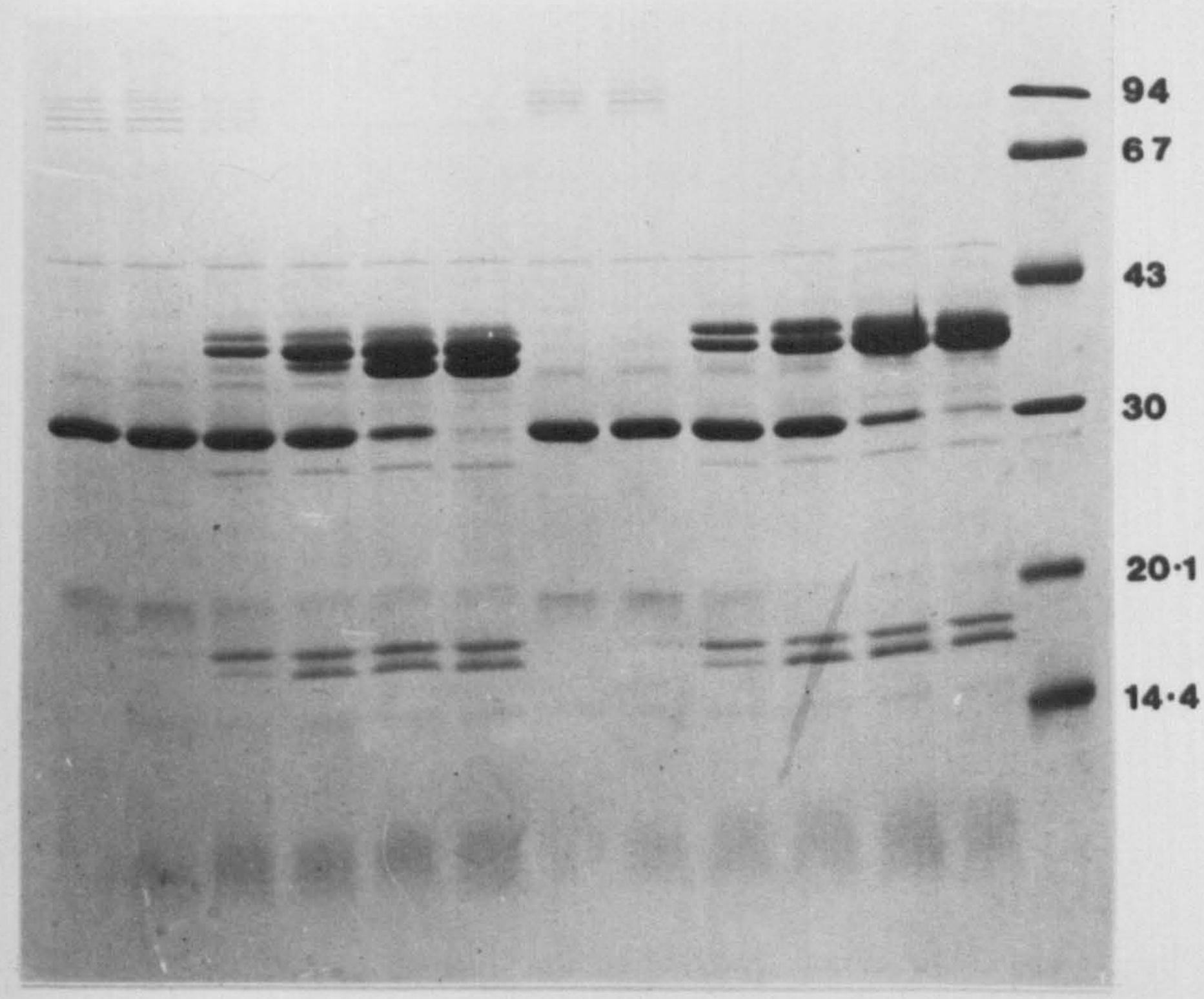
Second, at 70°C and above two or three additional major proteins, in the molecular weight range 36 to 40.5 KDa were also present. These proteins were probably the equivalent of the PAPs (porin proteins) described in E.coli and other members of the Enterobacteriaceae (Lugtenberg et al., 1977). At 37°C and 50°C the proteins remained tightly associated with peptidoglycan and were unable to pass into the gel; at 70°C and above, however, the proteins were released from the peptidoglycan and passed into the gel. All isolates examined possessed a PAP of molecular weight 39.5 KDa, although expression of this protein varied according to the growth phase (see above). There was, however, variation between isolates in the possession of other PAPs; isolates possessed either a PAP of molecular weight 38 KDa, e.g. isolates RD6, RD20, RD28, RD30, RD34, RD38, RD44, RD54, RD168 and RD176, or a PAP of molecular weight 36.5 KDa, e.g. isolates RD42, RD170, RD178, RD200 and RD210. Isolate RD52 was unusual in that it possessed a PAP of molecular weight 40.5 KDa in addition to the one of 39.5 KDa. In addition, some isolates possessed a third PAP of molecular weight 36 KDa, e.g. isolates RD6, RD54, RD168,

Figure 7. Effect of solubilisation temperature on the outer membrane protein profiles of *Y. ruckeri*.

(A). OMPs of isolates RD28 (lanes 1 - 6) and RD30 (lanes 7 - 12) after heat-treatment (5 min) at 37°C (lanes 1 and 7), 50°C (lanes 2 and 8), 70°C (lanes 3 and 9), 80°C (lanes 4 and 10), 90°C (lanes 5 and 11) and 100°C (lanes 6 and 12). Molecular weight standards (KDa) are shown in lane 13.

(B). OMPs of isolates RD168 (lanes 1 - 5) and RD170 (lanes 6 - 10) after heat-treatment (5 min) at 37°C (lanes 1 and 6), 50°C (lanes 2 and 7), 70°C (lanes 3 and 8), 100°C (lanes 4 and 9) and 100°C for 15 min (lanes 5 and 10). Molecular weight standards (KDa) are shown in lane 11.

A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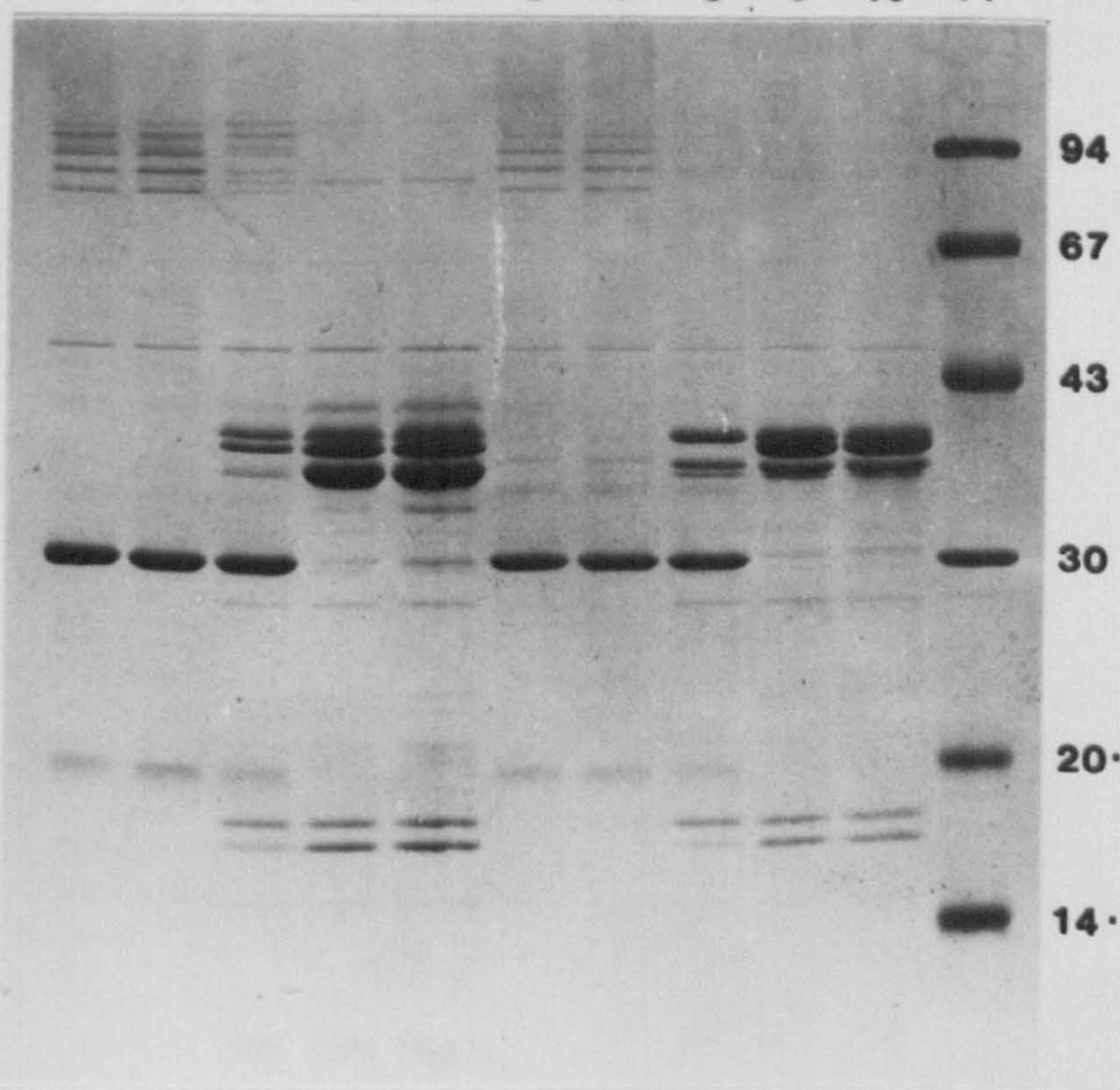
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Figure 8. Effect of solubilisation temperature on the outer membrane protein profiles of *Y. ruckeri*.

(A). Lanes 1 - 6 represent isolate RD6; lanes 7 - 12 represent isolate RD20.

(B). Lanes 1 - 6 represent isolate RD34; lanes 7 - 12 represent isolate RD38.

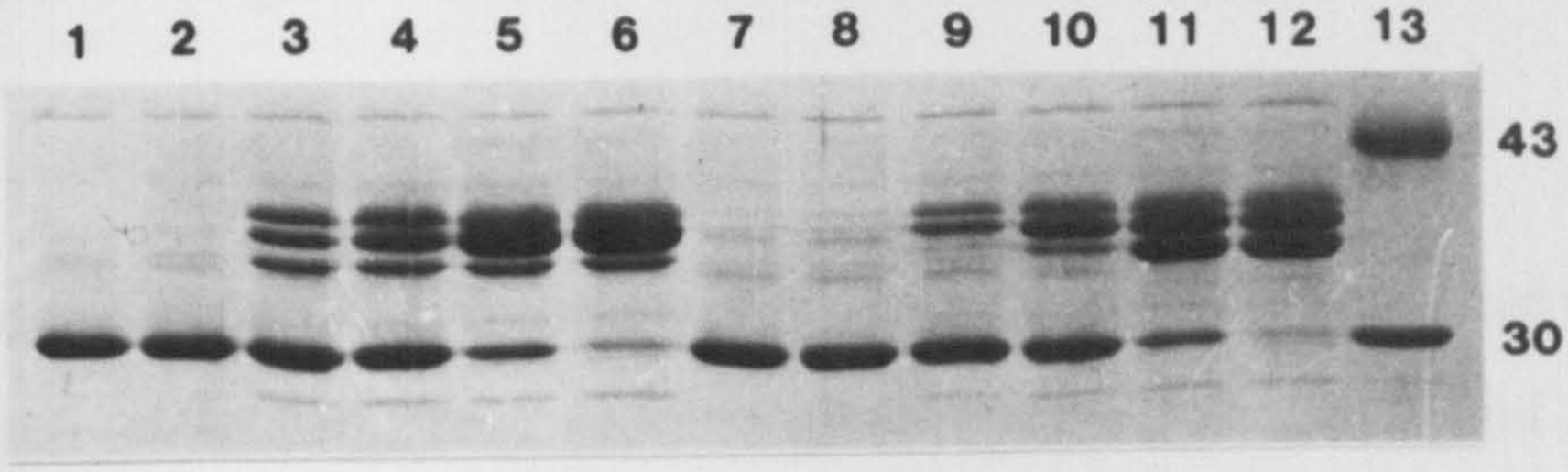
(C). Lanes 1 - 6 represent isolate RD200; lanes 7 - 12 represent isolate RD210.

(D). Lanes 1 - 6 represent isolate RD52; lanes 7 - 12 represent isolate RD54.

(E). Lanes 1 - 6 represent isolate RD176; Lanes 7 - 12 represent isolate RD178.

OMP profiles were examined after heat-treatment for 5 min at 37°C (lanes 1 and 7), 50°C (lanes 2 and 8), 70°C (lanes 3 and 9), 80°C (lanes 4 and 10), 90°C (lanes 5 and 11) and 100°C (lanes 6 and 12). Molecular weight standards (KDa) are shown in lane 13.

A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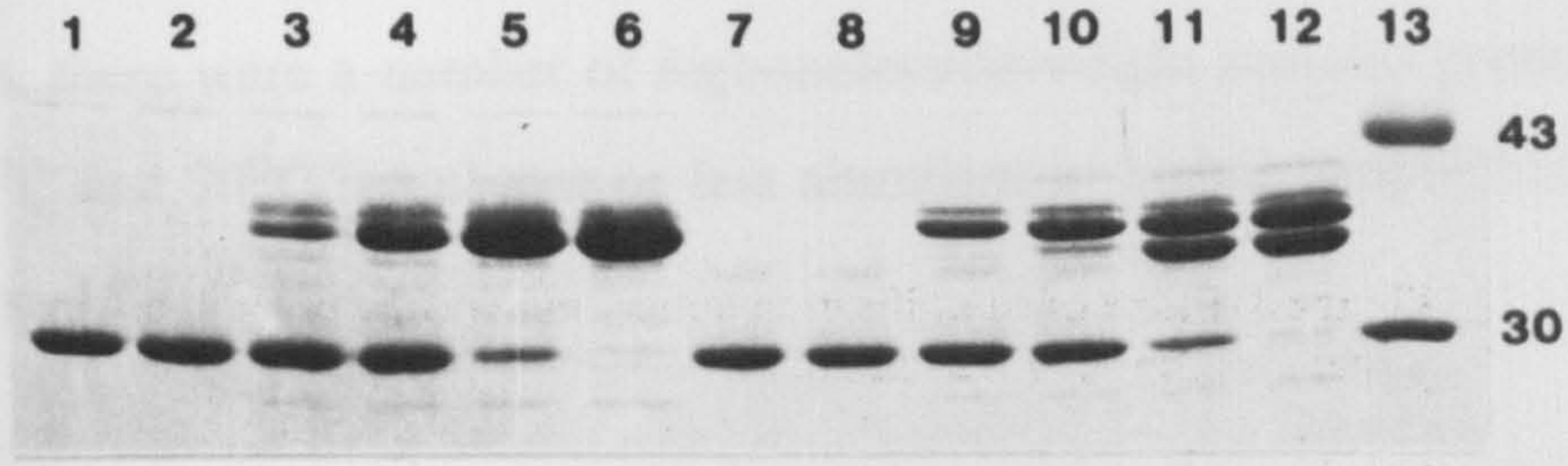
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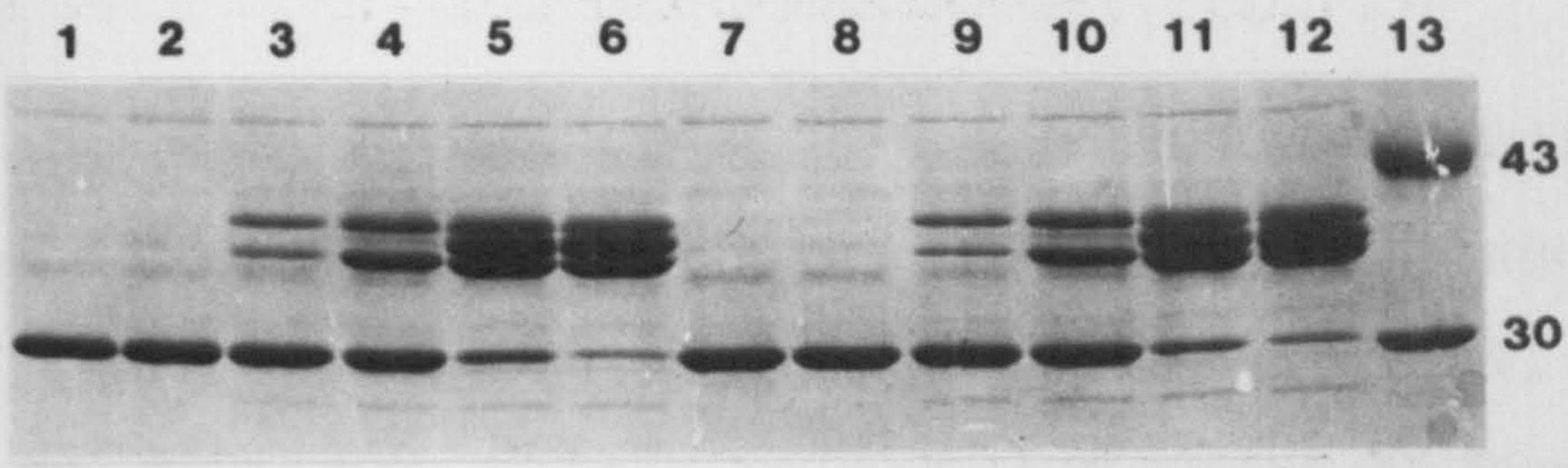
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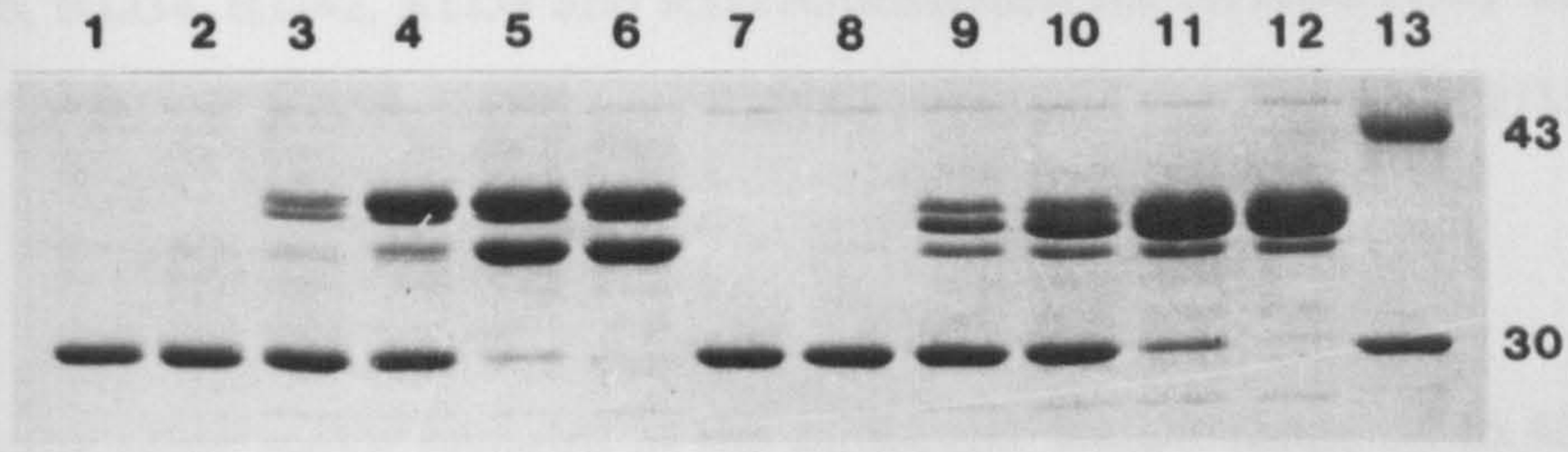
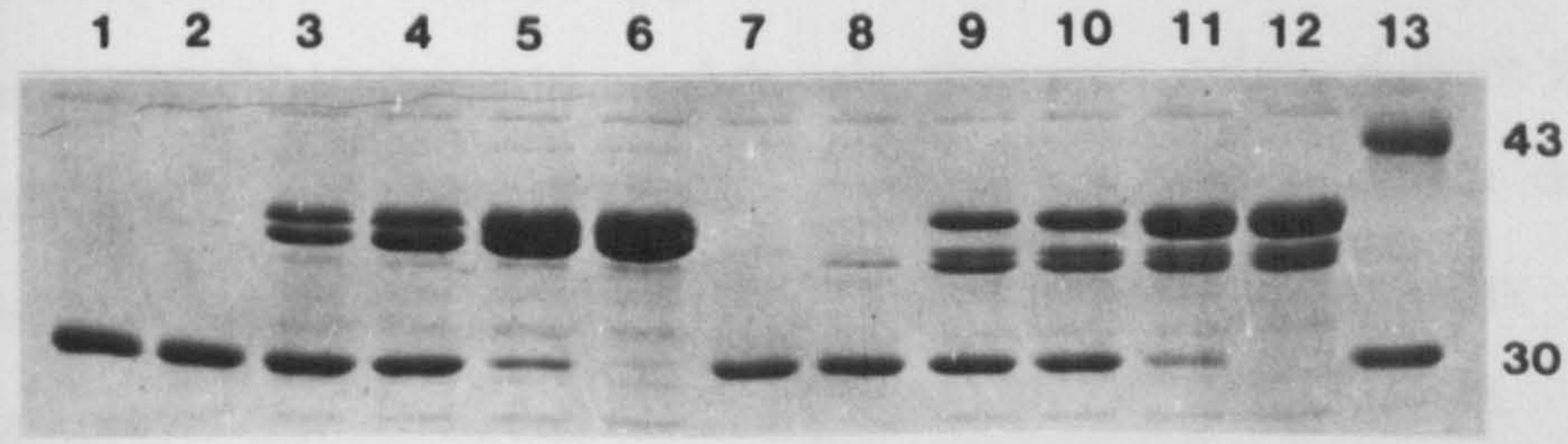
43
30

D


1 2 3 4 5 6 7 8 9 10 11 12 13
43
30

E


1 2 3 4 5 6 7 8 9 10 11 12 13
43
30

RD170 and RD178 (Figures 7 and 8). These results are summarised in Table 10.

In addition to the major proteins certain of the minor proteins also seemed to be peptidoglycan-associated. Thus, proteins of molecular weight 28, 18 and 17 KDa were present only when the samples were heated at 70^{o}C and above (Figures 7A and B). Whereas the 47 KDa minor protein was present at all temperatures, there were a number of high-molecular-weight proteins present at 37^{o}C, 50^{o}C and 70^{o}C but absent or less abundant at higher temperatures (Figures 7A and B).

4.3.3 Further analysis of the strain-variable heat-modifiable and peptidoglycan-associated outer membrane proteins

To confirm that the 38 and 36.5 KDa heat-modified HMPs were slightly different forms of the unheated 31 KDa HMP, the latter protein of various isolates was eluted from gel slices and re-run on gels after heating at 100^{o}C. It was shown that the 31 KDa protein was converted to one of two forms, depending on the isolate, of molecular weight either 38 or 36.5 KDa. Thus, isolates RD6, RD34, RD42, RD54 and RD170 possessed the 38 KDa HMP and isolates RD20, RD28, RD38, RD52 and RD168 possessed the 36.5 KDa HMP (Figure 9).

In addition, the other major proteins in the molecular weight range 36 to 40.5 KDa were shown to be peptidoglycan-associated. When heated at 37^{o}C, these proteins did not pass into the stacking gel but when they were eluted from gel slices, heated at 100^{o}C and re-run on slab gels, they were released from the peptidoglycan and passed into the gels. Furthermore, it was shown that different isolates possessed PAPs of different molecular weights as demonstrated previously. Thus, isolates RD6, RD20, RD28, RD34, RD38,

Table 10. Expression of the 38/36.5 KDa HMP and the 40.5, 38/36.5 and 36 KDa PAPs in selected isolates of Y. ruckeri.

Isolate	HMP/KDa		PAPs/KDa			
	38	36.5	40.5	38	36.5	36
RD6	+	-	-	+	-	+
RD20	-	+	-	+	-	-
RD28	-	+	-	+	-	-
RD30	+	-	-	+	-	-
RD34	+	-	-	+	-	-
RD38	-	+	-	+	-	-
RD42	+	-	-	-	+	-
RD44	+	-	-	+	-	-
RD52	-	+	+	-	-	-
RD54	+	-	-	+	-	+
RD168	-	+	-	+	-	+
RD170	+	-	-	-	+	+
RD176	+	-	-	+	-	-
RD178	+	-	-	-	+	+
RD200	+	-	-	-	+	-
RD210	+	-	-	-	+	-

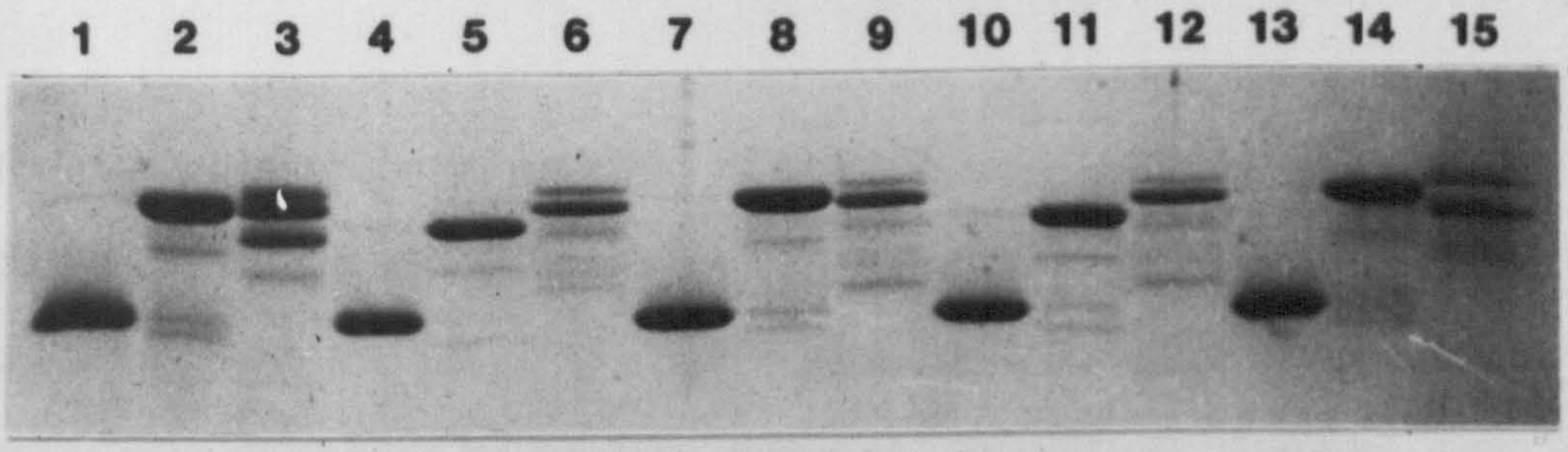
Figure 9. Demonstration of heat-modifiable and peptidoglycan-associated outer membrane proteins of *Y. ruckeri*.

HMP and PAP protein-bands, after no heat-treatment, were cut from first gel and re-run on second gel after being heated at 100°C for 5 min.

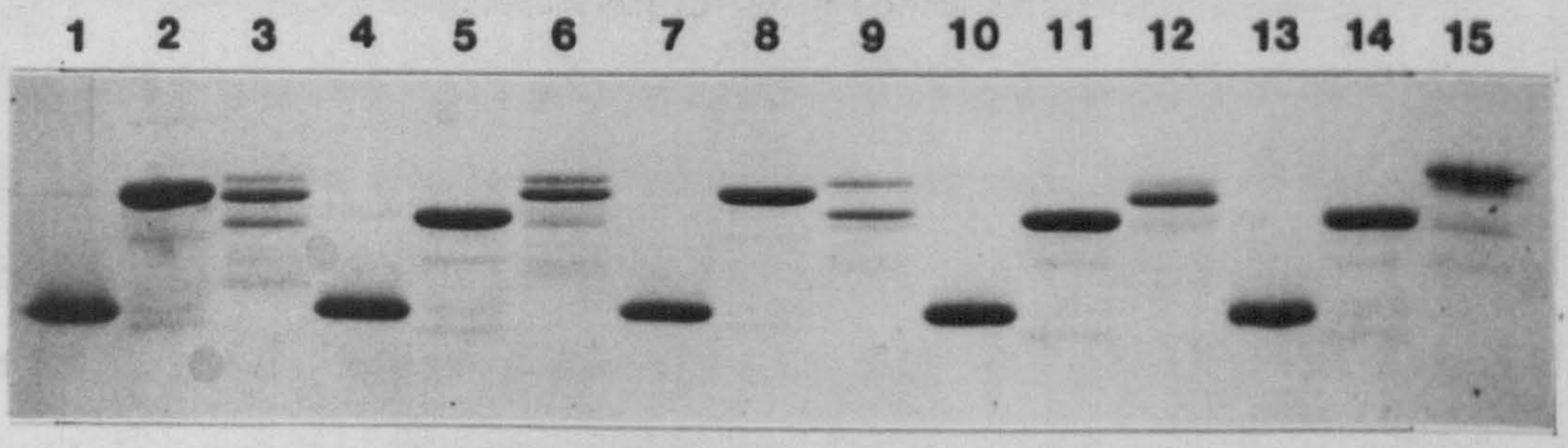
(A). Isolates RD6 (lanes 1 - 3), RD20 (lanes 4 - 6), RD34 (lanes 7 - 9), RD28 (lanes 10 - 12) and RD42 (lanes 13 - 15). Unheated HMPs (lanes 1, 4, 7, 10 and 13); HMPs after heating at 100°C (lanes 2, 5, 8, 11 and14); PAPs after heating at 100°C (lanes 3, 6, 9, 12 and 15).

(B). Isolates RD54 (lanes 1 - 3), RD168 (lanes 4 - 6), RD170 (lanes 7 - 9), RD38 (lanes 10 - 12) and RD52 (lanes 13 - 15). Unheated HMPs (lanes 1, 4, 7, 10 and 13); HMPs after heating at 100°C (lanes 2, 5, 8, 11 and 14); PAPs after heating at 100°C (lanes 3, 6, 9, 12 and 15).

A



B



RD54 and RD168 possessed PAPs of molecular weight 39.5 and 38 KDa; isolates RD6, RD54 and RD168 possessed an additional PAP of 36 KDa. Isolates RD42 and RD170 possessed PAPs of molecular weight 39.5 and 36.5 KDa; isolate RD170 possessed an additional PAP of 36 KDa. Isolate RD52 possessed PAPs of molecular weight 40.5, 39.5 and 36 KDa (Figure 9). These results confirmed the results described in the previous section and are summarised in Table 10.

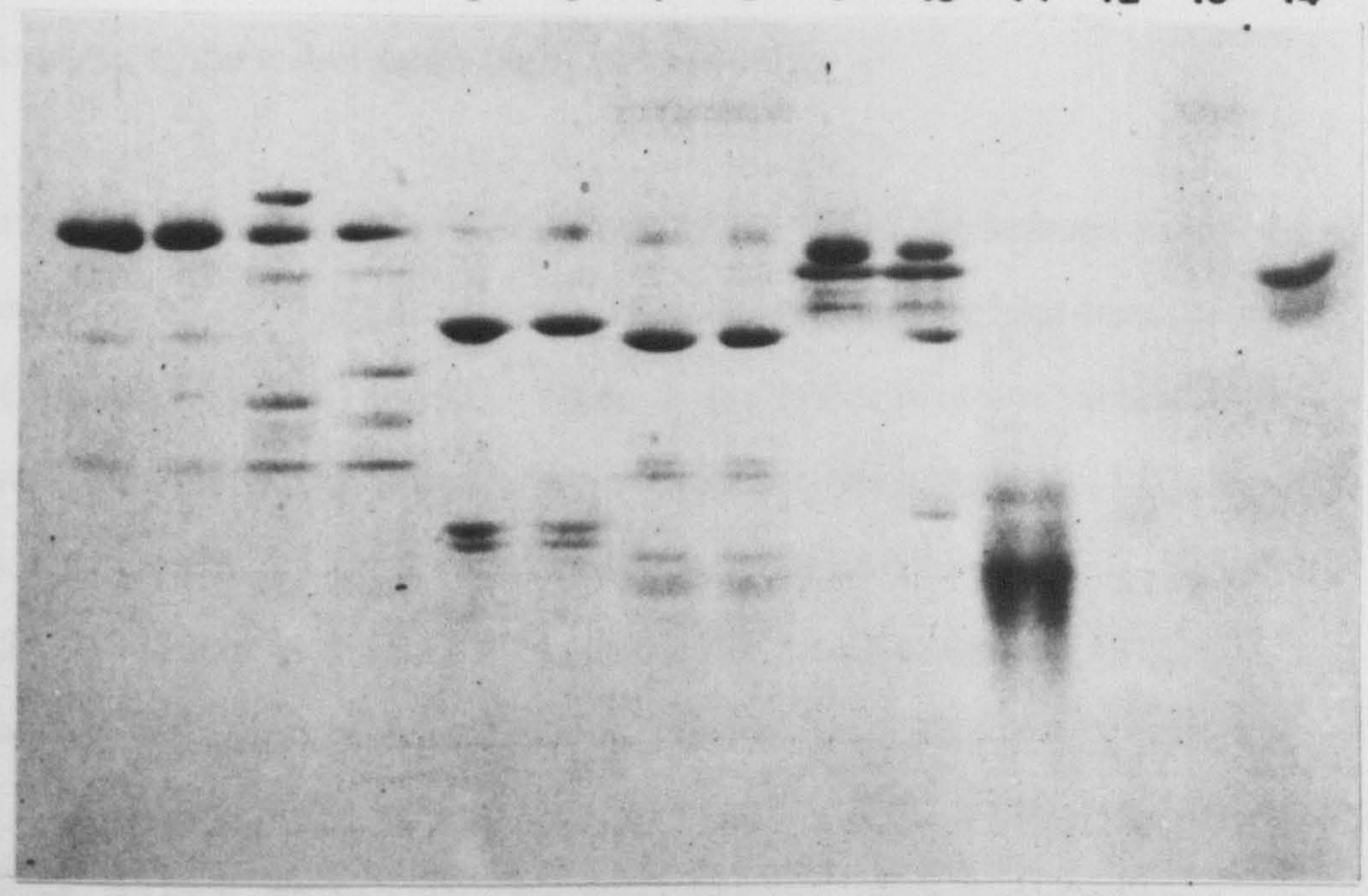
To determine whether the 38 and 36.5 KDa PAPs were different proteins or whether they were slightly different forms of the same protein, they were examined by peptide mapping. The two high-molecular-weight forms of the HMP were also examined by peptide mapping. Proteolytic cleavage, with chymotrypsin, papain and V8 protease, of the two heat-modified HMPs resulted in very similar peptide maps (Figure 10A). The molecular weights of the peptide products could not be determined, however, because the molecular weight standards were degraded also. The peptide maps resulting from digestion of the PAPs were not as clear as were those of the HMPs (Figure 10B). Nevertheless, careful examination of the original gel indicated that the peptide maps of the two PAPs were very similar. These results indicated that the 38 and 36.5 KDa HMPs were two slightly different forms of the same protein, and that the 38 and 36.5 KDa PAPs were also slightly different forms of the same protein. Although the 40.5 KDa PAP of isolate RD52 was not examined, it is possible that this protein may be a third form of the 38/36.5 KDa PAP. In some isolates (Section 4.3.5) a fourth slightly different form of this protein appeared to exist having a molecular weight of 37.5 KDa. When the OMP-profiles of selected isolates were examined using linear gradient resolving gels it became apparent that the 38 KDa HMP and the 38 KDa PAP actually had slightly different molecular weights. Thus, in those isolates possessing both of these proteins, two closely spaced proteins were resolved in

Figure 10. Peptide mapping of the heat-modifiable and peptidoglycan-associated proteins of *Y. ruckeri*.

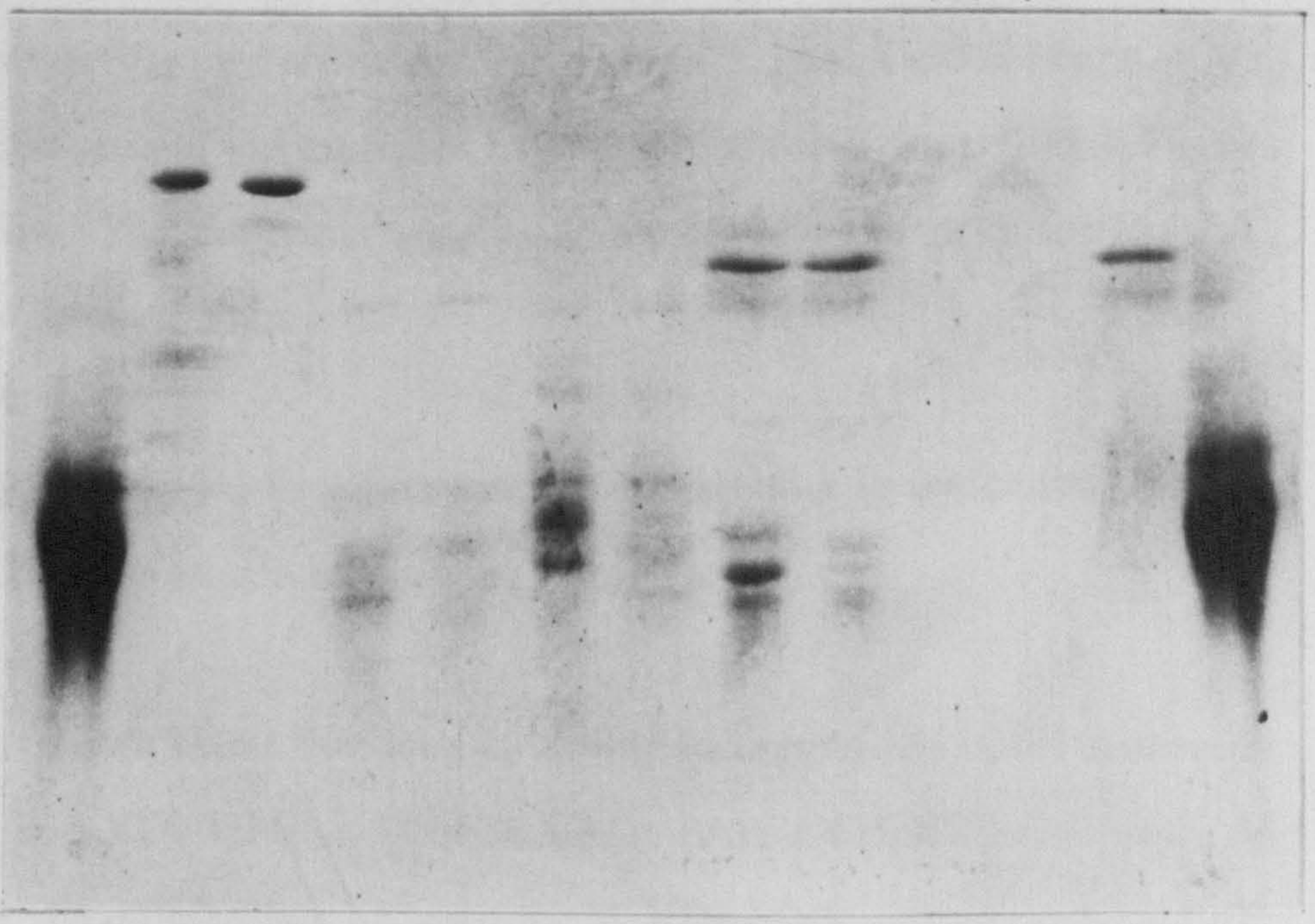
(A). HMP (36.5 KDa) of isolate RD26 (lanes 1, 3, 5, 7 and 9); HMP (38 KDa) of isolate RD74 (lanes 2, 4, 6, 8 and 10). Unheated proteins (lanes 1 and 2); proteins heated at 100°C for 5 min (lanes 3 and 4); proteins digested with chymotrypsin (lanes 5 and 6); proteins digested with papain (lanes 7 and 8); proteins digested with V8 protease (lanes 9 and 10); molecular weight standards (KDa) (lane 11); chymotrypsin (lane 12); papain (lane 13); V8 protease (lane 14).

(B). PAP (38 KDa) of isolate RD26 (lanes 2, 4, 6 and 8); PAP (36.5 KDa) of isolate RD74 (lanes 3, 5, 7 and 9); molecular weight standards (KDa) (lanes 1 and 13); untreated proteins (lanes 2 and 3); proteins digested with chymotrypsin (lanes 4 and 5); proteins digested with papain (lanes 6 and 7); proteins digested with V8 protease (lanes 8 and 9); chymotrypsin (lane 10); papain (lane 11); V8 protease (lane 12).

A



B



gradient gels whereas only a single protein was apparent in non-gradient gels (Figure 15).

4.3.4 Analysis of the minor outer membrane proteins

In addition to the major proteins described above, all of the isolates examined possessed from 15 to 20 minor proteins when examined after heat-treatment at 100^{o}C (Figures 11A ,B ,C and D). Whereas the major proteins showed inter-strain variation the minor proteins were extremely homogeneous. Thus, all isolates possessed minor proteins of molecular weights 47, 44.5, 41.5, 32.5, 30, 28, 23, 21, 18, 17 and 11.5 KDa. Faint protein bands of molecular weight 55.5, 43, 34 and 15 KDa were also observed in some isolates. In some isolates, e.g. RD56, RD58, RD64 and RD68 (Figure 11A), four high-molecular-weight proteins of 71, 68.5, 67 and 63 KDa were present; these proteins were not present in other isolates, e.g. RD60, RD62, RD66, RD70, RD72, RD74, RD76 and RD78 (Figure 11A), although these latter isolates had two (or four) uneven high-molecular-weight bands. These differences seemed to be artefactual. Some isolates, e.g. RD60, RD64 and RD68, possessed additional minor proteins (Figure 11A) and in a number of isolates, e.g. RD74 and RD88, the 41.5 KDa protein was expressed in greater abundance than normal (Figures 11A and B). The molecular weights of the major and minor OMPs of Y. ruckeri are shown in Figure 12.

4.3.5 A typing scheme based on inter-strain variation in the heat-modifiable and peptidoglycan-associated outer membrane proteins

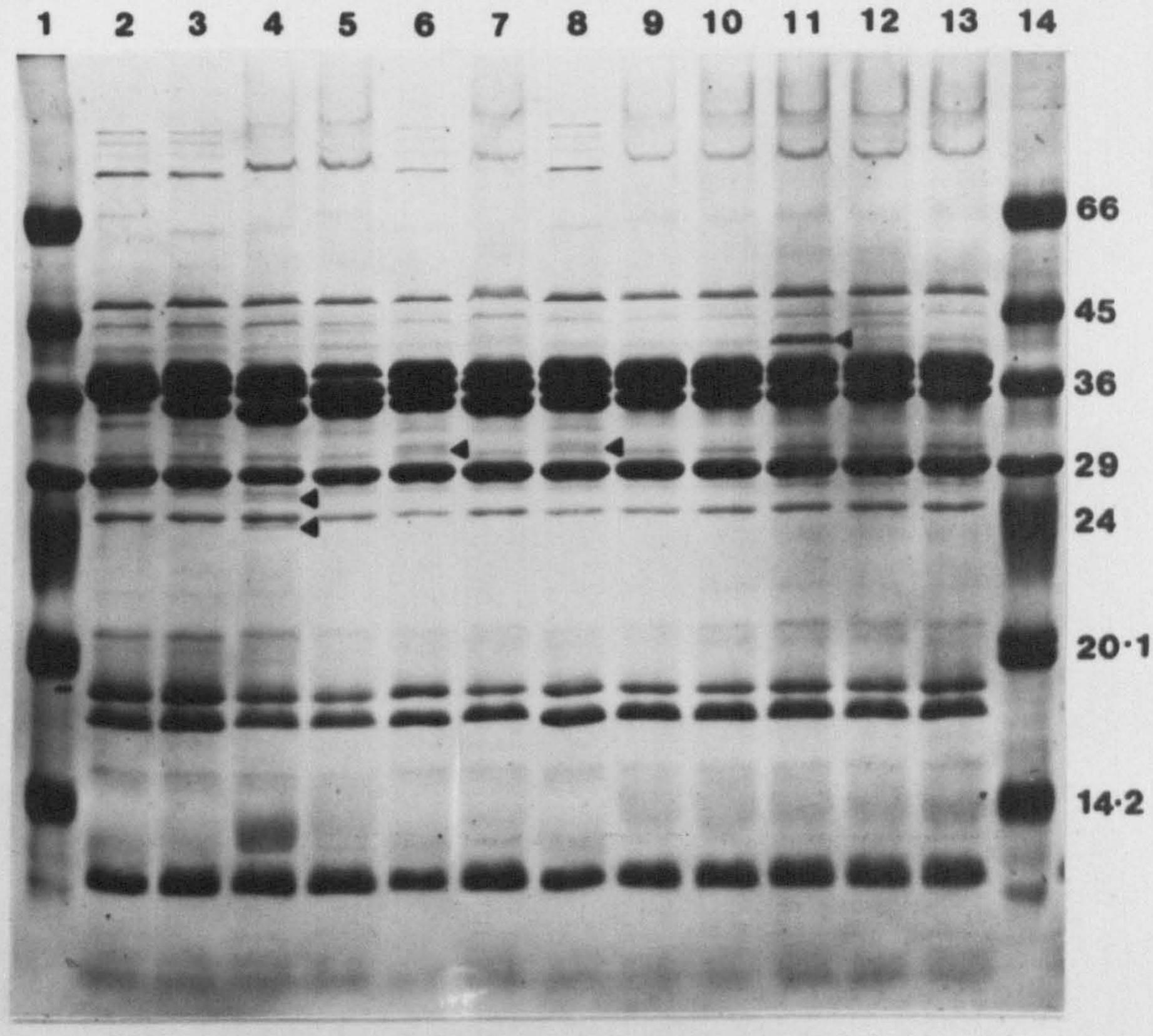
It has been shown above that inter-strain variation occurred in the possession of a 38 or 36.5 KDa HMP, a 38 or 36.5 KDa PAP, a 40.5 KDa PAP and a 36 KDa PAP. To differentiate between the 38 KDa HMP and the 38 KDa PAP, and between the 36.5 KDa HMP and the 36.5 KDa PAP, it was necessary to

Figure 11. Outer membrane protein profiles of *Y. ruckeri* isolates showing major and minor proteins after heat-treatment at 100°C.

(A). Lanes 2 - 13 represent isolates RD56, RD58, RD60, RD62, RD64, RD66, RD68, RD70, RD72, RD74, RD76 and RD78 respectively; molecular weight standards (KDa) are shown in lanes 1 and 14. Arrows indicate variation in the expression of minor proteins.

(B). Lanes 2 - 13 represent isolates RD80, RD82, RD84, RD86, RD88, RD90, RD92, RD94, RD96, RD98, RD100 and RD102 respectively; molecular weight standards (KDa) are shown in lanes 1 and 14. Arrows indicate variation in the expression of minor proteins.

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14
66
45
36
29
24
20·1
14·2

B

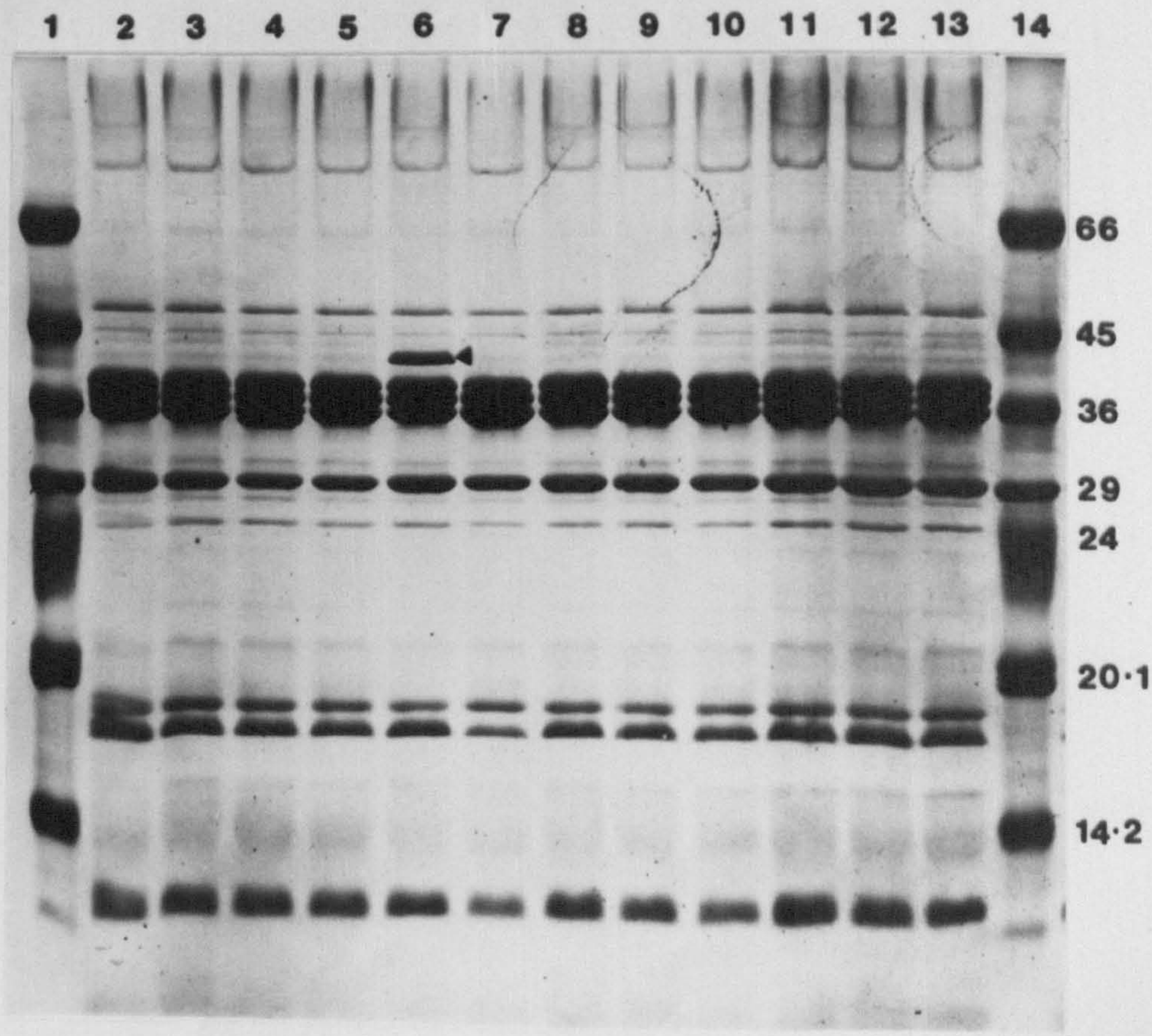
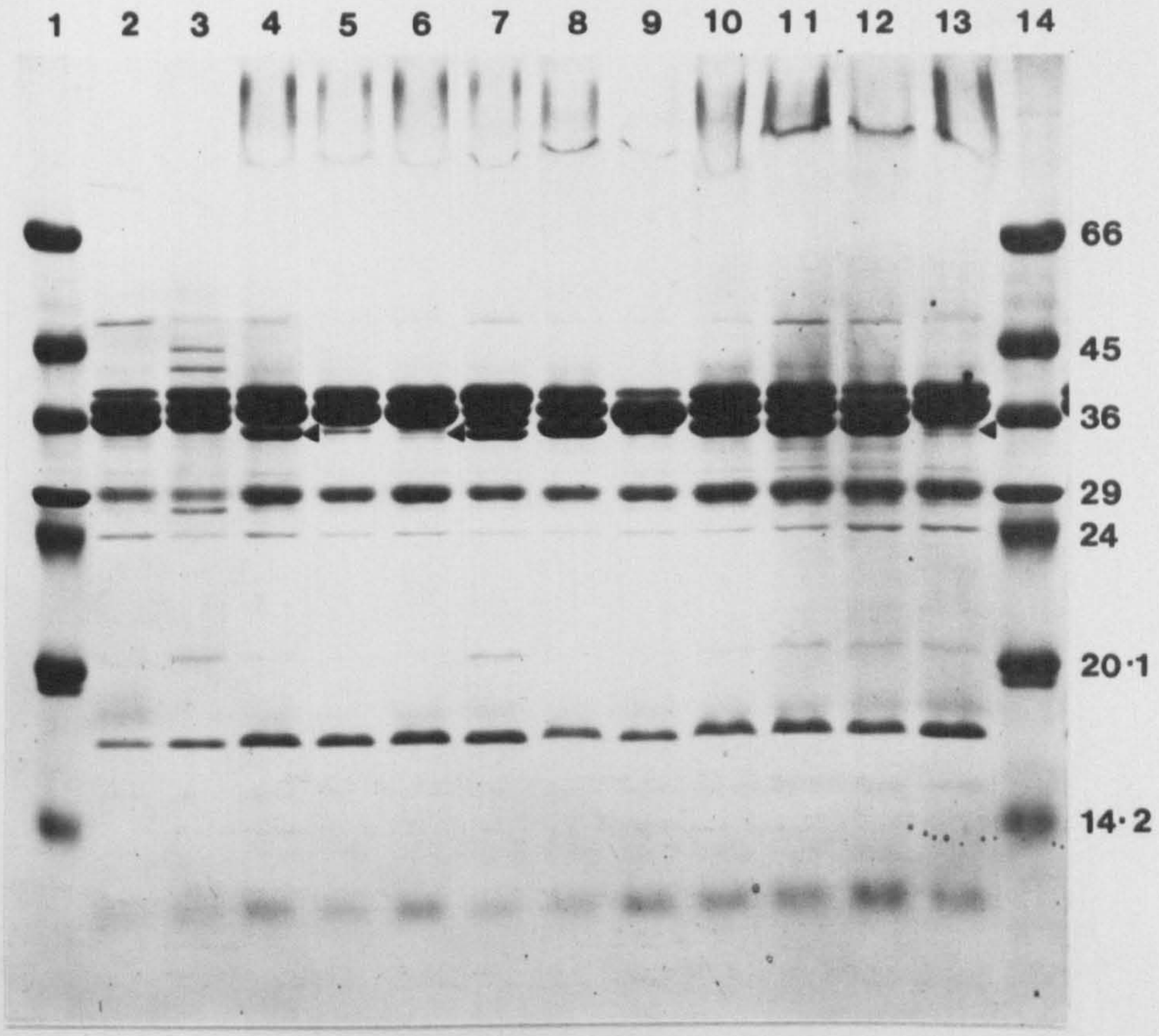

1 2 3 4 5 6 7 8 9 10 11 12 13 14
66
45
36
29
24
20·1
14·2

Figure 11 (continued). Outer membrane protein profiles of *Y. ruckeri* isolates showing major and minor proteins after heat-treatment at 100°C.

(C). Lanes 2 - 13 represent isolates RD2, RD4, RD6, RD10, RD14, RD16, RD20, RD22, RD24, RD26, RD28 and RD30; molecular weight standards (KDa) are shown in lanes 1 and 14. Arrows indicate variation in the expression of the 36 KDa PAP.

(D). Lanes 2 - 13 represent isolates RD32, RD34, RD36, RD38, RD40, RD42, RD44, RD46, RD48, RD50, RD52 and RD54; molecular weight standards (KDa) are shown in lanes 1 and 14.

C


1 2 3 4 5 6 7 8 9 10 11 12 13 14
66
45
36
29
24
20·1
14·2

D

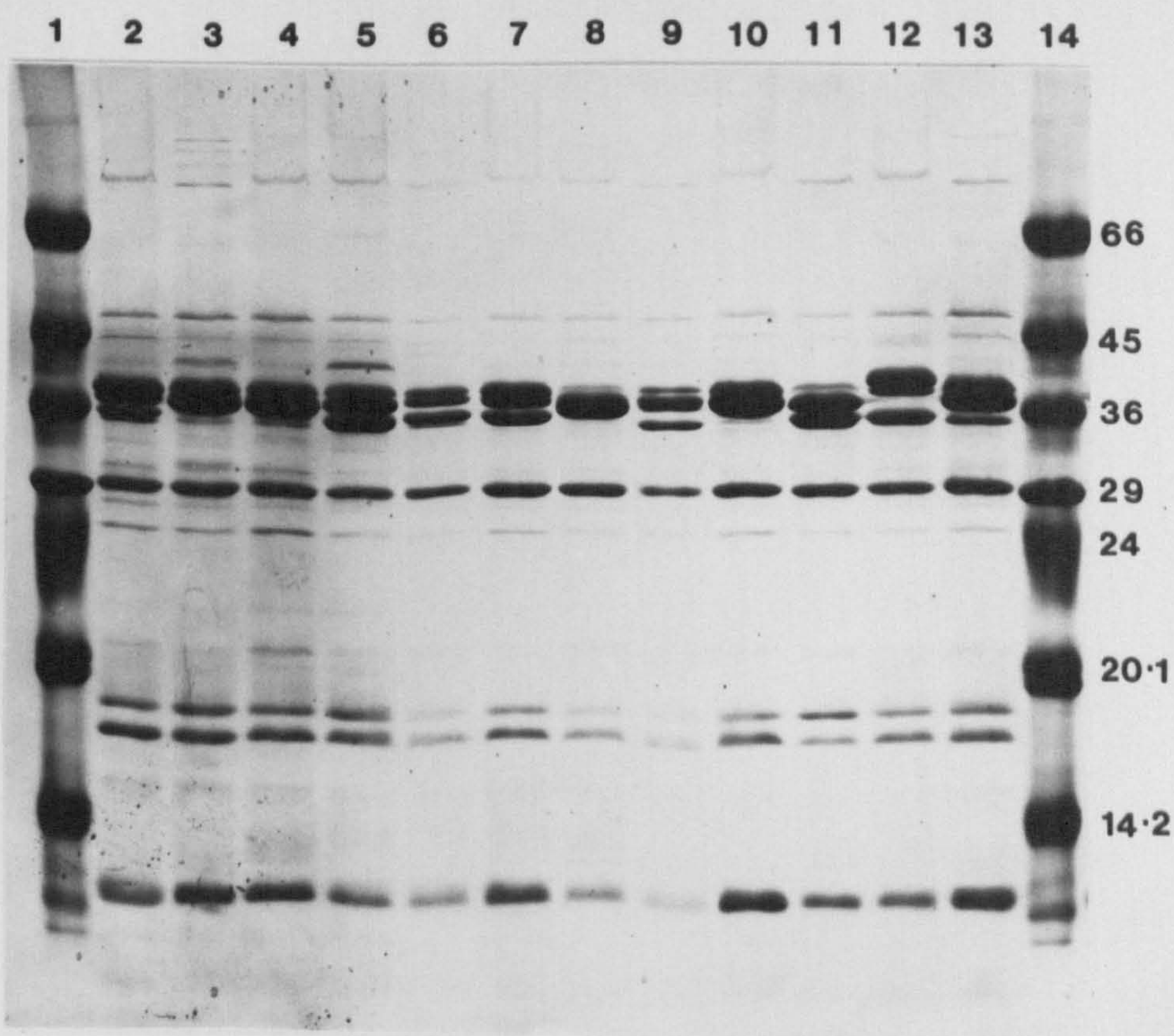
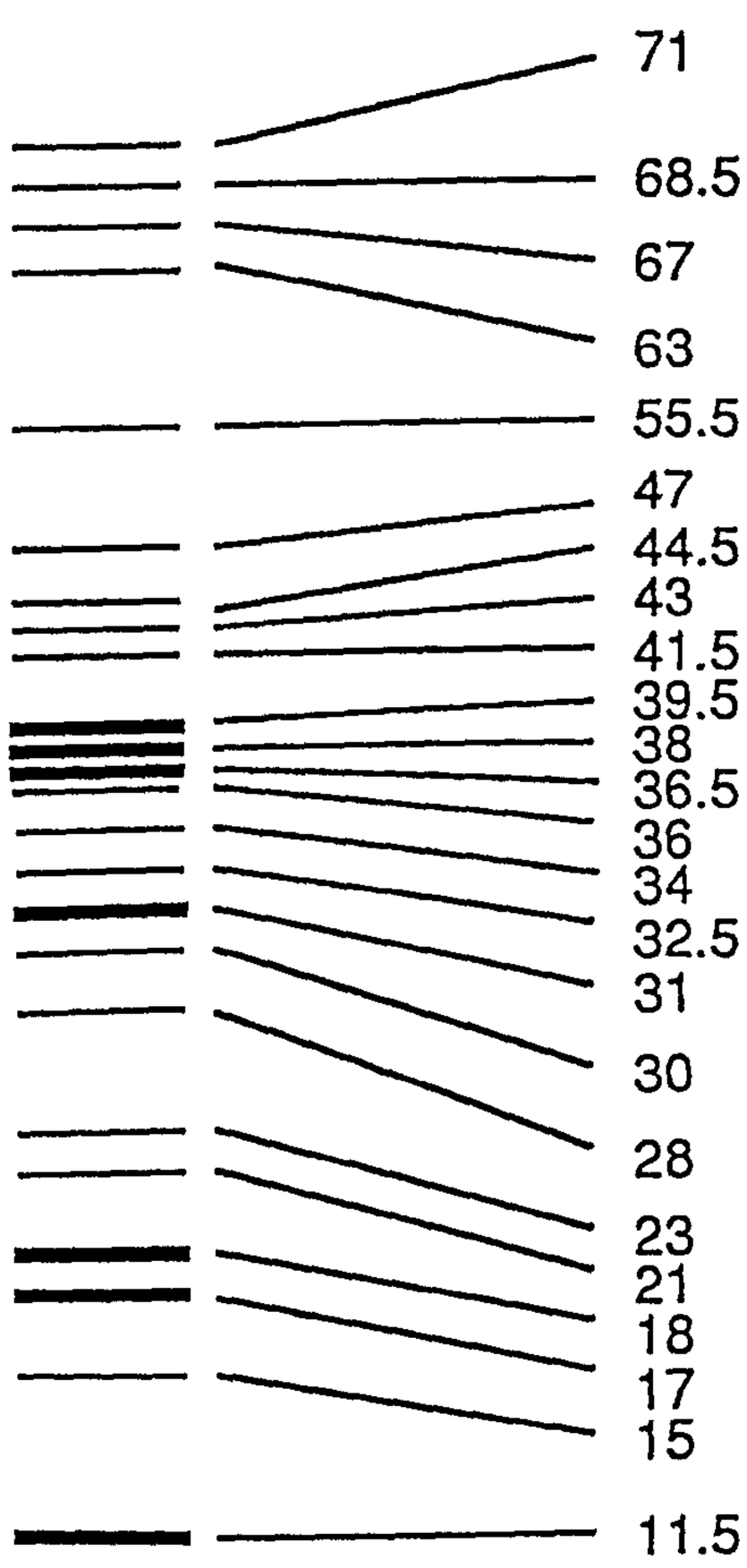

1 2 3 4 5 6 7 8 9 10 11 12 13 14
66
45
36
29
24
20·1
14·2

Figure 12. Molecular weights (KDa) of the outer membrane proteins of *Y. ruckeri.*


71
68.5
67
63
55.5
47
44.5
43
41.5
39.5
38
36.5
36
34
32.5
31
30
28
23
21
18
17
15
11.5

examine the OMP profiles of all isolates after heating the samples at both 80°C and 100°C. Thus, at 80°C the HMP was present in the low-molecular-weight (31 KDa) form and the PAPs could be distinguished; at 100°C the HMP was present in the heat-modified high-molecular-weight (38 or 36.5 KDa) form and its molecular weight could be determined. The major OMPs of all the isolates examined (except those of isolates RD220 and RD230) after heating at 100°C are shown in Figure 13, and the major OMPs of selected isolates after heating at 80°C are shown in Figure 14. The OMP profiles of selected isolates were also examined in gradient gels, to obtain better resolution of the proteins (in particular the major proteins), and these are shown in Figure 15.

Based on inter-strain variation in these major OMPs, five OMP-types (designated OMP-types 1, 2, 3, 4 and 5) were recognized among the 137 isolates of Y. ruckeri examined, including three major (OMP-types 1, 2 and 3) and two minor (OMP-types 4 and 5) OMP-types. Isolates of OMP-type 1 possessed a 38 KDa HMP and a 38 KDa PAP; isolates of OMP-type 2 possessed a 36.5 KDa HMP and a 38 KDa PAP; isolates of OMP-type 3 possessed a 38 KDa HMP and a 36.5 KDa PAP; isolates of OMP-type 4 possessed a 38 KDa HMP and a 37.5 KDa PAP and isolates of OMP-type 5 possessed a 36.5 KDa HMP and a 40.5 KDa PAP. The OMP-type 4 profile was very similar to the OMP-type 1 profile. However, the molecular weight of the type 4 PAP was slightly less, at about 37.5 KDa, than the molecular weight of the type 1 PAP. This difference is best seen by comparing Figures 14 A and E. This PAP type therefore constituted a fourth type in addition to the three described above (i.e. 38, 36.5 and 40.5 KDa PAPs).

Within each of the three major OMP-types, i.e. types 1, 2 and 3, variation occurred in the possession of a 36 KDa PAP. Based on the absence or presence of this protein, OMP-subtypes 1a and 1b, 2a and 2b and 3a and 3b

Figure 13. Major outer membrane proteins of isolates of *Y. ruckeri* after heat-treatment at 100°C .

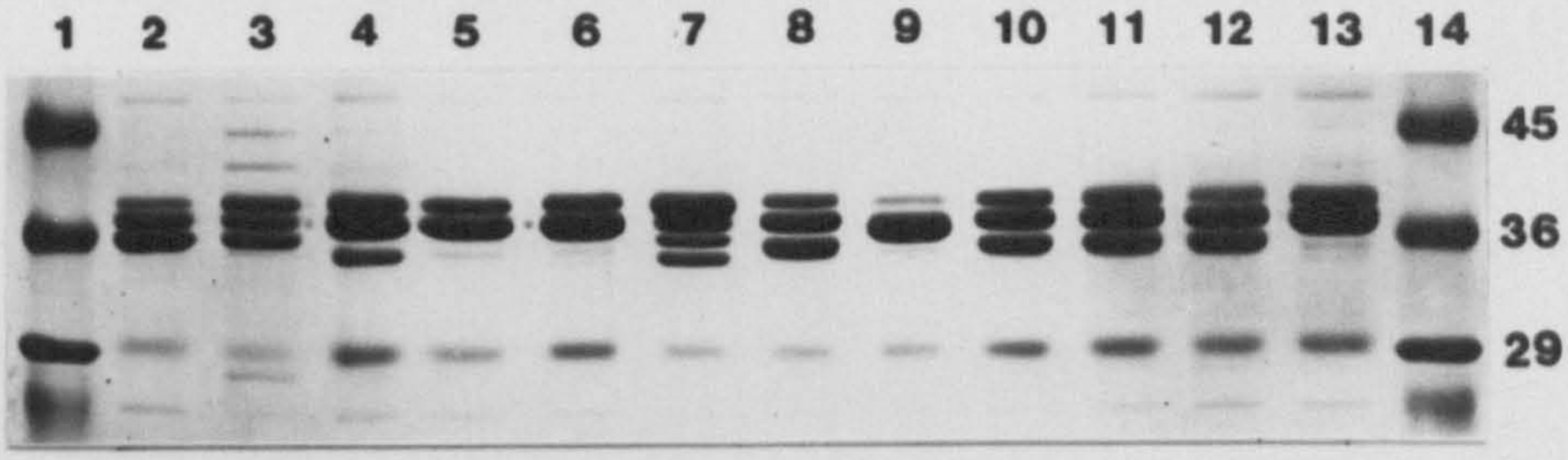
(A). Lanes 2 - 13 represent isolates RD2, RD4, RD6, RD10, RD14, RD16, RD20, RD22, RD24, RD26, RD28 and RD30; molecular weight standards (KDa) are shown in lanes 1 and 14.

(B). Lanes 2 - 13 represent isolates RD32, RD34, RD36, RD38, RD40, RD42, RD44, RD46, RD48, RD50, RD52 and RD54; molecular weight standards (KDa) are shown in lanes 1 and 14.

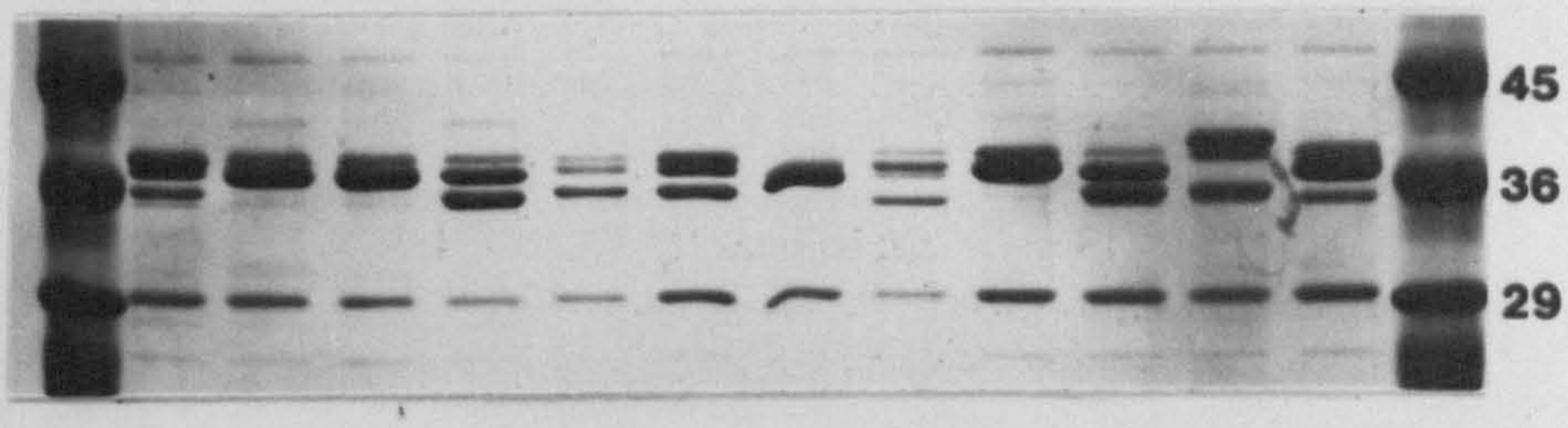
(C). Lanes 2 - 13 represent isolates RD56, RD58, RD60, RD62, RD64, RD66, RD68, RD70, RD72, RD74, RD76 and RD78; molecular weight standards (KDa) are shown in lanes 1 and 14.

(D). Lanes 2 - 13 represent isolates RD80, RD82, RD84, RD86, RD88, RD90, RD92, RD94, RD96, RD98, RD100 and RD102; molecular weight standards (KDa) are shown in lanes 1 and 14.

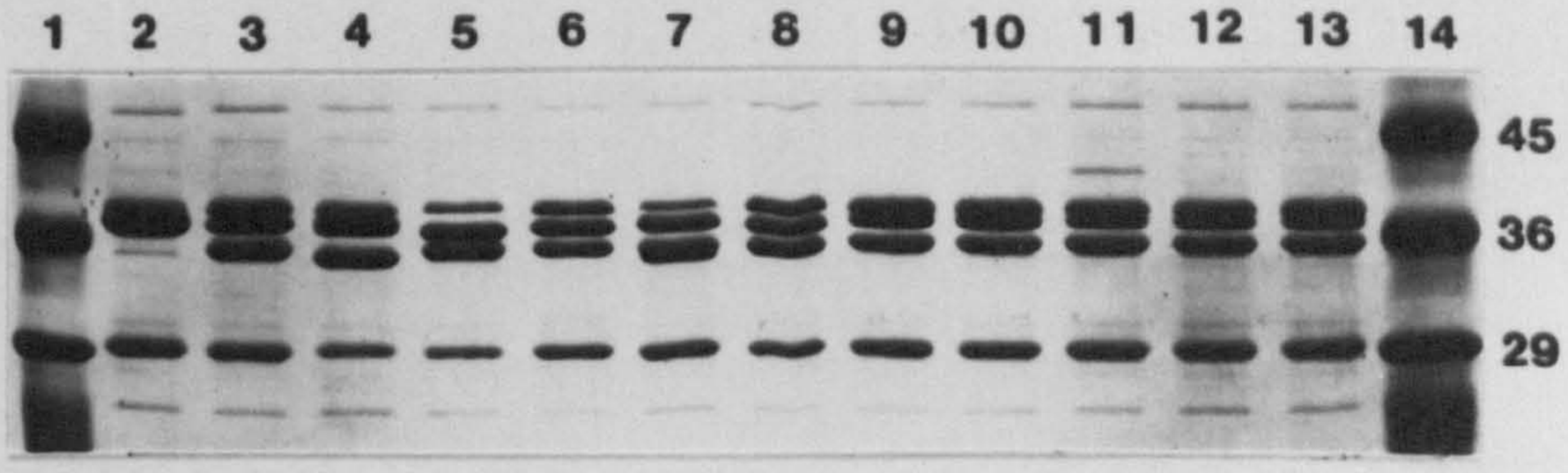
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29

C


1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29

D

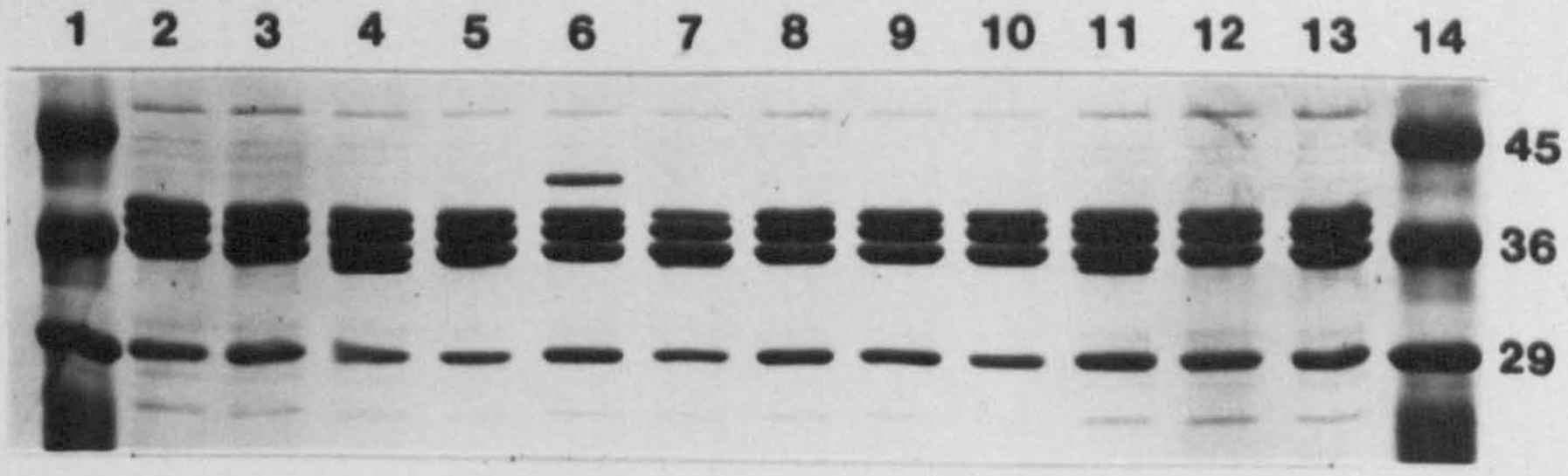

1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29

Figure 13 (continued). Major outer membrane proteins of isolates of *Y. ruckeri* after heat-treatment at 100°C.

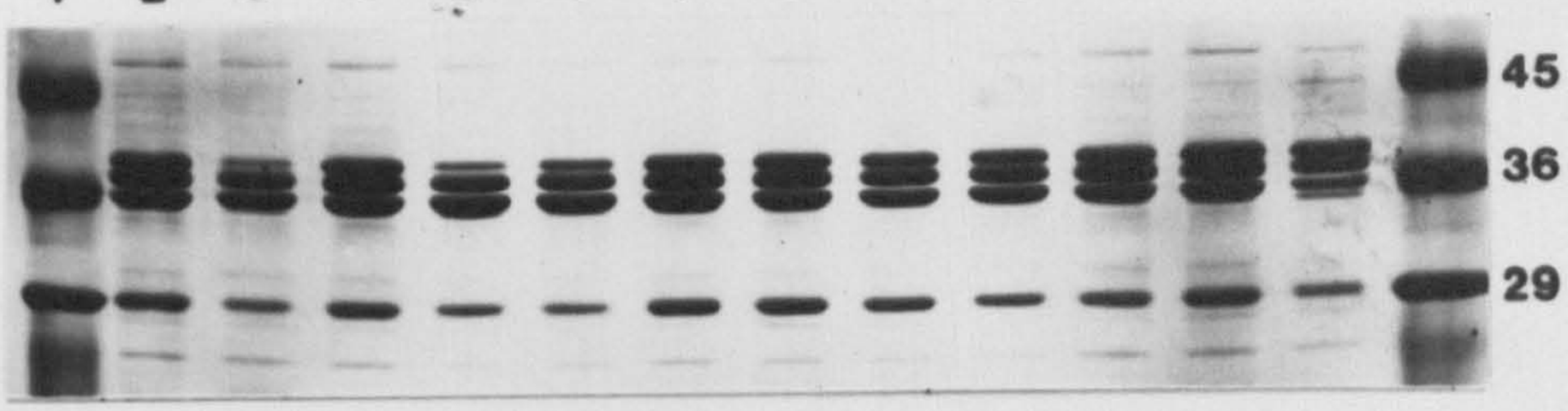
(E). Lanes 2 - 13 represent isolates RD104, RD106, RD108, RD110, RD112, RD114, RD116, RD118, RD120, RD122, RD124 and RD126; molecular weight standards (KDa) are shown in lanes 1 and 14.

(F). Lanes 2 - 13 represent isolates RD128, RD130, RD132, RD134, RD136, RD138, RD140, RD142, RD144, RD146, RD148 and RD150; molecular weight standards (KDa) are shown in lanes 1 and 14.

(G). Lanes 2 - 13 represent isolates RD152, RD154, RD156, RD158, RD160, RD162, RD164, RD166, RD168, RD170, RD172 and RD174; molecular weight standards (KDa) are shown in lanes 1 and 14.

(H). Lanes 2 - 13 represent isolates RD176, RD178, RD180, RD182, RD184, RD186, RD188, RD190, RD192, RD194, RD200 and RD210; molecular weight standards (KDa) are shown in lanes 1 and 14.

E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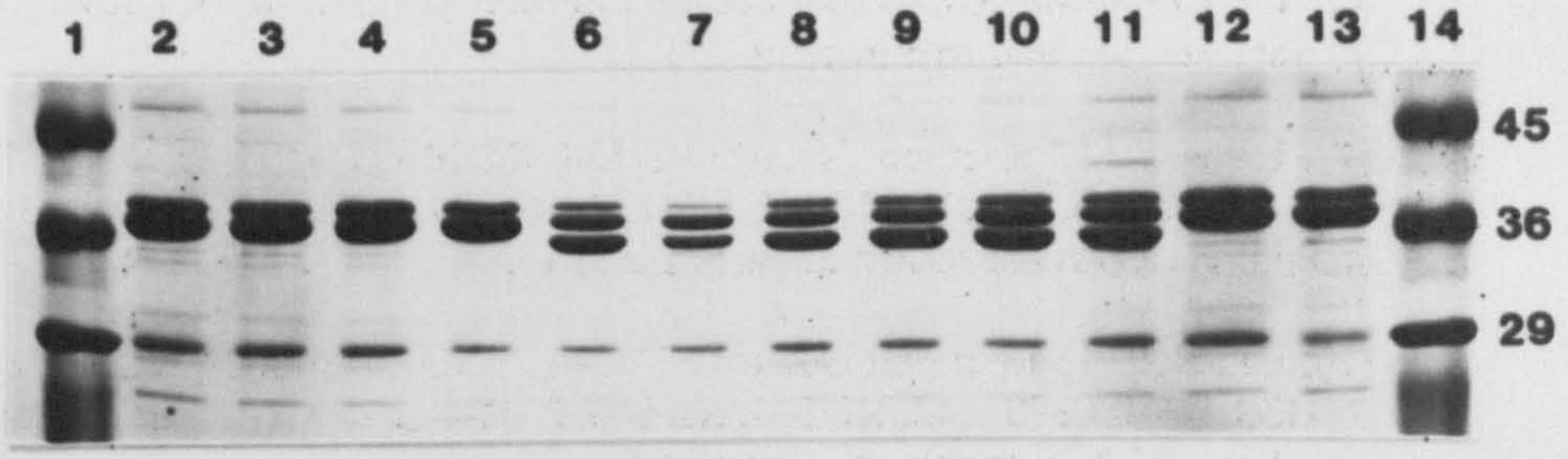
F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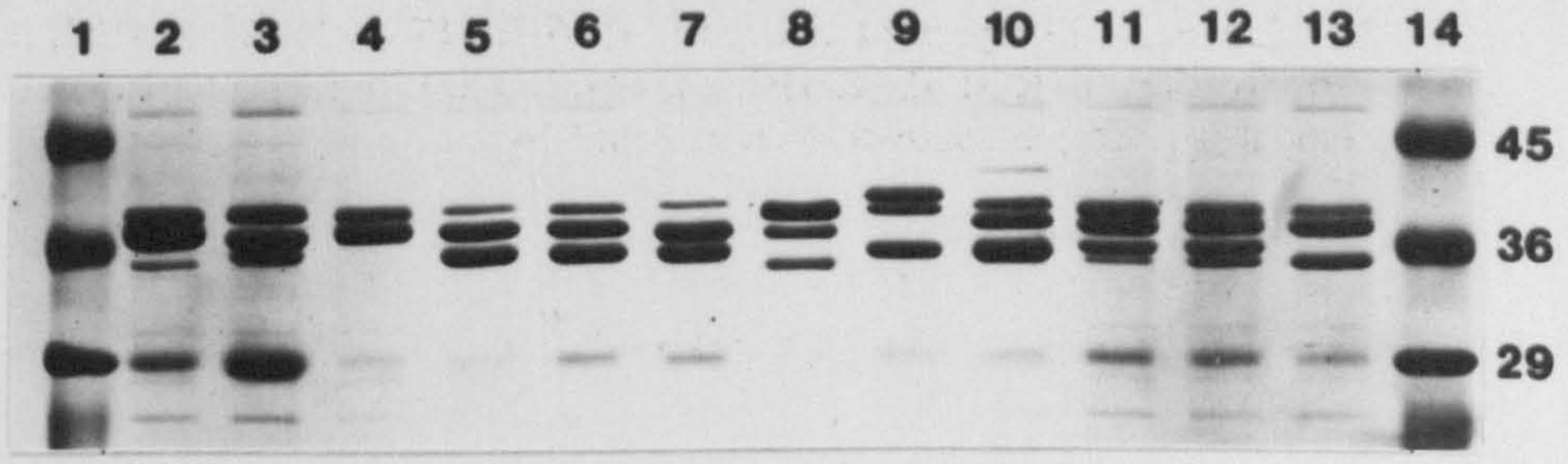
G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45
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29

H

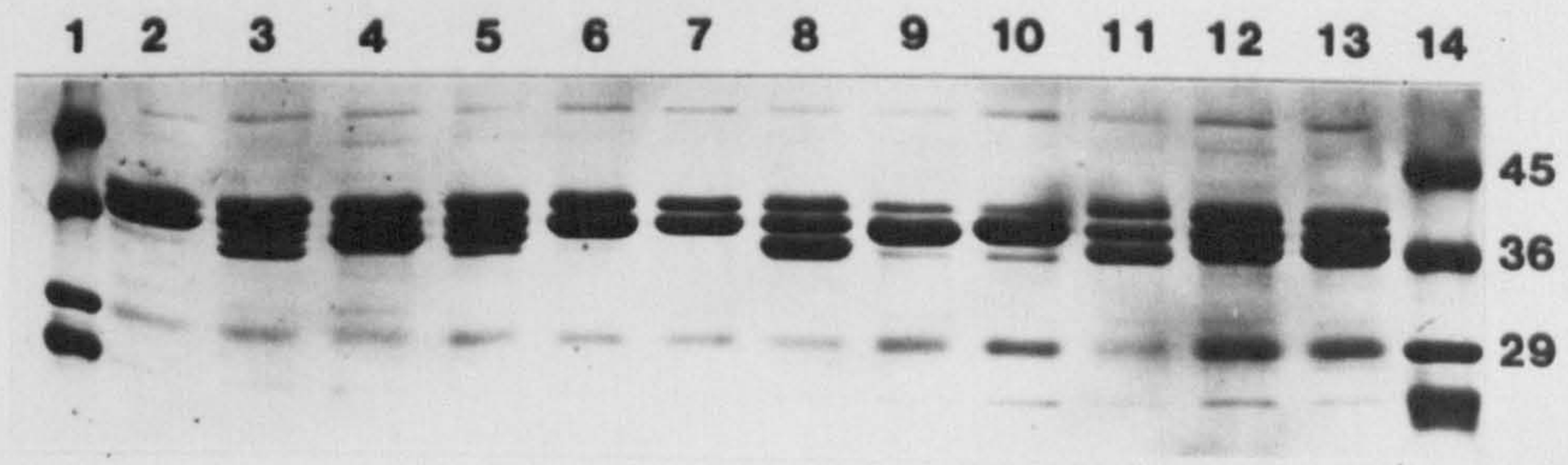

1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29

Figure 13 (continued). Major outer membrane proteins of isolates of *Y. ruckeri* after heat-treatment at 100°C.

(I). Lanes 1 - 14 represent isolates RD246, RD248, RD250, RD252, RD254, RD256, RD258, RD260, RD264, RD266, RD268, RD270, RD272 and RD274; molecular weight standards (KDa) are shown in lane 15.

(J). Lanes 1 - 13 represent isolates RD278, RD280, RD282, RD284, RD286, RD288, RD290, RD292, RD294, RD296, RD298, RD300 and RD302; molecular weight standards (KDa) are shown in lane 14.

(K). Lanes 1 - 13 represent isolates RD304, RD306, RD308, RD310, RD312, RD314, RD316, RD318, RD320, RD322, RD324, RD326 and RD328; molecular weight standards (KDa) are shown in lane 14.

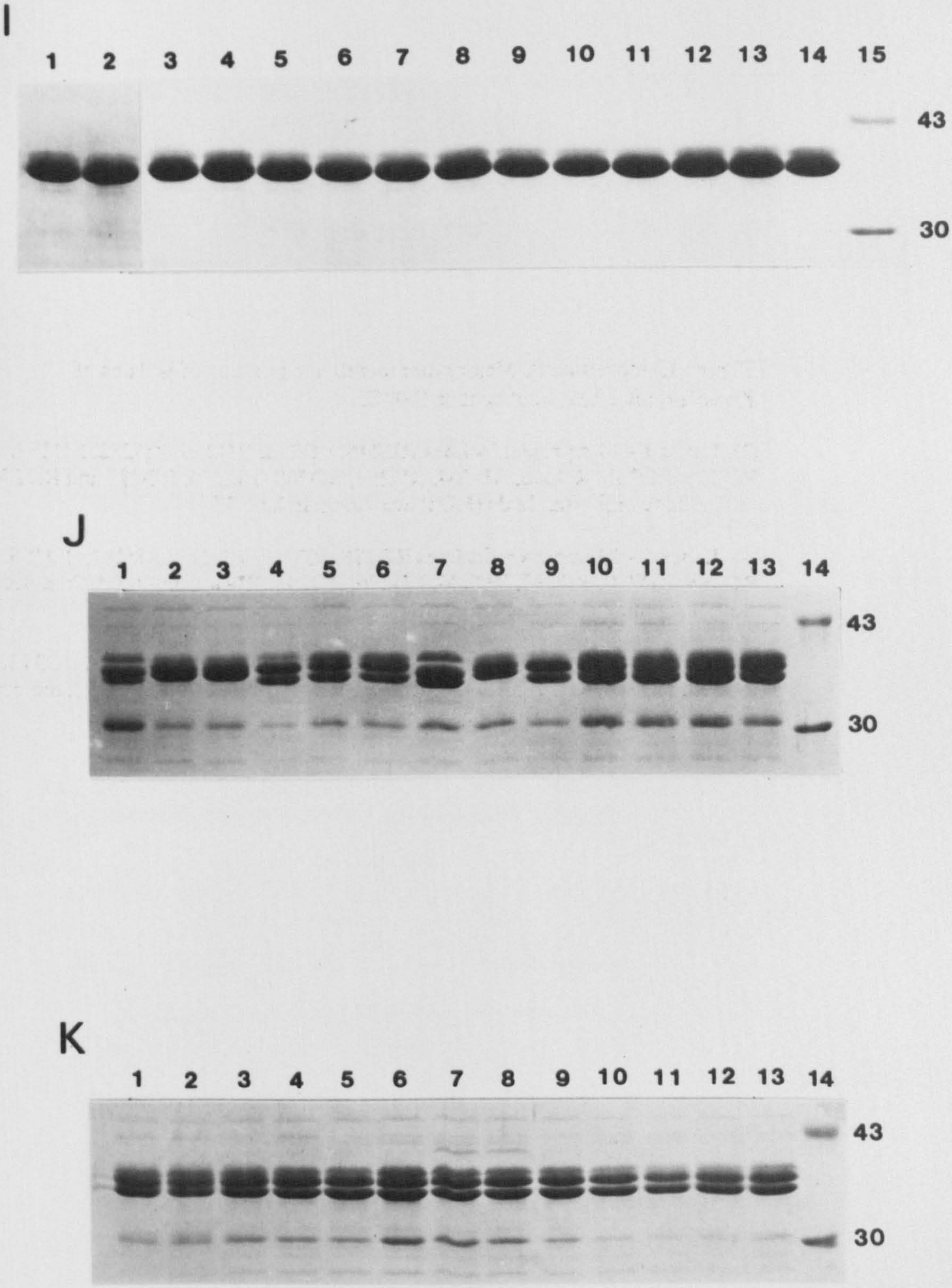

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
43
30
J
1 2 3 4 5 6 7 8 9 10 11 12 13 14
43
30
K
1 2 3 4 5 6 7 8 9 10 11 12 13 14
43
30

Figure 14. Major outer membrane proteins of isolates of *Y. ruckeri* after heat-treatment at 80°C.

(A). OMP-type 1: Lanes 1 - 17 represent isolates RD246, RD152, RD132, RD156, RD184, RD186, RD34, RD56, RD44, RD192, RD36, RD48, RD150, RD54, RD60, RD174 and RD46; molecular weight standards (KDa) are shown in lane 18.

(B). OMP-type 2: Lanes 1 - 17 represent isolates RD20, RD24, RD26, RD160, RD158, RD38, RD64, RD58, RD28, RD188, RD62, RD154, RD162, RD194, RD50, RD168 and RD66; molecular weight standards (KDa) are shown in lane 18.

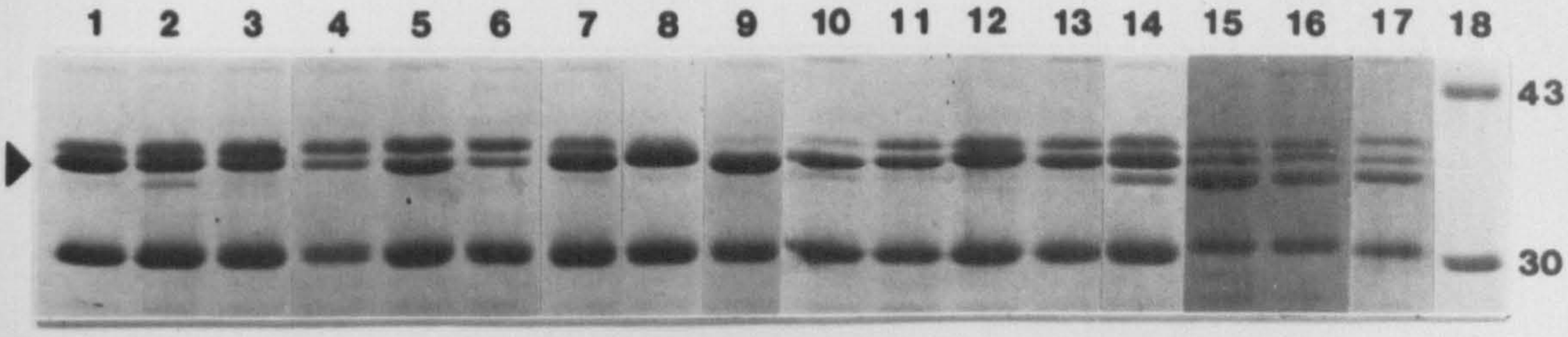
(C). OMP-type 3a: Lanes 1 - 10 represent isolates RD2, RD136, RD140, RD82, RD86, RD88, RD124, RD70, RD288 and RD42; molecular weight standards (KDa) are shown in lane 11.

(D). OMP-type 3b: Lanes 1 - 12 represent isolates RD4, RD32, RD180, RD126, RD16, RD84, RD170, RD178, RD182, RD172, RD40 and RD138; molecular weight standards (KDa) are shown in lane 13.

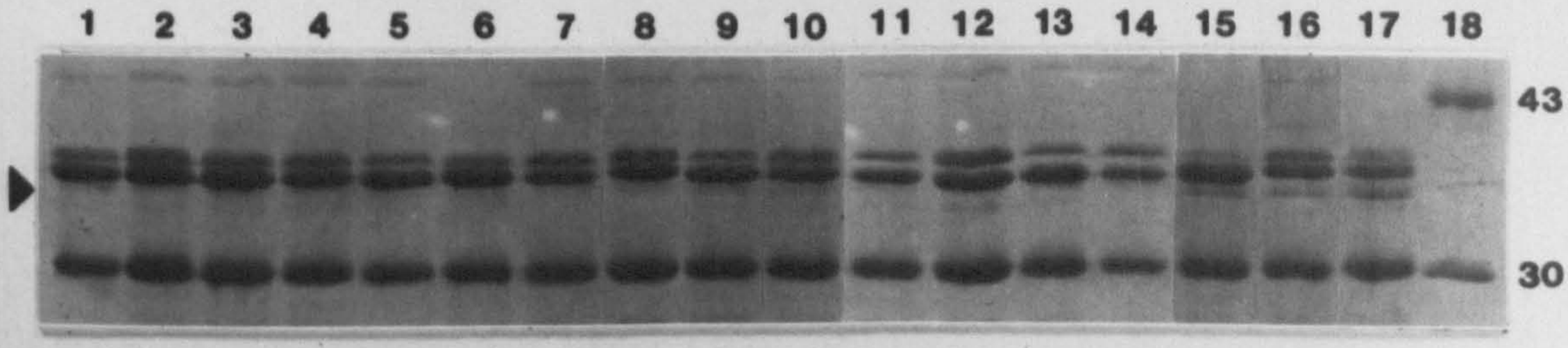
(E). OMP-type 4: Lanes 1 and 2 represent isolates RD22 and RD190. OMP-type 5: Lane 3 represents isolate RD52. Molecular weight standards (KDa) are shown lane 4.

Arrows indicate position of the high-molecular-weight forms of the heat-modifiable proteins, i.e. when proteins are heated at 100°C.

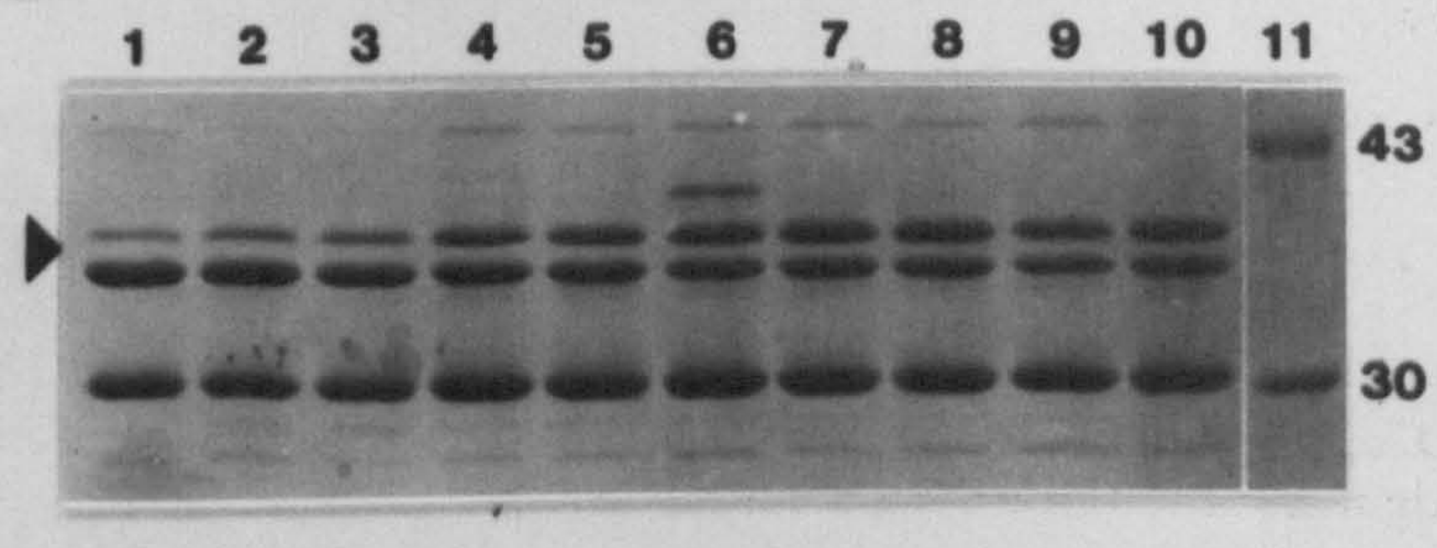
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
43
30

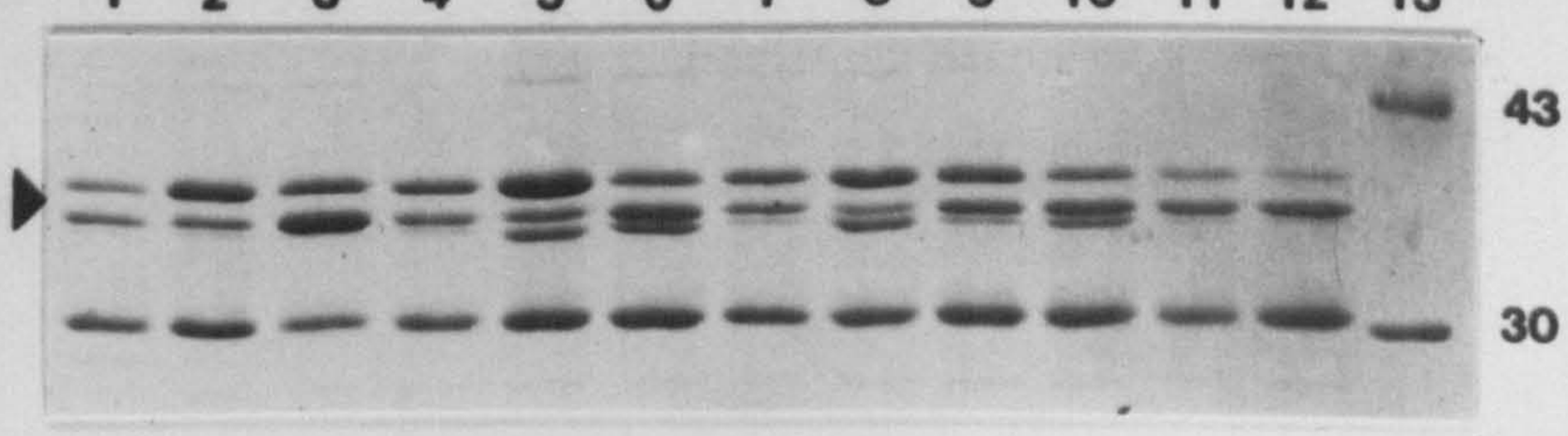
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
43
30

C


1 2 3 4 5 6 7 8 9 10 11
43
30

D


1 2 3 4 5 6 7 8 9 10 11 12 13
43
30

E

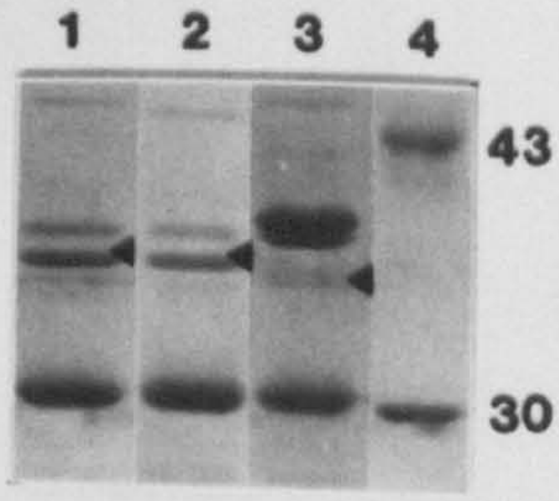

1 2 3 4
43
30

Figure 15. Outer membrane protein profiles of isolates of *Y. ruckeri* examined in 8-20% linear gradient gels showing increased resolution of proteins.

(A). Lanes 2 - 13 represent isolates RD2, RD4, RD6, RD10, RD14, RD16, RD20, RD22, RD24, RD26, RD28 and RD30; molecular weight standards (KDa) are shown in lanes 1 and 14.

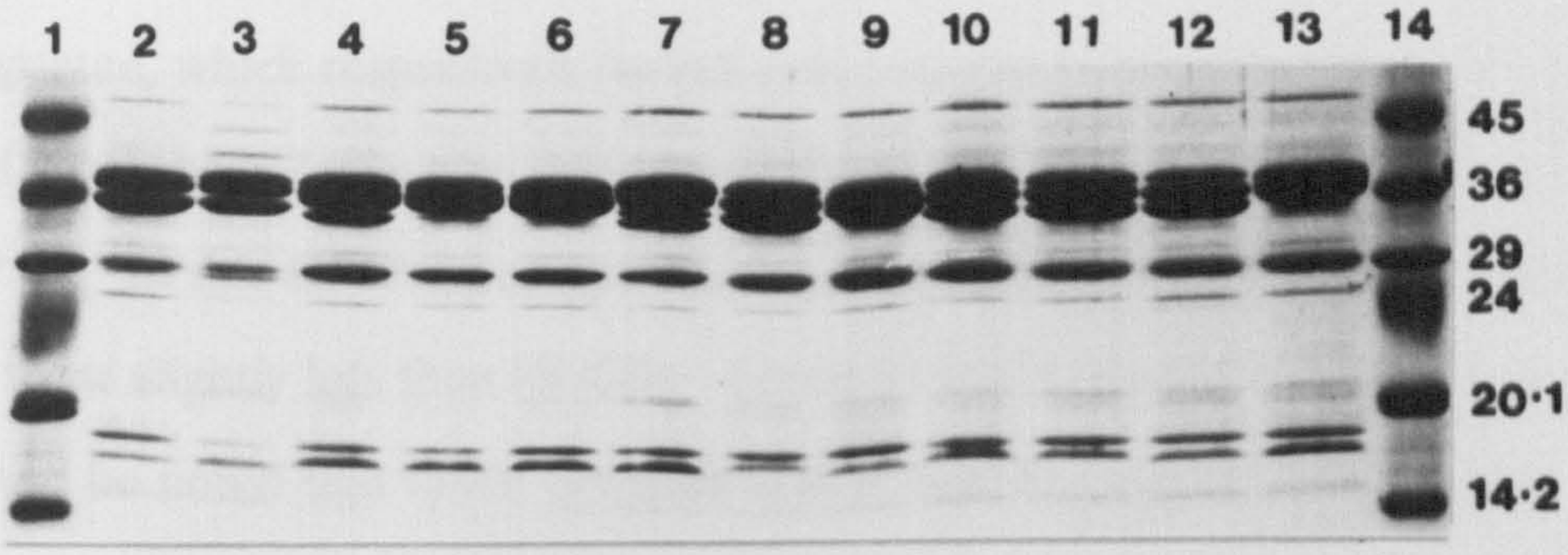
(B). Lanes 1 - 13 represent isolates RD34, RD32, RD34, RD36, RD38, RD40, RD42, RD44, RD46, RD48, RD50, RD52 and RD54; molecular weight standards (KDa) are shown in lane 14.

(C). Lanes 1 - 13 represent isolates RD102, RD104, RD106, RD108, RD110, RD112, RD114, RD116, RD118, RD120, RD122, RD124 and RD126; molecular weight standards (KDa) are shown in lane 14.

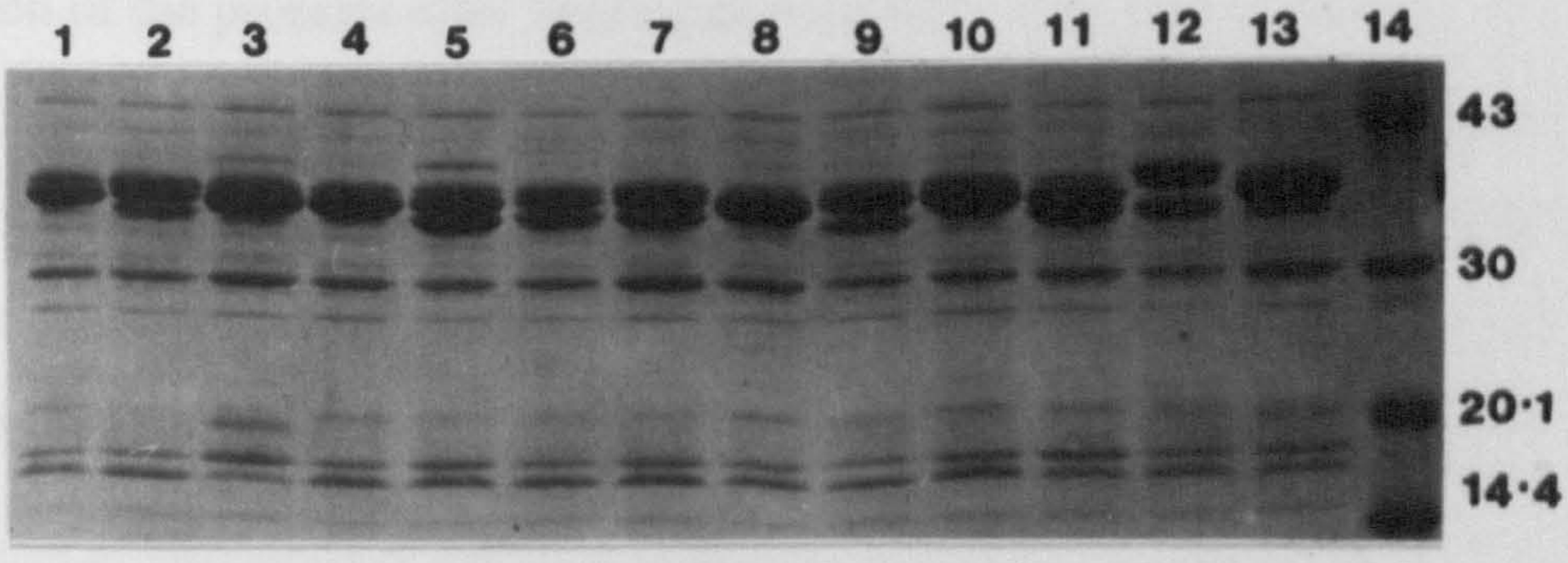
(D). Lanes 1 - 13 represent isolates RD128, RD128, RD130, RD132, RD134, RD136, RD138, RD140, RD142, RD144, RD146, RD148 and RD150; molecular weight standards (KDa) are shown in lane 14.

(E). Lanes 1-13 represent isolates RD180, RD176, RD178, RD180, RD182, RD184, RD186, RD188, RD190, RD192, RD194, RD200, RD210; molecular weight standards are shown in lane 14.

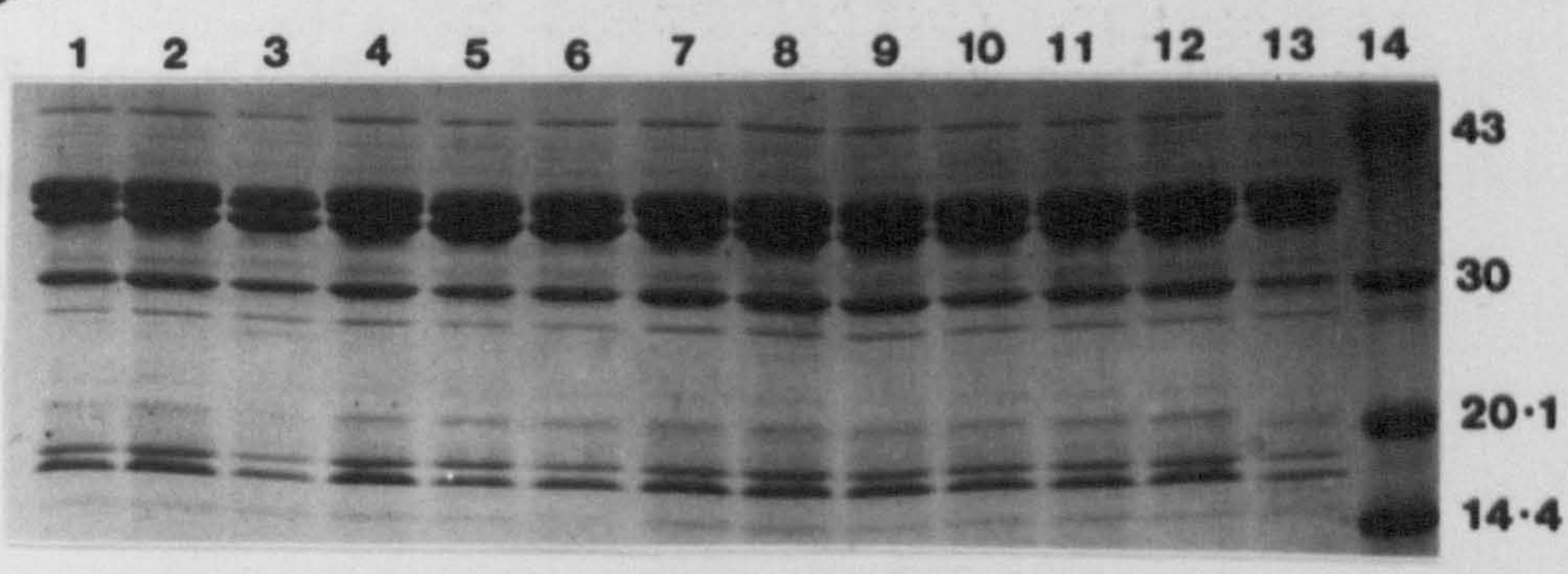
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29
24
20·1
14·2

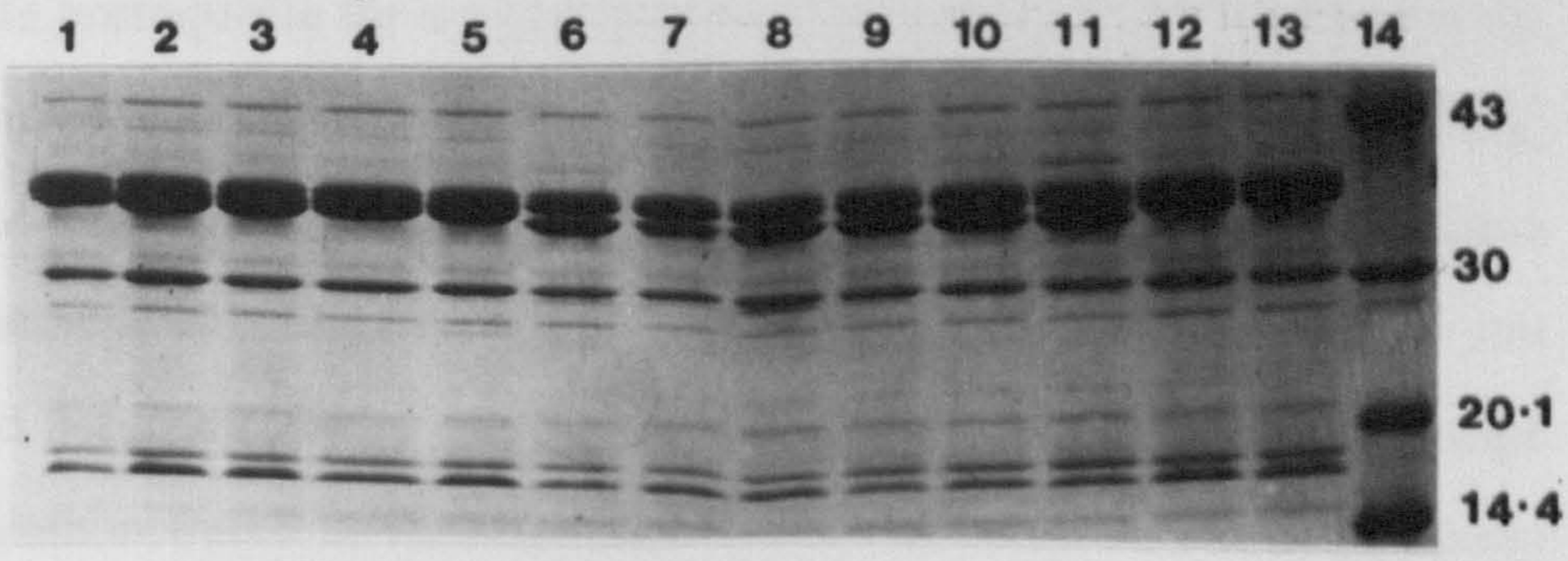
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14
43
30
20·1
14·4

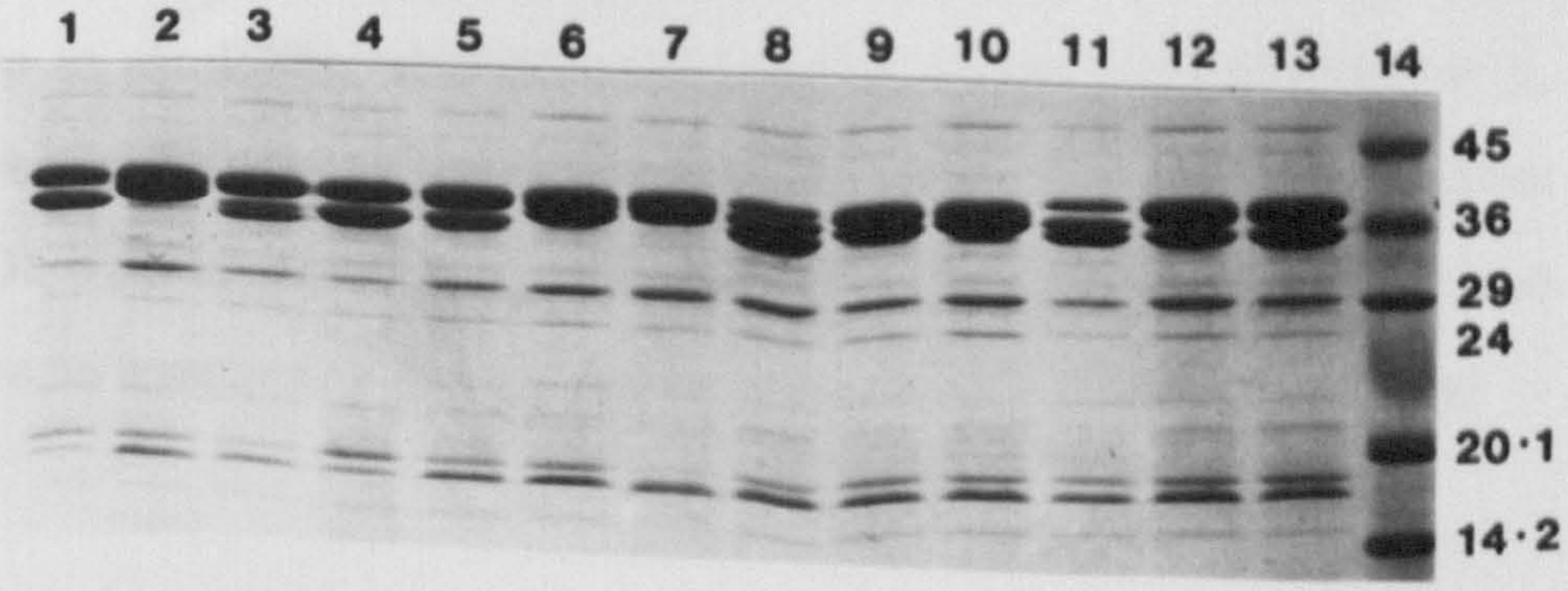
C


1 2 3 4 5 6 7 8 9 10 11 12 13 14
43
30
20·1
14·4

D


1 2 3 4 5 6 7 8 9 10 11 12 13 14
43
30
20·1
14·4

E


1 2 3 4 5 6 7 8 9 10 11 12 13 14
45
36
29
24
20·1
14·2

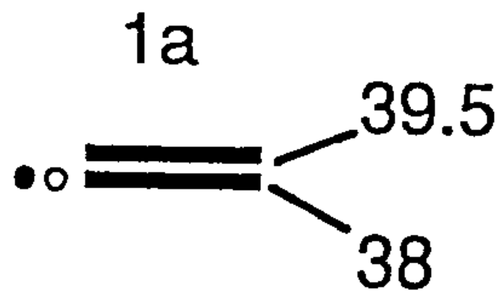
were designated, which respectively lacked and possessed this protein. A third subtype of OMP-type 2 was also designated, based on a slight variation of the 38 KDa PAP. In this OMP-type, type 2c, the molecular weight of this protein appeared to be slightly less than 38 KDa - about 37.5 KDa (compare OMP-type 4). It should be noted that OMP-types 2a and 3a and 2b and 3b were virtually identical after heating at 100^{o}C and could only be differentiated by examination of the proteins after heating at both 80^{o}C and 100^{o}C. For reasons discussed below OMP-subtypes probably do not indicate relatedness of isolates of a given OMP-type and their use here is meant to show observed differences and not to indicate possible relationships. A schematic representation of the OMP-types and OMP-subtypes is shown in Figure 16.

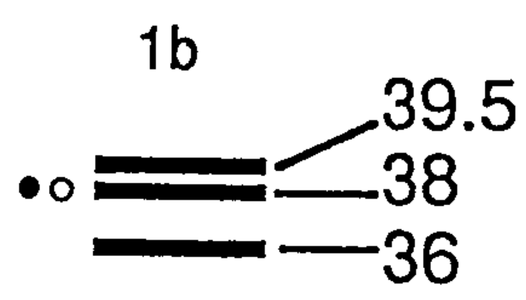
Of the 137 isolates examined thirty nine (28.4%) were of OMP-type 1, twenty two (16.1%) were of OMP-type 2, sixty nine (50.4%) were of OMP-type 3, five (3.6%) were of OMP-type 4 and two (1.5%) were of OMP-type 5. The OMP-types of the isolates examined are shown in Table 11.

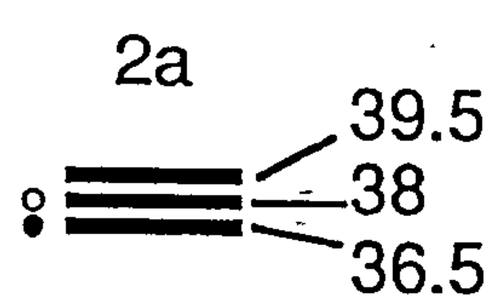
4.4 DISCUSSION

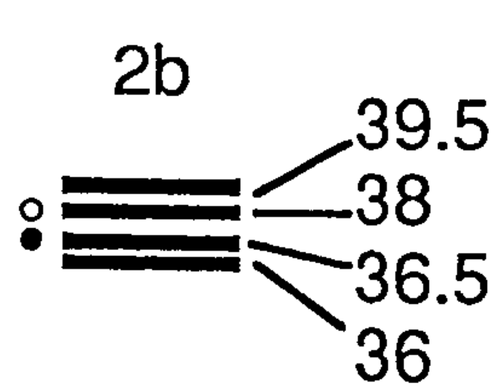
An absolute prerequisite for a typing scheme based on OMP profiles is that the OMPs remain stable after in vivo and in vitro passage. The OMP profiles of Y. ruckeri did not change after in vitro passage and there was no variation between individual colonies on solid medium. These findings were not unexpected because stability of OMPs after both in vivo and in vitro passage has been demonstrated in various other Gram-negative bacteria (Barenkamp et al., 1981; 1982; Mocca and Frasch, 1982; Blaser et al., 1983; Odumeru et al., 1983; Rapp et al., 1986). Although the stability of the OMP profiles of Y. ruckeri after in vivo passage was not examined, the fact that the European serotype O1 isolates of OMP-types 1 and 3 were geographically distinct and appeared to be temporally stable, suggested that the OMPs remain stable in

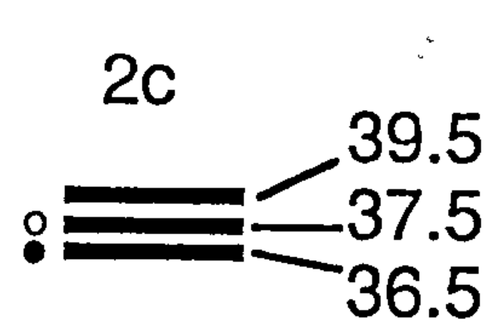
Figure 16. Outer membrane protein types of *Y. ruckeri*.

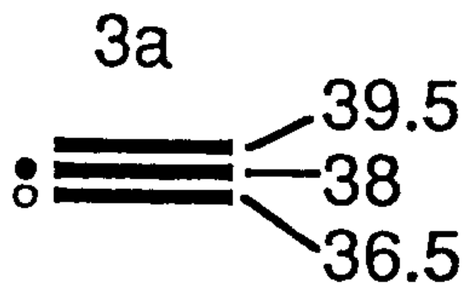

1a
39.5
38

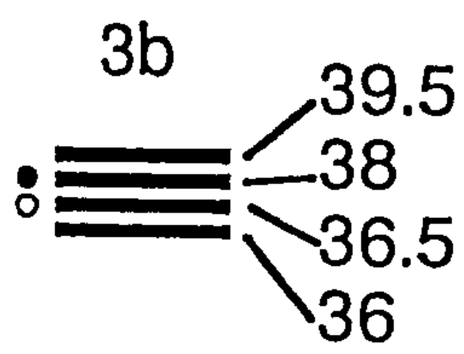

1b
39.5
38
36

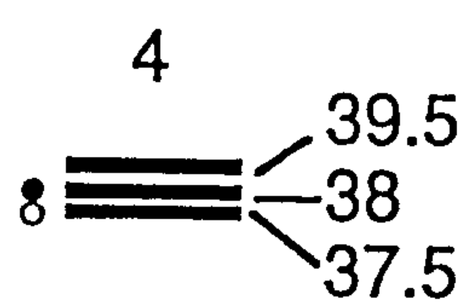

2a
39.5
38
36.5

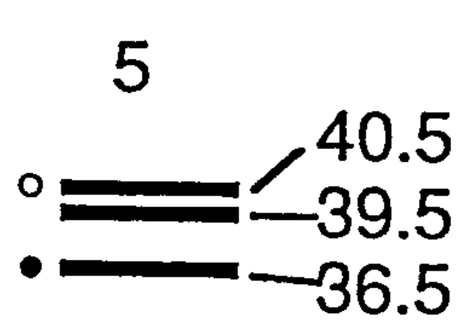

2b
39.5
38
36.5
36


2c
39.5
37.5
36.5


3a
39.5
38
36.5


3b
39.5
38
36.5
36


4
39.5
38
37.5


5
40.5
39.5
36.5

● = HMP

o = PAP

Table 11. Outer membrane protein types of isolates of Y. ruckeri.

OMP-type	Isolates
1a	RD10,RD14,RD30,RD34,RD36,RD44,RD48,RD56,RD128,RD130,RD132, RD134,RD148,RD150,RD156,RD176,RD184,RD186,RD192,RD246,RD248, RD250,RD252,RD256,RD258,RD260,RD264,RD266,RD268,RD270,RD272, RD274
1b	RD6,RD46,RD54,RD60,RD152,RD174
2a	RD20,RD24,RD26,RD28,RD38,RD58,RD64,RD68,RD158,RD160,RD188, RD284,RD294
2b	RD50,RD66,RD168
2c	RD62,RD154,RD162,RD194,RD278,RD290
3a	RD2,RD42,RD70,RD72,RD74,RD76,RD78,RD80,RD82,RD86,RD88,RD92, RD94,RD96,RD100,RD102,RD104,RD106,RD108,RD110,RD112,RD114, RD116,RD118,RD120,RD122,RD124,RD136,RD140,RD142,RD144,RD146, RD200,RD210,RD220,RD230,RD286,RD288,RD296,RD298,RD300,RD302, RD304,RD306,RD308,RD310,RD312,RD314,RD316,RD318,RD320,RD322, RD324,RD326,RD328
3b	RD4,RD16,RD32,RD40,RD84,RD90,RD98,RD126,RD138,RD170,RD172, RD178,RD180,RD182
4	RD22,RD190,RD280,RD282,RD292
5	RD52,RD166

vivo. There was, however, variation in the expression of a 39.5 KDa PAP according to the phase of the growth cycle. This porin protein was probably expressed in response to nutrient limitation since it was more abundant during stationary growth phase (when nutrients are a limiting factor) than during logarithmic growth phase. This variation also was not unexpected because variation in the expression of OMPs due to the phase of growth has been described in other Gram-negative bacteria (Loeb and Smith, 1980; Kelly and Parker, 1981; Blaser et al., 1983; Ferreira and Almeida, 1987).

Thus, the OMPs of Y. ruckeri were demonstrated to be stable enough for a typing scheme, based on inter-strain variation of certain proteins, to be considered practical. Variation between isolates in the expression of the 39.5 KDa PAP was avoided by harvesting the cells at approximately the same stage of the growth cycle. Slight variation, between isolates, in the abundance of this protein was perhaps due to slight differences in growth rate since the cells used for OMP analysis were harvested after a fixed period of time (i.e. overnight growth) and not at a fixed point of the growth cycle.

In addition to iron limitation (Section 7), phosphate limitation has also been shown to induce specific OMPs in Gram-negative bacteria. In E.coli K12, an OMP (the Pho E protein) is produced under conditions of phosphate limitation which has a similar molecular weight to the 39.5 KDa PAP in Y. ruckeri (Overbeeke and Lugtenburg, 1980b; Korteland et al., 1982). Like the Y. ruckeri PAP, the E.coli Pho E protein is also peptidoglycan-associated (Overbeeke and Lugtenberg, 1980b). It is possible, therefore, that the 39.5 KDa PAP of Y. ruckeri is the equivalent of the E.coli K12 Pho E phosphate-limited protein. This would need to be proven by further experimentation including the OMP-analysis of Y. ruckeri after growth under phosphate-limited conditions and comparison of the two proteins by immunological methods.

Inter-strain variation occurred in the possession of a 38/36.5 KDa HMP and in the possession of a 38/36.5 KDa PAP. The two molecular weight forms of each of these proteins were shown, by peptide mapping, to be virtually identical. These proteins were, therefore, slightly different forms of the same protein rather than different proteins. In some isolates (e.g. those of OMP-types 2c, 4 and 5) the 38/36.5 KDa PAP was replaced by one of 37.5 or 40.5 KDa although it was not confirmed whether these proteins were related to the 38/36.5 KDa PAPs. The change in electrophoretic mobility of the HMP from 31 KDa to 38 or 36.5 KDa was probably due to a conformational change in the protein after heating (Nakamura and Mizushima, 1976). At low temperature the protein probably has a β-structure but this probably changes to a α-helix structure (which binds more SDS and migrates more slowly through the gel) after heating. Presumably, the β-structure configuration of the 31 KDa protein is very much the same in all isolates of Y. ruckeri but the α-helix structure is slightly different in the 38 and 36.5 KDa forms of the protein. These facts indicate that the proteins differ only slightly, perhaps by only a few amino acids. Based on variation in the HMP and PAPs, isolates were assigned to one of five OMP-types, of which OMP-types 1, 2 and 3 comprised the majority (95%) of the isolates.

There was evidence to suggest that variation in the expression of the 36 KDa PAP was quantitative rather than absolute. First, the protein was abundant in some isolates but was present as only a faint band in other closely related isolates, e.g. Italian OMP-type 3a and 3b isolates and British OMP-type 1a and 1b isolates (Figures 11B and C). Second, isolates RD54 and RD176, and RD36 and RD60, which were different subcultures (from different laboratories) of the same original isolate (see Appendix 1), showed variation in the expression of the 36 KDa protein (Figures 13B and H, and Figures 13B and C). Third, OMPs of isolate RD6 prepared on different occasions showed variation in the expression of the 36 KDa protein (compare Figures 6A, B and C with Figure

13A). Expression of the 36 KDa OMP appeared, therefore, to be variable (intra-strain variation), and the protein probably has limited use in an OMP-typing scheme. It should be pointed out that this variation was not observed in the initial study of OMP-stability. The sub-division of OMP-types 1, 2 and 3 into subtypes, based on variation in the expression of this protein, should therefore be treated with reservation. Although the subdivision of OMP-types is maintained in Table 11, this is meant only to show observed differences within the OMP-types. The use of sub-type categories does not necessarily indicate relationships between isolates of a given OMP-type.

Unlike the major OMPs, which showed inter-strain variation, the minor OMPs (with a few exceptions) were shown to be extremely uniform. Variation in the minor OMPs of isolates RD64 and RD68, both serotype O2 isolates from West Germany, was useful in distinguishing these isolates from other isolates of the same serotype and OMP-type. Whereas variation in the major OMPs enabled the population structure of Y. ruckeri to be examined, homogeneity of the minor proteins was useful in distinguishing Y. ruckeri isolates from other species. For example, isolate RD164, originally described as a serotype IV isolate of Y. ruckeri (Stevenson and Airdrie, 1984a) but later shown not to be Y. ruckeri (De Grandis et al., 1984; 1988), possessed three major OMPs in the same molecular weight range as those of Y. ruckeri but had totally different minor OMPs (Figure 13G). Thus, OMP analysis may be useful not only for epizootiological studies of Y. ruckeri but may also be useful in taxonomic studies.

The relationship between biotypes, serotypes, and OMP-types of isolates of Y. ruckeri, the identification of clonal groups based on these relationships, and the use of these characteristics in the study of the epizootiology of Y. ruckeri, are discussed in the next Section. The relationships of these characteristics, and in particular the relationships of clonal groups based on these characteristics, to

the susceptibility of isolates to the bactericidal effects of rainbow trout serum and to virulence are discussed in Sections 8 and 9 respectively.

Section 5

Clonal Analysis of Yersinia ruckeri based on Biotypes, Serotypes and OMP-types and the Combined Use of these Characteristics as an Epizootiological Tool

Clonal Analysis of Yerninia ruckeri based on Biotypes, Serotypes and OMP-types and the Combined Use of these Characteristics as an Epizootiological Tool

5.1 Introduction

The clone concept of bacterial population structure was put forward for E.coli to explain the specific association of a small number of O:K:H serotypes and biotypes among enterotoxigenic strains and because of the rarity of these serobiotypes among non-disease isolates (Ørskov et al., 1976). Subsequently, evidence for the clonal structure of E.coli came from two sources. First, multilocus enzyme electrophoretic analysis of large numbers of strains isolated over long periods of time showed that certain frequently-recovered genotypes represented bacterial clones which were stable over broad geographic areas (Selander and Levin, 1980; Caugant et al., 1981; Ochman and Selander, 1984). Second, an analysis of OMP patterns, supplemented by the use of traditional microbiological methods, of E.coli strains belonging to defined serotypes implicated in extra-intestinal disease processes demonstrated the existence of distinct geographically widespread, temporally stable clones within certain serotypes (Achtman et al., 1983). These conclusions were later confirmed by enzyme electrophoretic analysis (Ochman and Selander, 1984). Multilocus enzyme electrophoresis has subsequently been used for the clonal analysis of populations of Bordetella spp. (Musser et al., 1986), H. influenzae (Musser et al., 1985; Porras et al., 1986), H. pleuropneumoniae (Musser et al., 1987), L. pneumophila (Selander et al., 1985), N. meningitidis (Caugant et al., 1986a ; 1986b) as well as Y. ruckeri (Schill et al., 1984). In addition, OMP analysis has been used to demonstrate the clonal nature of enteropathogenic E.coli strains

(Stenderup and Orskov, 1983), E.coli O2:K1 strains (Achtman et al., 1986), H. influenzae (Porras et al., 1986), H. pleuropneumoniae (Rapp et al., 1986; Musser et al., 1987) and N. meningitidis (Caugant et al., 1987).

It was suggested by Achtman et al. (1983) that OMP patterns were better suited for recognizing bacterial clones than other traditional methods such as metabolic properties, production of haemolysin and colicin, and plasmid content. However, after a clonal analysis of E.coli O2:K1 strains from diseased humans and animals it was concluded that clonal groupings were best recognized by the combination of OMP and electrophoretic enzyme patterns (Achtman et al., 1986). In a study of the population structure of H. influenzae Porras et al. (1986) compared multilocus enzyme electrophoresis, OMP profiles, capsule antigens and biochemical characteristics (biotypes), and concluded that none of the methods alone would allow unambiguous identification of clones although multilocus enzyme electrophoresis was the most discriminating method. Clonal diversity in H. pleuropneumoniae was also investigated using a combination of OMP profiles, enzyme electrophoresis patterns and serotypes (Musser et al., 1987). The relative merits of isoenzyme analysis and OMP patterns in the study of bacterial populations was discussed by Achtman and Pluschke (1986). Although both methods have their advantages and disadvantages these authors strongly recommended including OMP analysis in population studies.

In a multilocus isoenzyme electrophoretic study of 47 isolates of Y. ruckeri from widespread geographic locations in North America, as well as from Australia, Schill et al. (1984) concluded that the population structure was clonal with one clone, accounting for 42 of the isolates, predominating. Each electrophoretic type differed at no more than two of the 15 loci examined and 42 isolates were of the same electrophoretic type; in total there were only four electrophoretic types and variation occurred in only three of the 15 loci

examined. By comparison, in 46 E.coli O2:K1 strains variation was described in nine of the 15 enzyme loci examined and there were 15 electrophoretic types (Achtman et al., 1986). Also, in 135 isolates of H. pleuropneumoniae variation occurred in 13 of 15 enzyme loci and 32 electrophoretic types were distinguished (Musser et al., 1987). Multilocus enzyme electrophoretic analysis therefore has limited potential as an epizootiological tool in the study of Y. ruckeri.

An analysis of the biotypes, serotypes and OMP-types of Y. ruckeri was carried out with the aims of demonstrating relatedness between isolates of Y. ruckeri and of identifying clonal groups. The geographic distribution of clonal groups (or otherwise related isolates) could then be examined, thus leading to a better understanding of the epizootiology of this organism. In addition, the identification of virulent and avirulent clonal groups would further our understanding of the virulence mechanisms of this bacterium. The analysis of biotypes, serotypes and OMP-types is based on the results of Sections 1, 2 and 4.

5.2 Relationship between biotypes, serotypes and OMP-types

Whereas OMP-types 3 and 4 were associated only with serotype O1 and OMP-type 5 was associated only with serotype O7, this was not the case for OMP-types 1 and 2. Thus, OMP-type 1 included isolates of serotypes O1, O2, O5, O6 and O7, and OMP-type 2 included isolates of serotypes O1, O2, O5 and O6 (Table 12). These observations were not unexpected because similar work with E.coli has shown that isolates having the same OMP pattern have different O-serotypes and isolates having the same O-serotype have different OMP-patterns (Jann and Jann, 1980; Overbeeke and Lugtenberg, 1980a; Achtman et al., 1983). It was interesting that OMP-types 3 and 4 were associated only with serotype O1 whereas OMP-types 1 and 2 were associated with the full range of

Table 12. Geographic origins, biotypes, serotypes and OMP-types of isolates of Y. ruckeri.

Geographic Origin	Biotype	Serotype	OMP type	No. of isolates	Isolates
Australia	1	01	1a	4	RD44,RD156,RD184,RD186
Canada	1	02	1a	1	RD176
	1	05	1a	1	RD192
Denmark	1	07	1a	1	RD150
U.K.	2	01	1a	22	RD10,RD14,RD30,RD128,RD130,RD132,RD134, RD148,RD246,RD248,RD250,RD252,RD254,RD256, RD258,RD260,RD264,RD266,RD268,RD270,RD272, RD274
	1	02	1a	1	RD34
U.S.A.	1	02	1a	1	RD56
	1	06	1a	1	RD48
	1	07	1a	1	RD36
Australia	1	UT	1b	1	RD46
Canada	1	02	1b	1	RD54
	2	02	1b	1	RD174
U.K.	2	01	1b	2	RD6,RD152
U.S.A.	1	07	1b	1	RD60
Australia	1	01	2a	1	RD158
Finland	1	01	2a	4	RD20,RD24,RD26,RD160
France	1	02	2a	1	RD284
Norway	1	02	2a	1	RD294
U.K.	1	05	2a	1	RD28
U.S.A.	1	01	2a	1	RD38
	1	02	2a	1	RD58
	1	05	2a	1	RD188
West Germany	1	02	2a	2	RD64,RD68
U.S.A.	1	02	2b	1	RD168
	1	05	2b	1	RD50
West Germany	1	06	2b	1	RD66
Canada	1	06	2c	1	RD194
Finland	1	06	2c	1	RD162
France	1	01	2c	1	RD278
Norway	2	01	2c	1	RD154
U.K.	1	05	2c	1	RD290
West Germany	1	01	2c	1	RD62
Bulgaria	1	01	3a	13	RD296,RD298,RD300,RD302,RD304,RD306,RD308, RD310,RD312,RD314,RD316,RD318,RD320
Denmark	1	01	3a	7	RD112,RD114,RD116,RD118,RD120,RD122,RD124
France	1	01	3a	4	RD136,RD140,RD142,RD144
Italy	1	01	3a	21	RD74,RD76,RD78,RD80,RD82,RD86,RD88,RD92, RD94,RD96,RD100,RD102,RD104,RD106,RD108, RD110,RD146,RD200,RD210,RD220,RD230
South Africa	1	01	3a	2	RD286,RD288
Switzerland	1	01	3a	4	RD322,RD324,RD326,RD328
U.S.A.	1	01	3a	1	RD42
West Germany	1	01	3a	2	RD70,RD72
Reference	2	01	3a	1	RD2

Canada	1	01	3b	2	RD178,RD182
France	1	01	3b	1	RD138
Italy	1	01	3b	3	RD84,RD90,RD98
U.K.	2	01	3b	1	RD16
U.S.A.	1	01	3b	3	RD40,RD170,RD172
Reference	1	01	3b	3	RD4,RD32,RD180
Vaccine	1	01	3b	1	RD126
Canada	1	01	4	1	RD190
Finland	1	01	4	1	RD22
France	1	01	4	2	RD280,RD282
Norway	1	01	4	1	RD292
Canada	1	07	5	2	RD52,RD166

serotypes. This could suggest a direct association between types 3 and 4 OMPs and type O1 LPS. This is especially significant in the case of serotype O1, OMP-type 3 isolates which are highly virulent (Section 9).

Outer membrane protein analysis was able to differentiate between isolates of a given serotype. Thus, serotype O1 isolates consisted of four OMP-types (see below), serotype O2 isolates consisted of five isolates of OMP-type 1 and six isolates of OMP-type 2, serotype O5 isolates consisted of one isolate of OMP-type 1 and four isolates of OMP-type 2, serotype O6 isolates consisted of one isolate of OMP-type 1 and three isolates of OMP-type 2 and serotype O7 isolates consisted of three isolates of OMP-type 1 and two isolates of OMP-type 5 (Table 12).

When biotype was taken into consideration serotype O1 isolates of OMP-type 1 could be differentiated into two distinct groups, and serotype O1, OMP-type 2 isolates, similarly, could be divided into two groups (Table 12). This will be discussed in more detail below.

5.3 The clonal nature of serotype O1 isolates

Serotype O1 isolates consisted of four OMP-types (OMP-types 1, 2, 3, and 4). In addition, two groups of serotype O1 isolates within each of the OMP-types 1 and 2 could be differentiated on the basis of having different biotypes, i.e. biotypes 1 and 2. These six groups of serotype O1 isolates were considered as individual clonal groups which were designated clones 1 to 6. In addition to similarities in biotype, serotype and OMP-type, isolates of these clonal groups were also related in their susceptibility to the bactericidal effect of rainbow trout serum (Section 8) and in their virulence (Section 9). Thus, isolates of clones 1, 3, 4 and 6 were serum-sensitive and avirulent whereas isolates of clones 2 and 5 were serum-resistant and virulent. This will be discussed in

more detail later (Sections 8 and 9). It should also be pointed out that variation of serotype O1 isolates, in bacteriophage sensitivity and in resistance to polymyxin B (Stevenson and Airdrie, 1984b; De Grandis and Stevenson, 1985), may be related to the clonal variation described above. The clonal relationships of the serotype O1 isolates are shown in Table 13.

That the population structure of Y. ruckeri is clonal was suggested by the facts that three of the clones (clones 3, 5 and 6) were geographically widespread in distribution and had been isolated over a relatively long period of time (at least ten years in the case of clone 3 and 20 years in the case of clone 5). Widespread geographic distribution and temporal stability are strong indicators of the clonal nature of bacterial populations (Achtman et al., 1986; Caugant et al., 1987; Musser et al., 1987). Although isolates of clone 2 were isolated only in the U.K., the facts that the U.K. is geographically isolated and that there are strict restrictions on the import and export of fish (which will reduce the spread of the organism), could account for this. Furthermore, a relatively small number of North American serotype O1 isolates were examined, and this clone could also be present in North America. It was perhaps surprising that clone 1 isolates were identified only in Australia. This may indicate that this variant has arisen in Australia (and is not present elsewhere), or that it is present elsewhere but was not included in the isolates studied. The single clone 4 isolate may also be a recent variant. The low virulence of these latter two clones could also account for their apparent limited distribution although isolates of clone 6 were also of low virulence (Section 9).

Isolates of OMP-types 1 and 2 belonging to serotypes other than serotype O1 were not included in the clonal grouping scheme because the precise clonal relationship of these isolates to serotype O1 isolates was not known. In addition to differences in LPS structure (Section 3) these serotypes are also known to differ from serotype O1 isolates in their plasmid content (Cook and Gemski,

Table 13. Clonal relationship and geographic origins of serotype O1 isolates of Y. ruckeri based on biotype and OMP-type.

Clone designation	Biotype	OMP-type	No. of isolates	Geographic origin
1	1	1	4	Australia
2	2	1	24	U.K.
3	1	2	1	Australia
			4	Finland
			1	France
			1	West Germany
			1	U.S.A.
4	2	2	1	Norway
5	1	3	13	Bulgaria
			7	Denmark
			5	France
			24	Italy
			4	Switzerland
			2	West Germany
			2	South Africa
			2	Canada
			4	U.S.A.
6	1	4	1	Finland
			2	France
			1	Norway
			1	Canada

1982; De Grandis and Stevenson, 1982; Toranzo et al., 1983). In a clonal analysis of H. pleuropneumoniae, Rapp et al. (1986) demonstrated an association between serotype and OMP profile and suggested that these character combinations marked clones. Two bacterial clones possessing identical OMP patterns are not necessarily closely related (Achtman and Pluschke, 1986). In E.coli, however, all bacteria assigned to a common OMP pattern proved to be closely related even when their serotypes were totally different (Achtman et al., 1983; 1986). In the case of Y. ruckeri, therefore, isolates of OMP-types 1 and 2 belonging to different serotypes may or may not be closely related. The relationship of these isolates to each other and to serotype O1 isolates requires further investigation.

5.4 The epizootiology of Y. ruckeri based on variations in biotype, serotype and OMP-type

Outer membrane protein analysis combined with biochemical analysis (biotyping) and serological analysis (serotyping) enabled the identification of six clonal groups among serotype O1 isolates. Isolates belonging to clone 1 were restricted to Australia; no representative isolates of this clone were identified in North America or Europe. Isolates belonging to clone 2 were identified only in the U.K. Isolates belonging to clone 3 were identified in north-west Europe as well as in Australia and the U.S.A. Isolates belonging to this clone were obtained from Finland, France and West Germany, but not from Bulgaria, Denmark, Italy, Norway or Switzerland. A single clone 4 isolate was identified from Norway. With the exception of six isolates from Canada and the U.S.A. all of the isolates belonging to clone 5 originated from mainland Europe, including Bulgaria, Denmark, France, Italy, Switzerland and West Germany, as well as from South Africa. No clone 5 isolates were obtained from Australia, Finland, Norway or the U.K. The larger number of clone 5 isolates from Europe, compared to the number from North America, was due

to the method of selection of the latter isolates and did not represent the actual distribution of this clonal group (which is probably very abundant in North America also). Clone 6 comprised, with one exception, sorbitol-fermenting serotype O1 isolates from Canada, Finland and France. The geographic distribution of the clonal groups is summarised in Table 13.

The geographic distribution of serotype O1 isolates of Y. ruckeri in Europe therefore appears to have three major components. First, the highly virulent clone 2 appears to be restricted to the U.K. where it has been responsible for the majority of disease outbreaks and has spread rapidly. Second, the highly virulent clone 5, which has been responsible for most of the disease outbreaks in Europe, is restricted to mainland Europe but appears to be limited (so far) in its distribution to Bulgaria, Denmark, France, Italy, Switzerland and West Germany. With the exception of Bulgaria these countries all share common borders and it is relatively easy for the organism to be spread from one country to another. It is noteworthy that clone 5 isolates have not been responsible (to date) for any significant disease outbreaks in Norway, Finland or the U.K. Third, the avirulent clone 3 is restricted to north-west Europe including Finland, France and West Germany. Compared to the previous two clones, however, it has not been responsible for any significant disease outbreaks. The avirulent clones 4 and 6 are relatively unimportant clones which have not been responsible for significant disease outbreaks. It is notable, however, that the geographic distribution of clone 6 (it has been isolated in Finland, France and Norway) is very similar to that of clone 3. Not surprisingly the recent outbreak of disease in South Africa was caused by a virulent clone 5 organism, whereas Australia seems to have its own unique avirulent clone, clone 1. Considering the biochemical homogeneity of Y. ruckeri isolates, which suggests that the Australian strains probably did not originate there, it is surprising that the Australian strains are avirulent and are not found elsewhere (according to this study). The observations confirmed the findings of Humphrey et al. (1987) who

suggested that the virulent "Hagerman" strain (clone 5) of Y. ruckeri is exotic to Australian salmonids.

Outer membrane protein analysis was able to distinguish between a biotype 2, serotype O1 Norwegian isolate (RD154) (clone 4) and the biotype 2, serotype O1 U.K. isolates (clone 2). This observation was extremely relevant in view of fish movements between the U.K. and Norway in the past which have been blamed for the introduction of furunculosis into Norway. Whereas biochemical and classical serological methods indicated that these strains were the same, LPS and OMP analysis indicated that they were different. In addition, the U.K. serotype O2 isolate (RD34) was shown to be of a different OMP type (OMP-type 1) from other European serotype O2 isolates (RD64, RD68, RD284, RD294) which were of OMP-type 2. These observations, together with the facts that the clone 2 isolates were restricted to the U.K., that serotype O5 isolates were identified in the U.K. but not in any other part of Europe (Section 2), and that clone 5 isolates were abundant in mainland Europe but were not recovered from the U.K. suggests that the origin and epizootiology of Y. ruckeri in the U.K. is different from that in the rest of Europe.

Although OMP analysis was able to differentiate between isolates of a given serotype and was extremely useful in the study of serotype O1 isolates, in which clonal groups were identified, and for differentiating U.K. isolates from other European isolates, the technique was limited in its usefulness for the analysis of non-serotype O1 isolates. This was due to the fact that OMP-types 1 and 2 were associated with all five of the O-serotypes. However, this problem may have been due to the small number of non-serotype O1 isolates examined. Analysis of the OMP-types of non-serotype O1 isolates would probably have been more useful if a larger number of isolates had been studied. Then, OMP analysis may have been able to detect geographic variation in the distribution of non-serotype O1 isolates, as was the case for serotype O1 isolates.

5.5 Comparison of outer membrane protein analysis with multilocus isoenzyme electrophoresis in population studies of Y. ruckeri

Thirteen of the isolates examined by Schill et al. (1984) using multilocus enzyme electrophoresis were examined by OMP analysis in this study. It was demonstrated that of eight isolates belonging to Schill's clone 1, four were of OMP-type 1, two were of OMP-type 2 and two were of OMP-type 3; of two isolates belonging to Schill's clone 2, one was of OMP-type 2 and the other was of OMP-type 5. The remaining isolates of Schill's clone 3 (two isolates) and clone 4 (one isolate) were both of OMP-type 1 (Table 14). It seems, therefore, that OMP-analysis is more useful than multilocus enzyme electrophoresis in population studies of Y. ruckeri. Nevertheless, it would be interesting to use this technique in a further comparison of European isolates, and in particular to examine the six serotype O1 clonal groups.

Table 14. Comparison of isoenzyme electrophoretic types amd OMP-types of isolates of Y. ruckeri.

Isolate	Serotype	Clonal Group[a] (ET)	OMP-type
RD44	01	1	1
RD56	02	1	1
RD48	06	1	1
RD46	UT	1	1
RD58	02	1	2
RD50	05	1	2
RD40	01	1	3
RD42	01	1	3
RD38	01	2	2
RD52	07	2	5
RD36	07	3	1
RD60	07	3	1
RD54	02	4	1

[a] = clonal groups designated by Schill et al. (1984).

UT = untypable

ET = electrophoretic type

PART II

PATHOGENESIS

Section 6

Western-blot Analysis of the Antibody Response

in Rabbits and in Rainbow Trout to

Outer Membrane Antigens of Yersinia ruckeri

Western-blot Analysis of the Antibody Response

in Rabbits and in Rainbow Trout to

Outer Membrane Antigens of Yersinia ruckeri

6.1 INTRODUCTION

The surface components of pathogenic bacteria, including OMPs, LPS and capsule polysaccharides as well as pili and flagella, play important roles in their pathogenicity (Smith, 1977; Buchanan and Pearce, 1979). Surface structures are important in attachment, invasion, serum resistance, uptake of iron, and resistance to phagocytosis. Surface structures will be recognized by the host immune system and antibodies will be produced against them and, depending on the nature of the antigen, some of these antibodies will have a protective function.

Analysis of the immunogenicity of bacterial surface structures will identify those components which are immunogenic and which may generate protective antibodies. Such surface structures will be very important in the pathogenesis of infection and will also be relevant to the development of vaccines. For example, antibodies against both OMPs (Kuusi et al., 1979; Hansen et al., 1982; Montaraz et al., 1985; Udhayakumar and Muthukkaruppan, 1987) and LPS (Svenson et al., 1979; 1981; Saxen et al., 1986) of pathogenic bacteria are protective in animal models.

Antigenic heterogeneity of OMPs may also be used for serotyping bacteria and can be used in epidemiological investigations. Murphy and Apicella (1985) developed a serotyping system for nontypable H. influenzae based on antigenic

differences of OMPs and Erwin and Kenny (1984) examined OMP antigenicity in H. influenzae type b isolates and identified 13 antigenic groups. Antigenic variation in certain OMPs of N. meningitidis, identified by monoclonal antibodies, forms the basis of a complex serotyping system which is extremely important in epidemiological investigations (Abdillahi and Poolman, 1988).

The objective of this part of the study was to identify important antigenic components of the OM of Y. ruckeri, using Western-blotting, by examining the antibody response to whole cells in rabbits and in rainbow trout. The response to OMPs was examined in rabbits (the response to LPS is described in Section 3) and the response to both OMPs and LPS was examined in rainbow trout. An analysis of antigenic heterogeneity in isolates of different serotypes and OMP-types might account for the differences observed in the virulence of isolates (Section 9). In particular, differences in the immune response in rainbow trout to virulent and avirulent isolates could be important in identifying virulence determinants. Such an analysis could also have epizootiological significance.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial isolates

The immunogenicity in rabbits of the OMPs of 56 isolates was examined by Western-blotting using whole-cell antisera against isolates of each of the five O-serotypes. The isolates examined are shown in Table 15. In addition, the immunogenicity in rainbow trout of both the OMPs and LPS of isolates RD6, RD20, RD124, RD154, RD170, RD34, RD168, RD28 and RD66 was examined using homologous and heterologous whole-cell antisera.

6.2.2 Preparation of hyperimmune rabbit antisera

Preparation of hyperimmune rabbit antisera against whole cells of isolates of serotypes O1, O2, O5, O6 and O7 is described in Section 2.

6.2.3 Preparation of hyperimmune rainbow trout antisera

Rainbow trout of average weight 1200 g, maintained in dechlorinated mains water at ambient temperature (10-13^{o}C) and fed twice daily, were inoculated with isolates RD6, RD20, RD124, RD154, RD170 (serotype O1), RD34, RD168 (serotype O2), RD28 (serotype O5) and RD66 (serotype O6). Bacteria were grown overnight in 20 ml TSB at 22^{o}C and were killed by adding formalin to 0.5% (v/v) and allowing to stand for 24 h. Cells were washed twice and resuspended in 0.5 ml 0.85% saline, and were thoroughly mixed with the same volume of Freund's Complete Adjuvant (FCA) (Sigma). Fish were anaesthetized by placing them in aquarium water containing a 1:10,000 dilution of benzocaine (Sigma). After withdrawing 0.5 ml of blood by caudal venipuncture for preimmune sera, the fish were inoculated by intraperitoneal injection of 0.5 ml bacteria (approximately 10^{9} - 10^{10} cells) in FCA; two fish were injected with each isolate. A second identical injection was given six weeks later and the fish were bled by caudal venipuncture six weeks after that. Blood was allowed to clot at room temperature for 1 h and was centrifuged at 1000 x g for 5 min. The serum was removed, filter sterilized through a 0.45 μm Millipore filter, and stored in 1 ml aliquots at -70^{o}C.

6.2.4 Antibody response in rabbits to outer membrane proteins

Outer membrane proteins were separated by SDS-PAGE as described in Section 4. Twenty microlitres of OM sample (original OM preparation diluted 1:5), heated at 100^{o}C for 5 min, were applied to each lane. After completion

of electrophoresis proteins were transferred to nitrocellulose and probed with rabbit anti-Y. ruckeri antiserum as described in Section 3. Primary rabbit antisera were used at a dilution of 1:250 in antibody buffer and incubation was for 2 h at 22°C; preimmune rabbit antiserum was used as a control. Secondary antibody (conjugate) incubation and development were as described in Section 3. After electrophoretic transfer gels were stained with Coomassie blue and destained (Section 4) to assess the effectiveness of protein transfer. In some cases, nitrocellulose membranes were stained with Coomassie blue (5 min) and destained also to assess the effectiveness of protein transfer.

6.2.5 Antibody reponse in rainbow trout to lipopolysaccharides and outer membrane proteins

Lipopolysaccharides and OMPs were separated by SDS-PAGE as described in Sections 3 and 4 respectively. Twenty microlitres of sample were applied to each lane in both cases, and OMP samples were previously heated at 100°C for 5 min. After completion of electrophoresis LPS and OMPs were transferred to nitrocellulose as described in Section 3. After blocking, nitrocellulose membranes were incubated overnight at 22°C with a 1:100 dilution of primary rainbow trout antiserum; preimmune rainbow trout serum was used as a control. After two washes with TTBS, the membranes were incubated for 4 h at 22°C with a 1:250 dilution of rabbit anti-rainbow trout antibody (kindly provided by M. Tatner, Institute of Aquaculture). Subsequent reaction with horseradish peroxidase-labelled goat anti-rabbit IgG and development were as described in Section 3.

6.3 RESULTS

6.3.1 Effectiveness of electrophoretic transfer and comparison of preimmune and hyperimmune rabbit and rainbow trout antisera

Transfer of proteins to nitrocellulose was demonstrated to be incomplete, by Coomassie blue staining of the gels after electrophoretic transfer, although the number of proteins incompletely transferred was small (results not shown). In particular, transfer of the HMP may not have been complete. Staining strips of nitrocellulose membrane with Coomassie blue was not particularly effective for detecting proteins transferred to the membrane. This was later found to be due to incorrect procedure.

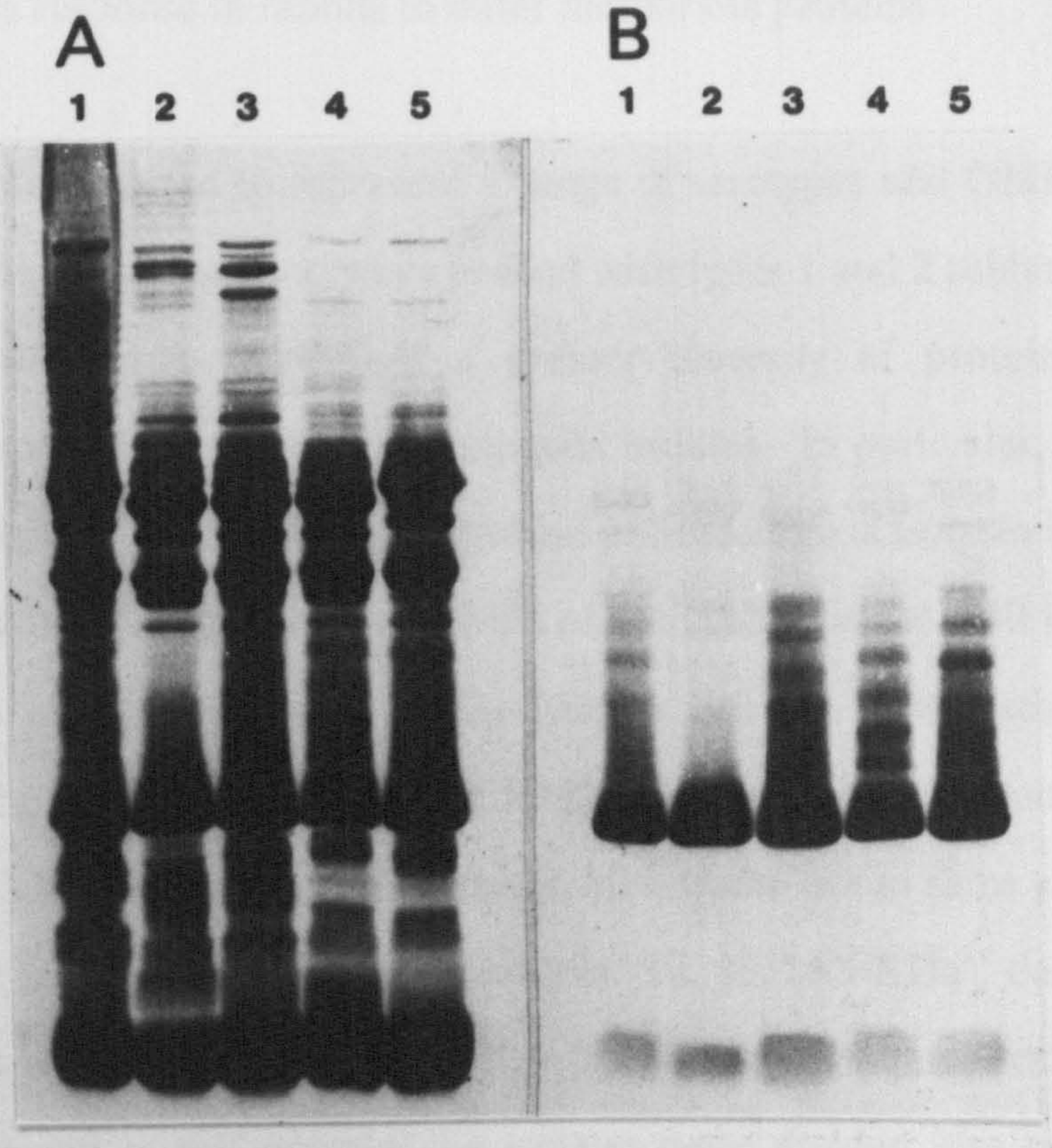
Western-blotting carried out with preimmune rabbit and rainbow trout sera revealed some reactivity against certain OM components including the major OMPs (Figure 17). In particular, there was strong activity in rabbit serum against protein components in the molecular weight range 20-30 KDa (estimated from Coomassie blue-stained gels). Reactions against the major OMPs were very weak in both the rabbit and the rainbow trout sera. There were also very weak reactions, in both rabbit and rainbow trout sera, to a low-molecular-weight component which was possibly the lipid A-oligosaccharide core region of LPS (Figures 17B and D). In hyperimmune type 1 rabbit and rainbow trout antisera, however, there was much more extensive reactivity with OMPs, including the major OMPs. In the homologous isolates there was also reactivity against LPS although this was much more evident in the rabbit antiserum than in the rainbow trout antiserum (Figures 17A and C).

Figure 17. Western-blots showing reactions of outer membrane components of representative isolates of serotypes 01, 02, 05, 06 and 07 with hyperimmune and preimmune rabbit and rainbow trout antisera.

(A) and (B) represent reactions with hyperimmune type 1 (A) and preimmune (B) rabbit antiserum.

(C) and (D) represent reactions with hyperimmune type 1 (C) and preimmune (D) rainbow trout antiserum.

Lanes 1 - 5 represent outer membranes of isolates RD170 (serotype 01, OMP-type 3), RD168 (serotype 02, OMP-type 2), RD28 (serotype 05, OMP-type 2), RD194 (serotype 06, OMP-type 2) and RD150 (serotype 07, OMP-type 1), respectively.


A
1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5
D
1 2 3 4 5

6.3.2 Antibody response in rabbits to outer membrane proteins

Fifty six isolates, selected to represent a range of serotypes and OMP-types as well as differences in virulence, were probed with types 1 and 2 rabbit antisera. The type 1 antiserum recognized a greater diversity of proteins in the homologous isolates than in the heterologous isolates. In particular, OMPs of molecular weight >40 KDa were recognized in homologous isolates but not in heterologous isolates. At least four OMPs of molecular weight >40 KDa were recognized in most of the serotype O1 isolates but not in isolates of other serotypes. The high-molecular-weight HMP, and to a lesser extent the low-molecular-weight HMP, were recognized in all isolates to the same extent and the molecular weight variation in this protein (i.e. 38/36.5 KDa), described in Section 4, was clearly evident (Figures 18A-G). Based on the molecular weight variation observed in this protein, the isolates were divided into one of two OMP-immunotypes which were designated OMP immunotypes 1 and 2 (Table 15). By contrast, type 2 antiserum recognized very few proteins of the heterologous isolates, particularly those proteins of molecular weight >40 KDa and the HMP, although the reactions with the homologous isolates were extensive (Figures 18H and I). In fact, type 2 antiserum recognized little more in the heterologous isolates than did the preimmune serum (compare Figures 17B and 18H). Type 5 antiserum was reacted with selected isolates and also recognized a large number of proteins in homologous isolates (Figure 18J) but the reactions with heterologous isolates were weaker. High-molecular-weight proteins were not recognized and the HMP was weakly antigenic (results not shown). Types 6 and 7 antisera were not used extensively but gave similar reactions to the type 1 antiserum (see below). The 39.5 and 38/36.5 KDa PAPs were not recognized by type 1 antiserum although there was some recognition of these proteins in homologous isolates by types 2, 5, 6 and 7 antisera (see below).

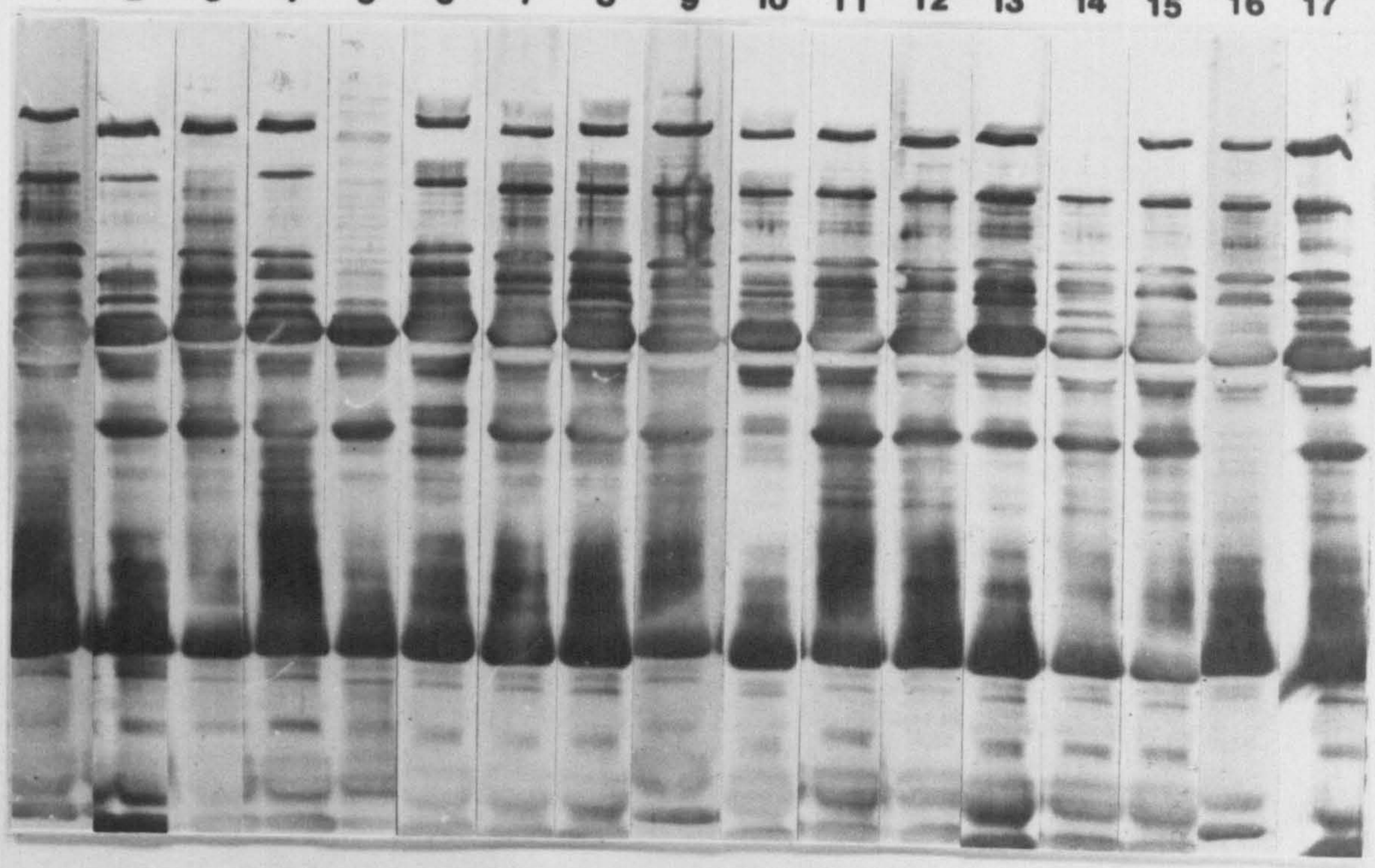
Figure 18. Western-blots showing reactions of outer membrane components of representative isolates of serotypes 01, 02, 05, 06 and 07 with types 1, 2 and 5 rabbit antisera.

(A). Reactions of serotype 01 isolates of OMP-types 1 (lanes 1 - 5) and 3 (lanes 6 - 17) with type 1 antiserum.
Lanes 1 - 17 represent isolates RD6, RD44, RD156, RD184, RD186, RD70, RD86, RD88, RD124, RD136, RD42, RD170, RD172, RD178, RD182, RD180 and RD288, respectively.

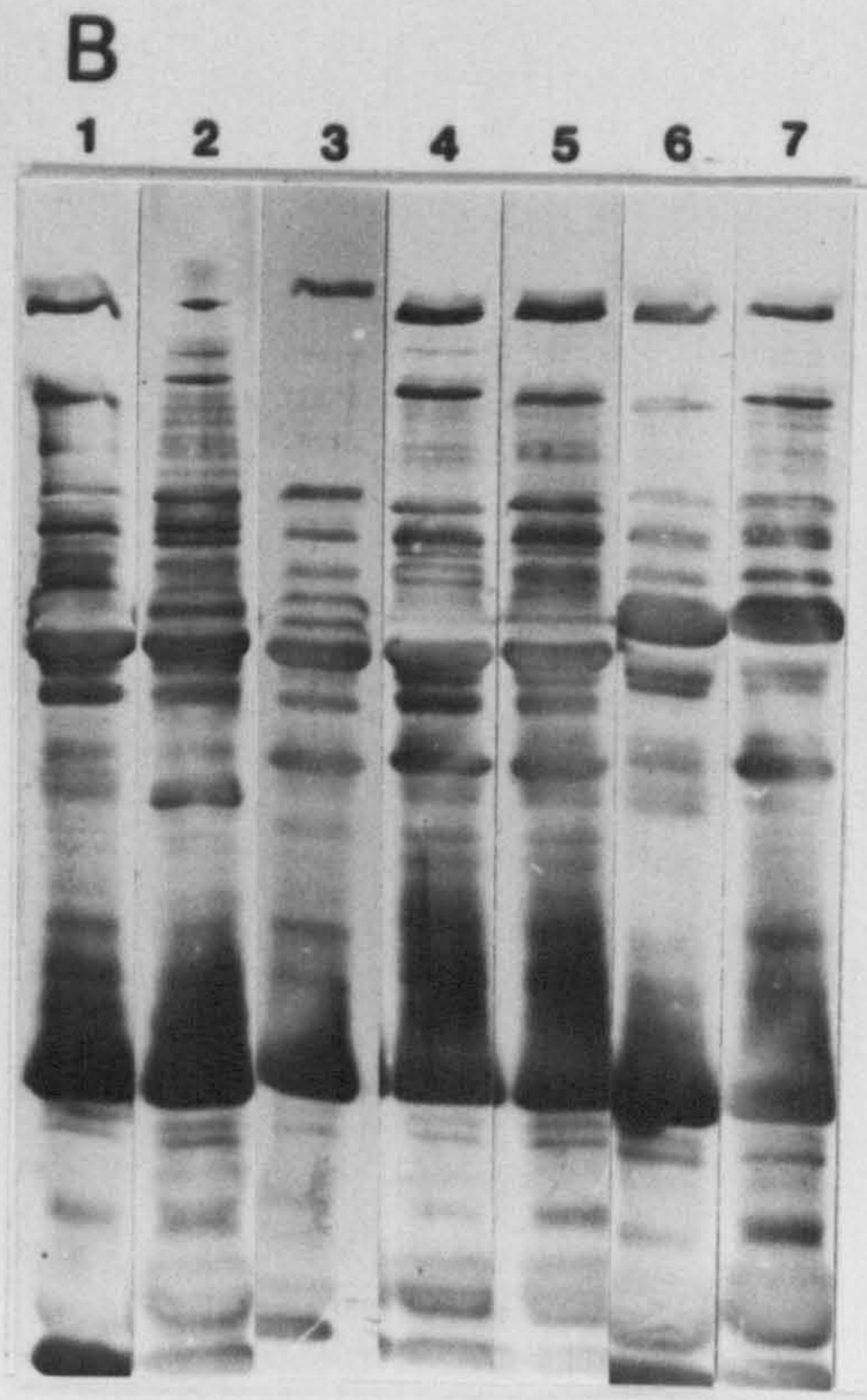
(B). Reactions of serotype 01 isolates of OMP-types 2 (lanes 1 - 5) and 4 (lanes 6 - 7) with type 1 antiserum.
Lanes 1 - 7 represent isolates RD20, RD62, RD154, RD38, RD158, RD22 and RD190, respectively.

(C). Reactions of serotype 02 isolates of OMP-types 1 (lanes 1 - 4) and 2 (lanes 5 - 7) with type 1 antiserum.
Lanes 1 - 7 represent isolates RD34, RD54, RD56, RD174, RD64, RD58 and RD168, respectively.

A



B



C

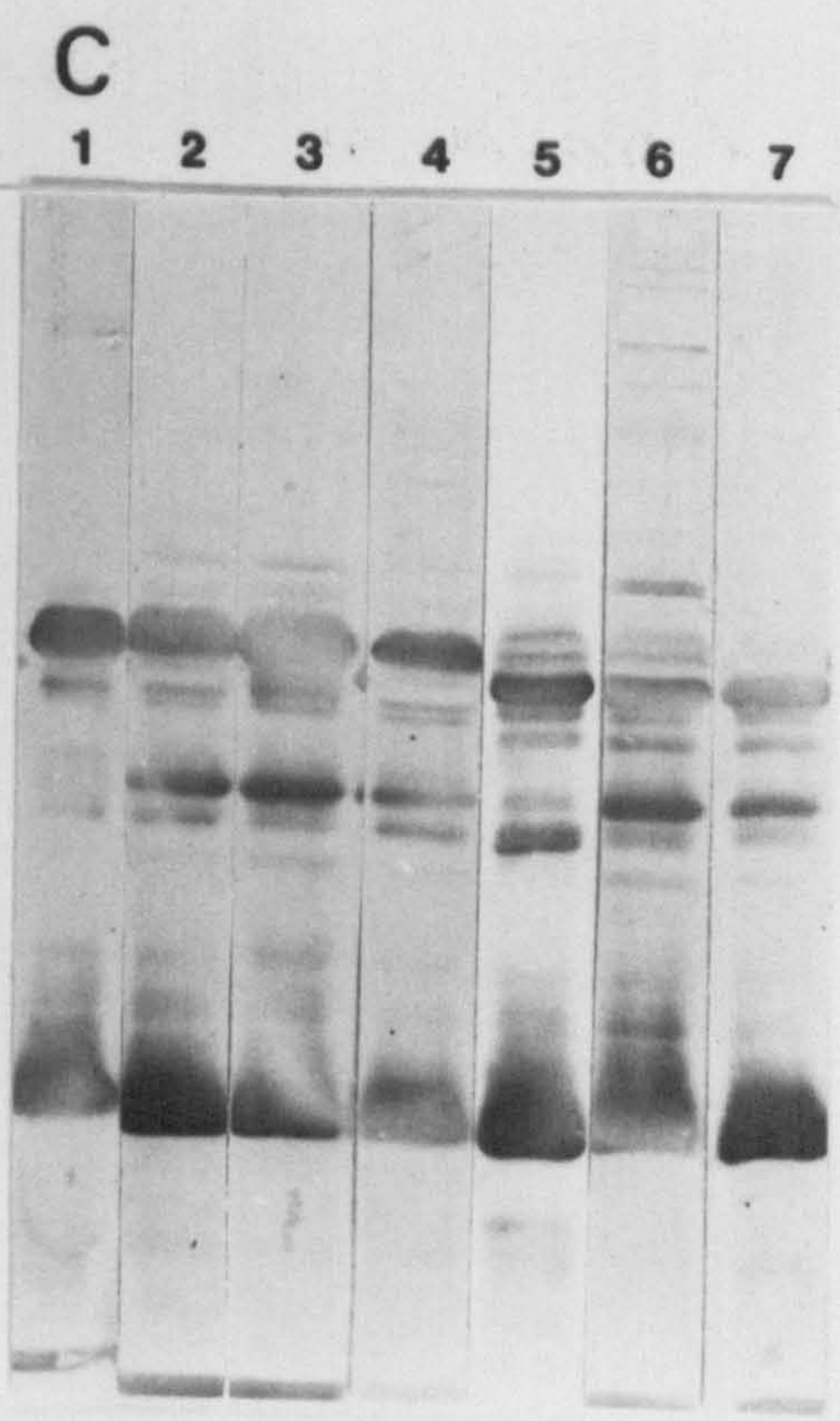


Figure 18 (continued). Western-blots showing reactions of outer membrane components of representative isolates of serotypes 01, 02, 05, 06 and 07 with types 1, 2 and 5 rabbit antisera.

(D). Reactions of serotype 05 isolates of OMP-types 2 (lanes 1 - 3) and 1 (lane 4) with type 1 antiserum.
Lanes 1 - 4 represent isolates RD28, RD50, RD188 and RD192, respectively.

(E). Reactions of serotype 06 isolates of OMP-types 2 (lanes 1 - 3) and 1 (lane 4) with type 1 antiserum.
Lanes 1 - 4 represent isolates RD162, RD66, RD194 and RD48, respectively.

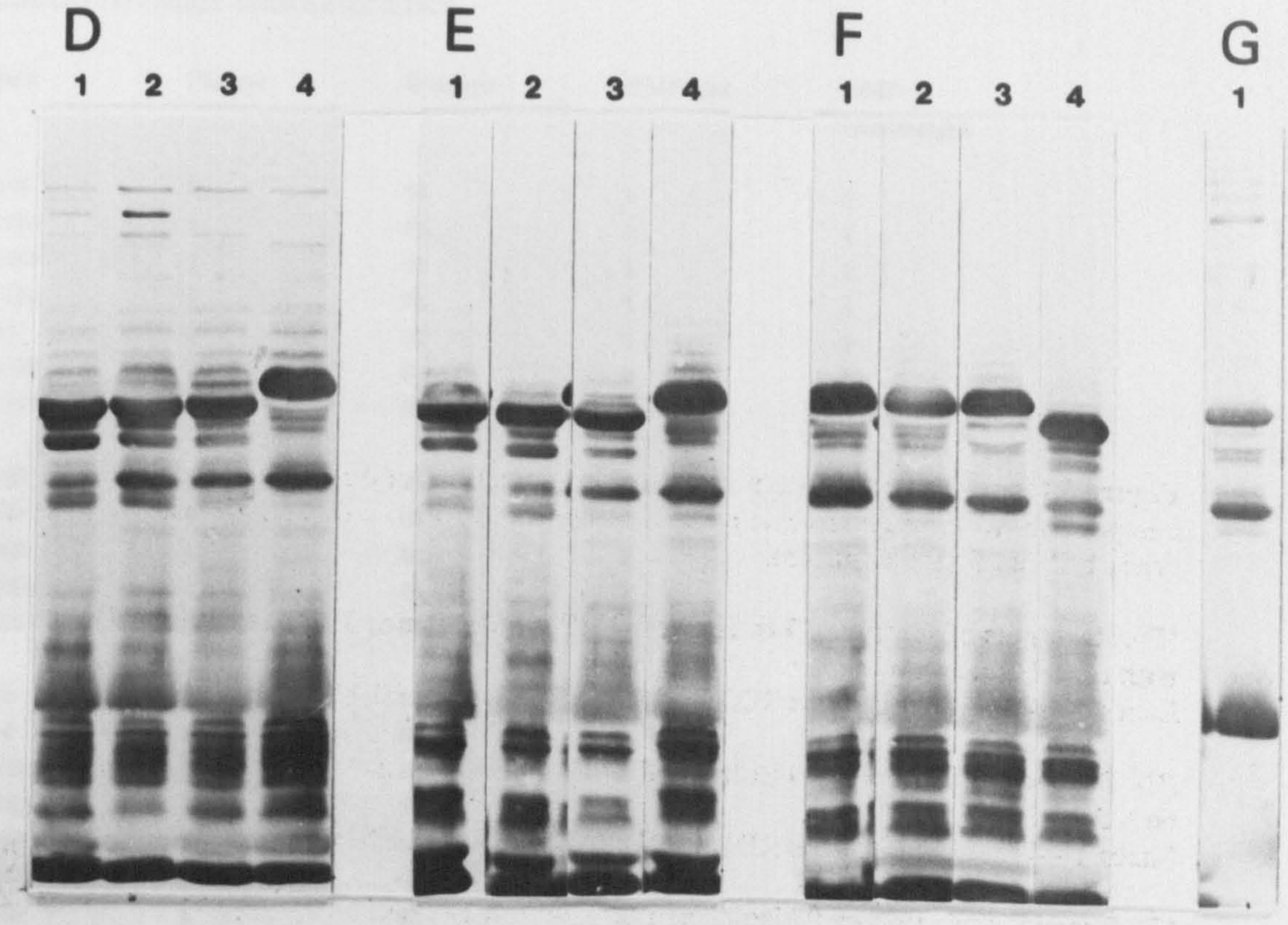
(F). Reactions of serotype 07 isolates of OMP-types 1 (lanes 1 - 3) and 5 (lane 4) with type 1 antiserum.
Lanes 1 - 4 represent isolates RD150, RD36, RD60 and RD52, respectively.

(G). Reaction of untypable isolate (RD46) of OMP-type 1 with type 1 antiserum.

(H). Reactions of serotype 01 isolates of OMP-types 3 with type 2 antiserum.
Lanes 1 - 4 represent isolates RD70, RD136, RD138 and RD140, respectively.

(I). Reactions of serotype 02 isolates of OMP-types 2 (lanes 1 and 4) and 1 (lanes 2 and 3) with type 2 antiserum.
Lanes 1 - 4 represent isolates RD168, RD54, RD56 and RD58, respectively.

(J). Reactions of serotype 05 isolates of OMP-types 2 (lanes 1 - 3) and 1 (lane 4) with type 5 antiserum.
Lanes 1 - 4 represent isolates RD28, RD50, RD188 and RD192, respectively.


D
1 2 3 4
E
1 2 3 4
F
1 2 3 4
G
1

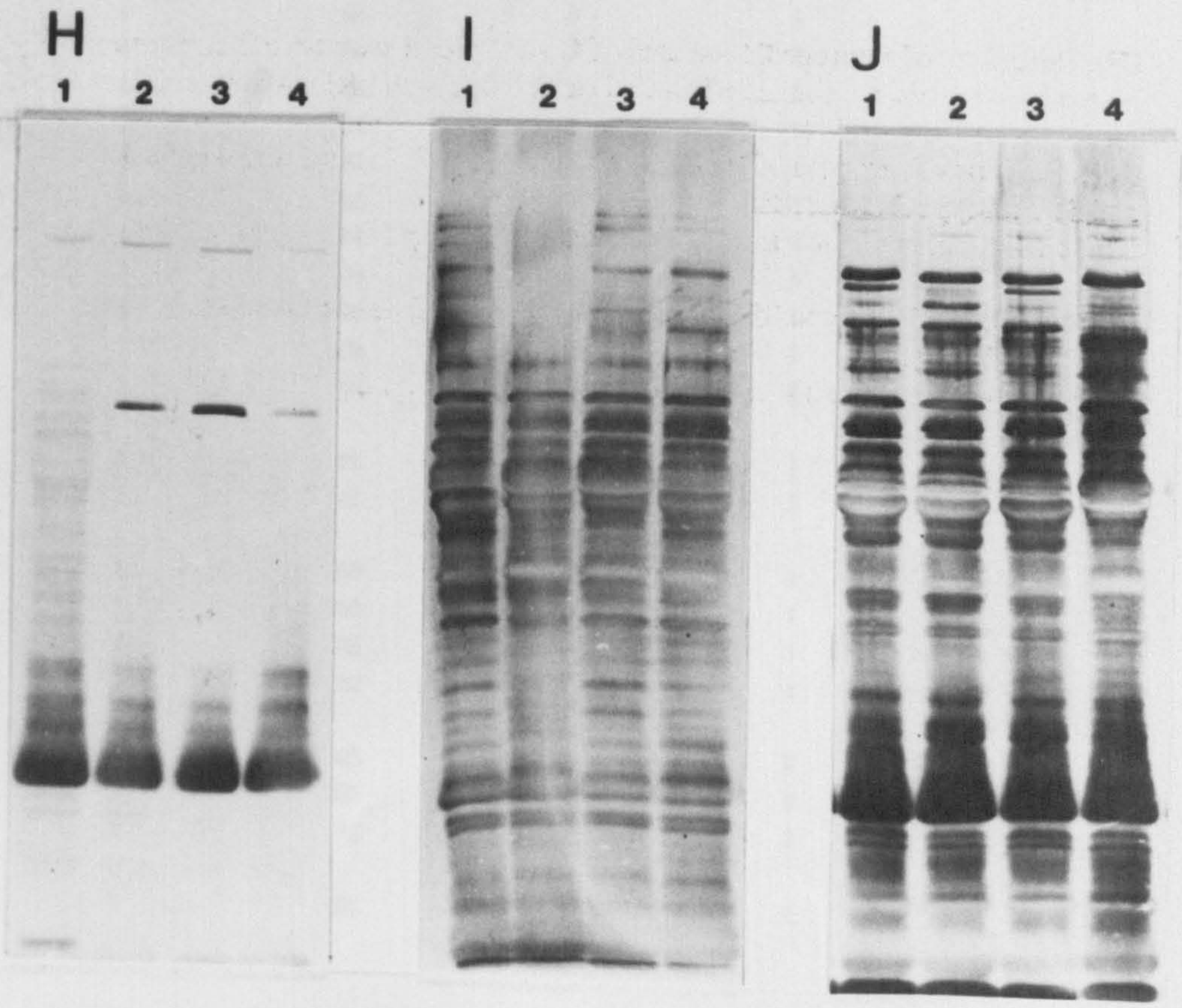

H
1 2 3 4
I
1 2 3 4
J
1 2 3 4

Table 15. Outer membrane protein immunotypes of isolates of Y. ruckeri in relation to other characteristics.

Isolate	Biotype	Serotype	OMP-type	OMP-immunotype
RD44	1	01	1	1
RD156	1	01	1	1
RD184	1	01	1	1
RD186	1	01	1	1
RD6	2	01	1	1
RD132	2	01	1	1
RD152	2	01	1	1
RD20	1	01	2	2
RD38	1	01	2	2
RD62	1	01	2	2
RD158	1	01	2	2
RD154	2	01	2	2
RD2	1	01	3	1
RD4	1	01	3	1
RD16	1	01	3	1
RD32	1	01	3	1
RD40	1	01	3	1
RD42	1	01	3	1
RD70	1	01	3	1
RD82	1	01	3	1
RD84	1	01	3	1
RD86	1	01	3	1
RD88	1	01	3	1
RD124	1	01	3	1
RD126	1	01	3	1
RD136	1	01	3	1
RD138	1	01	3	1
RD140	1	01	3	1
RD170	1	01	3	1
RD172	1	01	3	1
RD178	1	01	3	1
RD180	1	01	3	1
RD182	1	01	3	1
RD288	1	01	3	1
RD22	1	01	4	1
RD190	1	01	4	1
RD34	1	02	1	1
RD54	1	02	1	1
RD56	1	02	1	1
RD174	2	02	1	1
RD58	1	02	2	2
RD64	1	02	2	2
RD168	1	02	2	2
RD192	1	05	1	1

RD28	1	05	2	2
RD50	1	05	2	2
RD188	1	05	2	2
RD48	1	06	1	1
RD66	1	06	2	2
RD162	1	06	2	2
RD194	1	06	2	2
RD36	1	07	1	1
RD60	1	07	1	1
RD150	1	07	1	1
RD52	1	07	5	2
RD46	1	UT	1	1

UT = untypable

Antigenic cross-reactivities between types 1, 2, 5, 6 and 7 antisera and the homologous and heterologous isolates are compared in Figure 19. The cross-reactivity of type 1 antiserum with the high- and low-molecular-weight HMPs of heterologous isolates is shown in Figure 19A whereas the lack of cross-reactivity of these proteins with type 2 antiserum is shown in Figure 19B. Figure 19C demonstrates the weaker cross-reactivity of the HMPs of heterologous isolates with type 5 antiserum. Types 6 and 7 antisera also reacted extensively with heterologous isolates. In particular, both the low- and high-molecular-weight HMPs of heterologous isolates cross-reacted (Figures 19D and E). Although proteins in the molecular weight range 20-30 KDa were recognized by all five antisera in all five serotypes, this was also the case for preimmune sera. Simlarly, a low-molecular-weight component (possibly lipid A-oligosaccharide core) showed cross-reactivity with each of the antisera but was also recognised by preimmune serum. In the homologous reactions more proteins were recognized in all cases, including some reactivity with the 39.5 and 38/36.5 KDa porin proteins; reactivity with LPS was also apparent with homologous reactions. An attempt to remove LPS antibodies by absorbing type 1 antiserum with heat-denatured (100^{o}C for 2 h) serotype O1 cells was unsuccessful; antibodies against OMPs were removed as well.

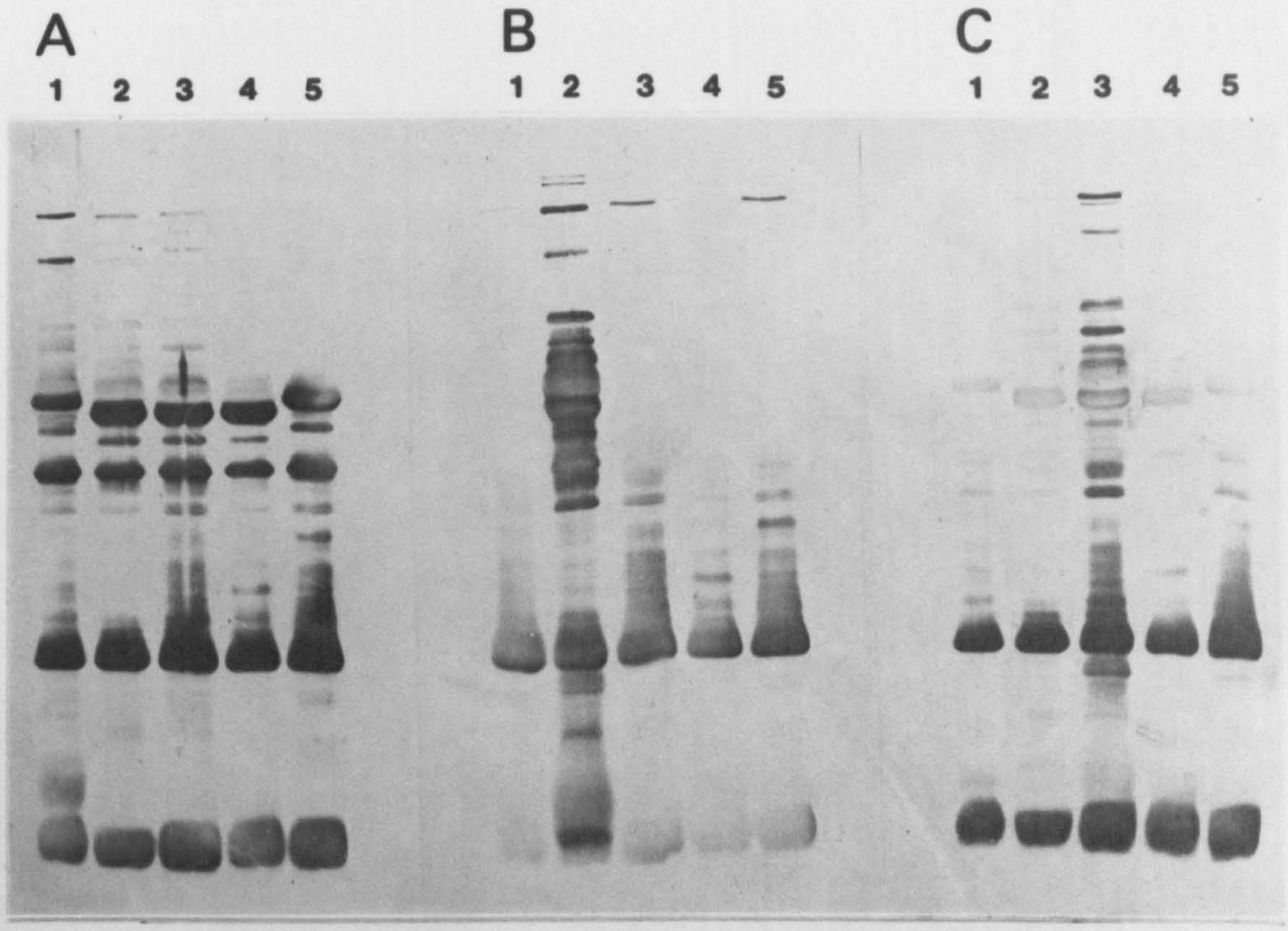
6.3.3 Antibody response in rainbow trout to lipopolysaccharides and outer membrane proteins

Reactions of rainbow trout types 1, 2, 5 and 6 antisera with homologous LPS and OMPs are shown in Figure 20. Type O2 and O6 LPS reacted strongly with the homologous antiserum; type O5 LPS reacted less strongly. The O1 LPS of isolates RD6, RD124 and RD170 reacted very weakly with their homologous antisera; the O1 LPS of isolates RD20 and RD154 reacted more strongly. The characteristic ladder-like LPS pattern was observed in each of the four LPS types, although it was weak in types O1 and O5 LPS. The lipid A-

Figure 19. Western-blots showing cross-reactions of outer membrane components of representative isolates of serotypes 01, 02, 05, 06 and 07 with types 1, 2, 5, 6 and 7 rabbit antisera.

(A) - (E) represent reactions with types 1, 2, 5, 6 and 7 antisera, respectively.

Lanes 1 - 5 represent outer membranes of isolates RD170 (serotype 01, OMP-type 3), RD168 (serotype 02, OMP-type 2), RD28 (serotype 05, OMP-type 2), RD194 (serotype 06, OMP-type 2) and RD150 (serotype 07, OMP-type 1), respectively.


A
1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5

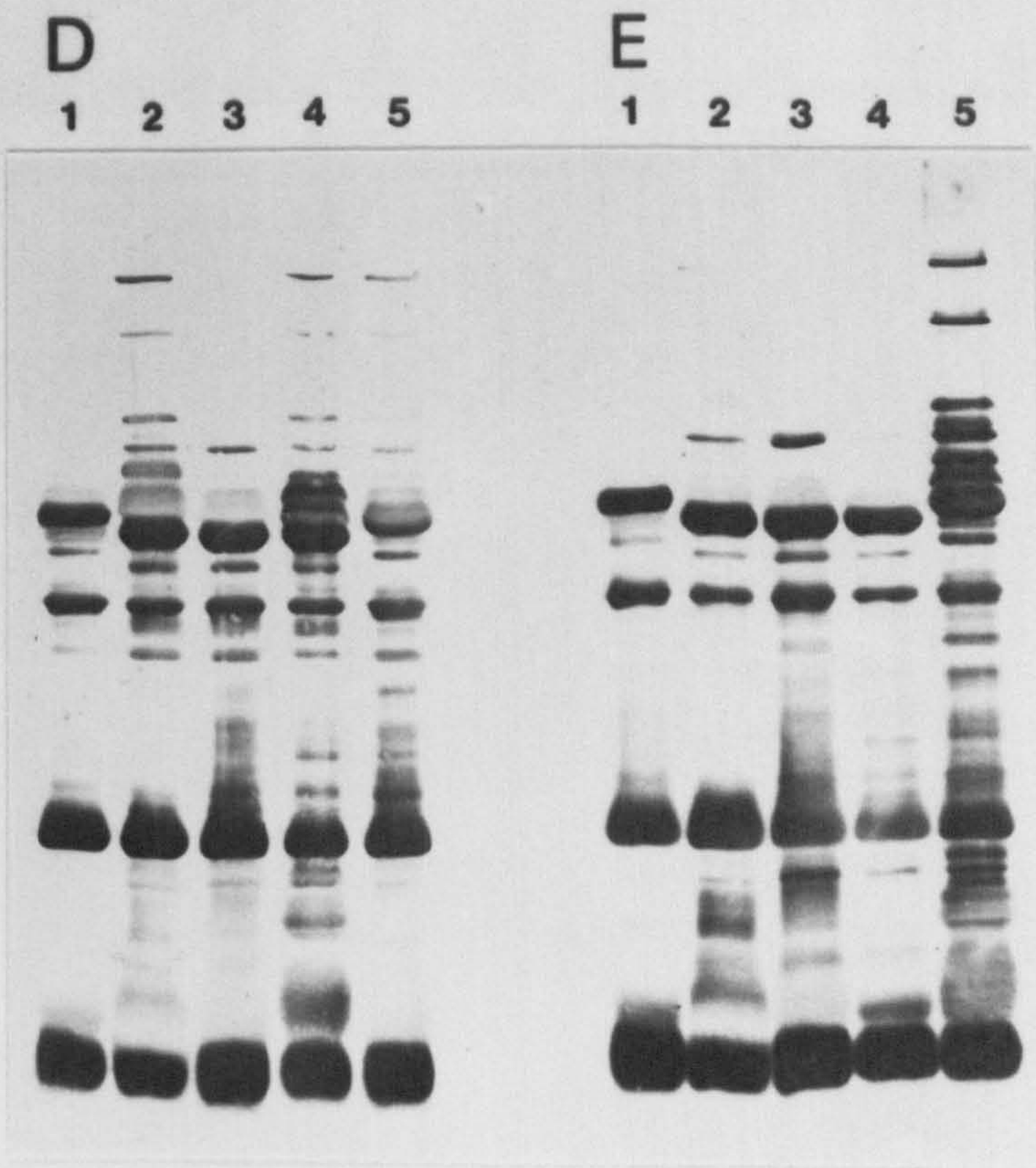
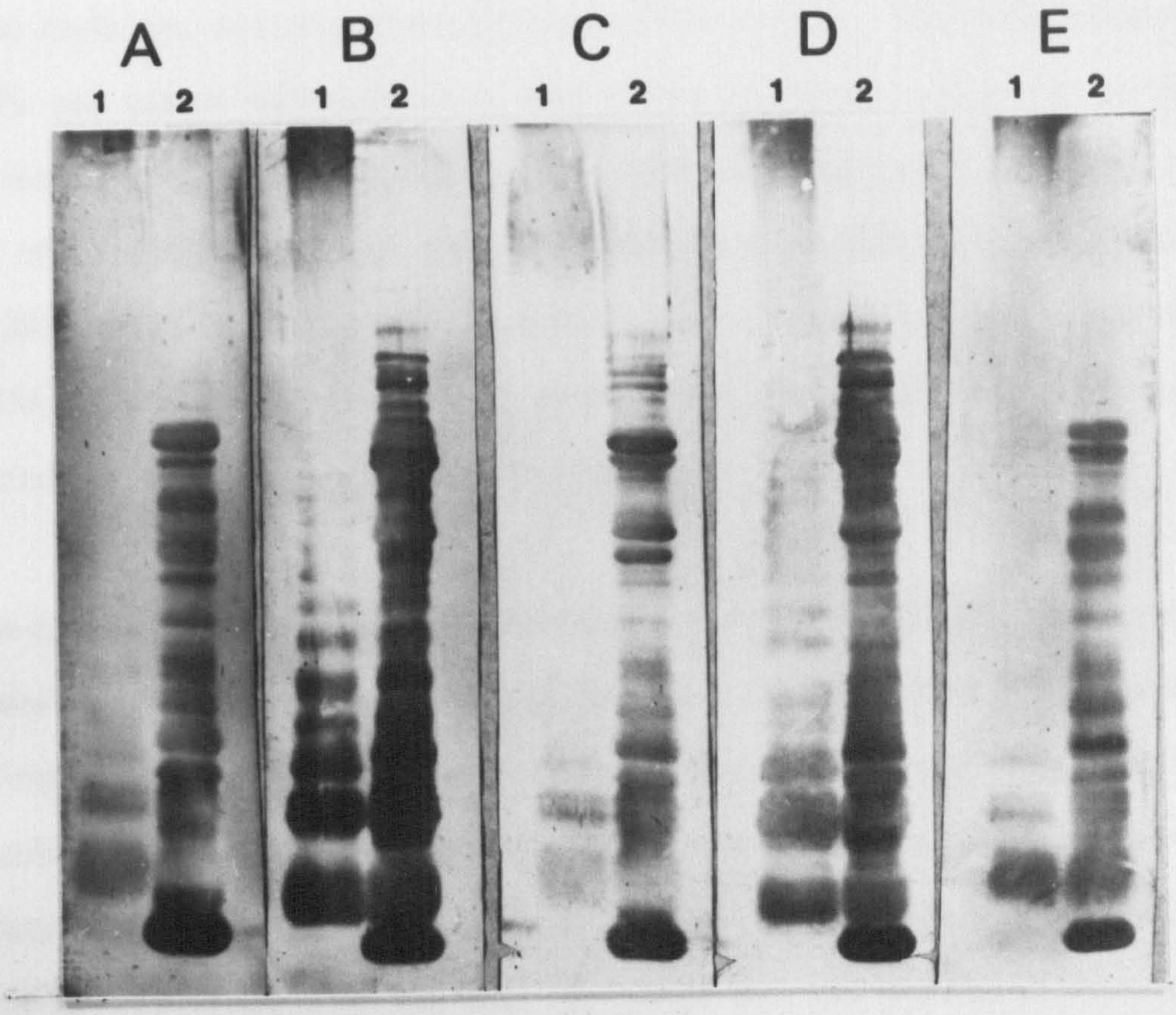
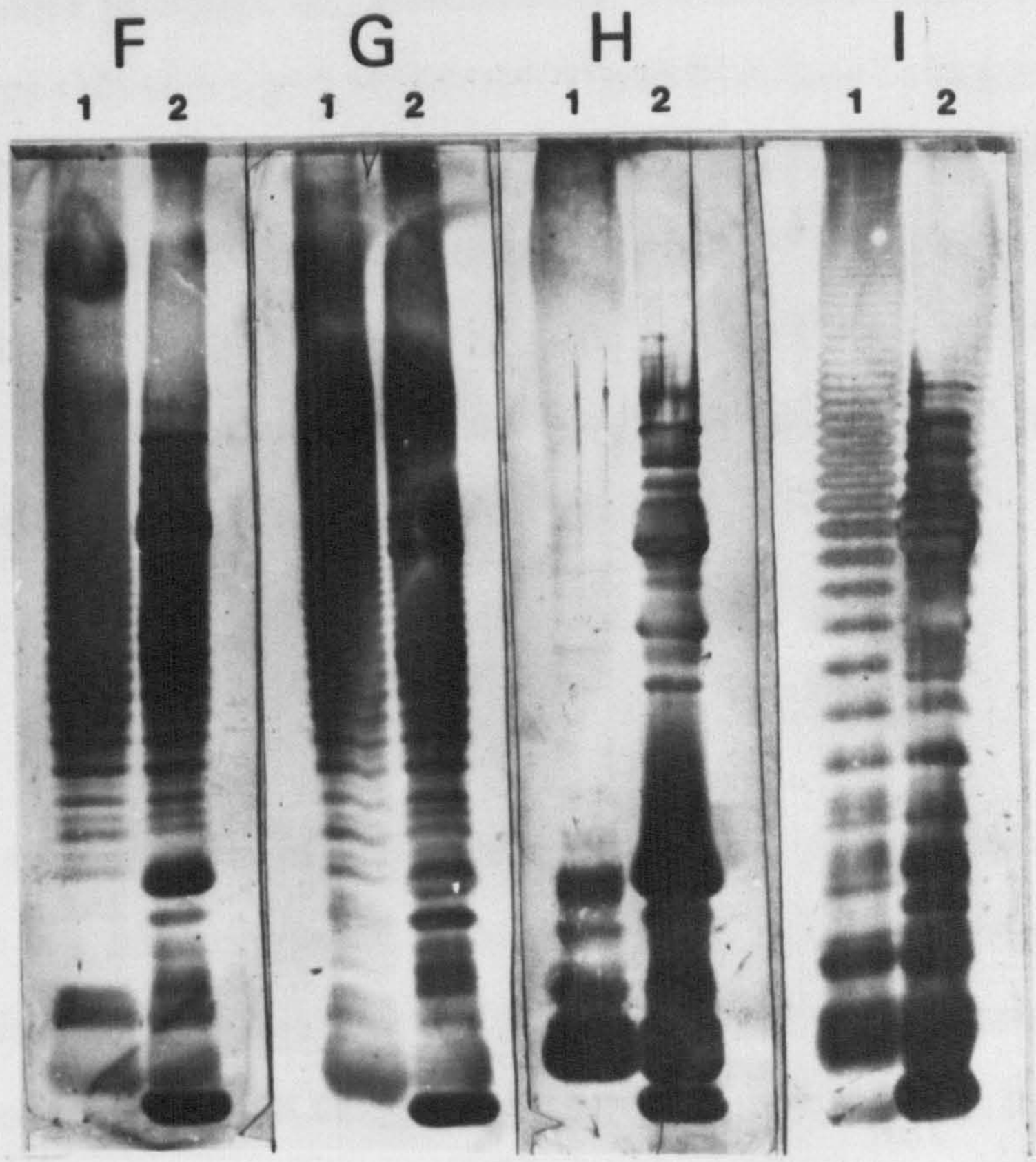

D
1 2 3 4 5
E
1 2 3 4 5

Figure 20. Western-blots showing reactions of lipopolysaccharides and outer membrane proteins of representative isolates of serotypes 01, 02, 05 and 06 with homologous rainbow trout antisera.

(A) - (I) represent reactions with isolates RD6 (serotype 01, OMP-type 1), RD20 (serotype 01, OMP-type 2), RD124 (serotype 01, OMP-type 3), RD154 (serotype 01, OMP-type 2), RD170 (serotype 01, OMP-type 3), RD34 (serotype 02, OMP-type 1), RD168 (serotype 02, OMP-type 2), RD28 (serotype 05, OMP-type 2) and RD66 (serotype 06, OMP-type 2), respectively.

Lanes 1 and 2 represent reactions with LPS and OMPs, respectively.


A
B
C
D
E
1 2
1 2
1 2
1 2
1 2


F
G
H
I
1 2
1 2
1 2
1 2

oligosaccharide core region of the LPS molecule, as well as the O-antigen part of the molecule, was recognized by each of the antisera. The recognition of OMPs was not as well defined as with rabbit antiserum, partly due to the interference of the LPS reactions. The HMPs appeared to be recognized by each of the antisera although other major OMPs were difficult to distinguish. The 39.5 and 38/36.5 KDa porin proteins appeared to be recognized in isolates RD154, RD28 and RD60. Most notable was the recognition of a low-molecular-weight protein in each of the isolates.

Cross-reactivity between rainbow trout antisera and heterologous isolates was demonstrated although the differences were not as pronounced as they were with rabbit antisera (Figure 21). Although major OMPs were recognized in heterologous isolates this was also the case in preimmune sera (although not to the same extent). The reactions shown in Figure 21 are not as strong as those in Figure 20. The reason for this may have been due to a decline in antibody activity because the reactions shown in Figure 21 were carried out some months after those in Figure 20. Furthermore, the strong reaction of isolate RD168 (serotype O2) with type 1 antiserum (Figure 21A, lane 2) suggested that the serum used in this reaction may have been derived from a fish accidently injected with serotype O2 cells instead of serotype O1 cells in the second booster inoculation. Cross-reactivity of both the HMP and the 39.5 and 38/36.5 KDa PAPs was apparent in rainbow trout sera, as well as cross-reactivity of the lipid A-oligosaccharide core region of LPS.

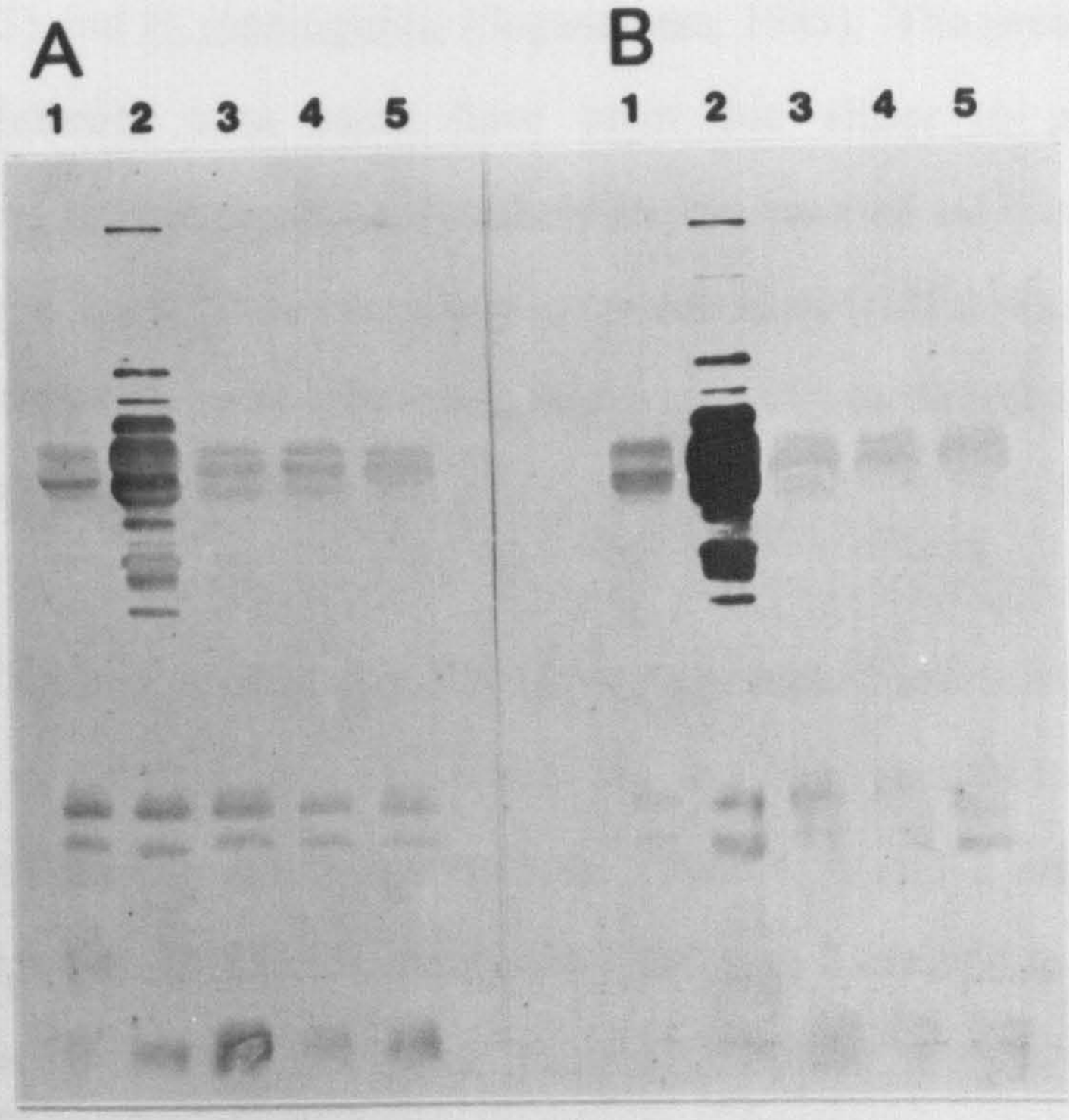
6.4 DISCUSSION

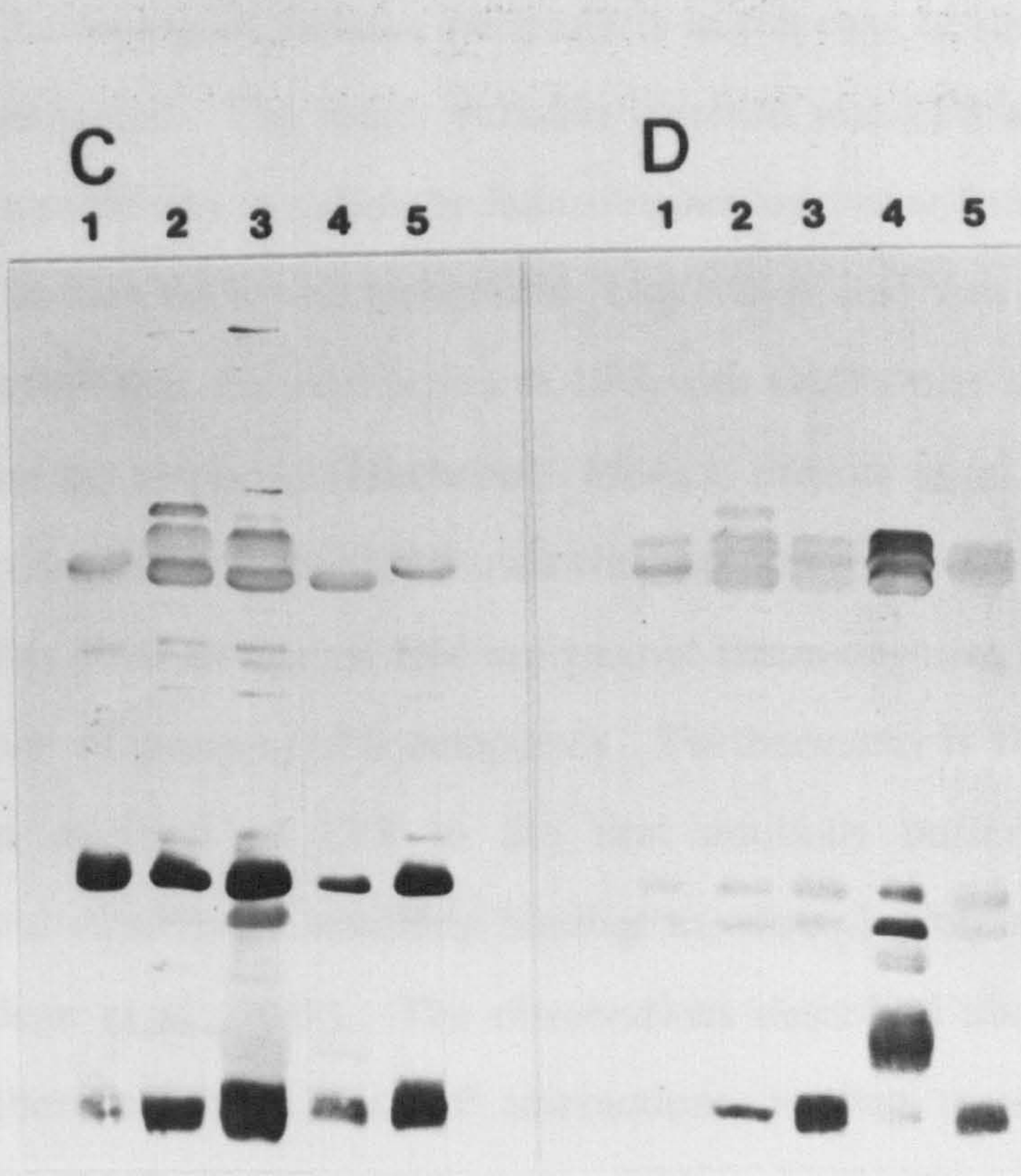
The presence of antibodies in preimmune rabbit and rainbow trout sera to certain OMPs was not unexpected since antibodies have been shown to be present in preimmune sera against the OMPs of various bacteria including C. jejuni (Mills and Bradbury, 1984), H. pleuropneumoniae (Rapp and Ross,

Figure 21. Western-blots showing cross-reactions of outer membrane components of representative isolates of serotypes 01, 02, 05, 06 and 07 with types 1, 2, 5 and 6 rainbow trout antisera.

(A) - (D) represent reactions with types 1, 2, 5 and 6 rainbow trout antisera, respectively.

Lanes 1 - 5 represent outer membranes of isolates RD170 (serotype 01, OMP-type 3), RD168 (serotype 02, OMP-type 2), RD28 (serotype 05, OMP-type 2), RD194 (serotype 06, OMP-type 2) and RD150 (serotype 07, OMP-type 1), respectively.


A
1 2 3 4 5
B
1 2 3 4 5


C
1 2 3 4 5
D
1 2 3 4 5

1986); H. influenzae type b (Loeb and Smith, 1982), non-typable H. influenzae (Hansen et al., 1985) and N. meningitidis (Sugasawara, 1985). The presence of antibodies in preimmune sera could have been due either to previous undetected exposure to the organism (unlikely in the case of rabbits) or to exposure to an organism with serologically cross-reacting OMPs. The cross-reactivity in preimmune sera was very weak, however, and was directed at only a small number of OM components.

Rabbit antibodies against each of the five serotypes reacted extensively with OMPs (and LPS) of the homologous isolates but did not react to the same extent with the OMPs of heterologous isolates. Type 1, 6 and 7 antibodies reacted strongly, type 5 antibodies reacted weakly and type 2 antibodies did not react at all with the HMP of heterologous isolates. With the exception of the major OMPs, the OMP profiles in Coomassie blue-stained gels of the isolates examined were remarkably uniform (Section 4) and so this lack of cross-reactivity between heterologous isolates, particularly in the case of the type 2 antiserum, was unexpected. The major variable involved was LPS and it is possible that LPS in some way modifies the heterologous reactions. Interaction of LPS and OMPs in the OM is well recognized (Lugtenberg and Van Alphen, 1983) and it is possible that the association of LPS with OMPs may alter the nature of the immune response (Hitchcock, 1984). Poxton et al. (1985) suggested that Western-blotting should be used with caution when undertaking studies on antibodies directed against OM antigens of Gram-negative bacteria due to the presence of protein/LPS complexes. Furthermore, it has been demonstrated that addition of LPS to the first antibody buffer causes renaturation of, and subsequent antibody binding to, class 2 proteins of N. meningitidis (Wedege et al., 1988). The observations described above may have been due, therefore, to LPS-OMP interactions, leading to antigenic modification of the OMPs in the whole cells and/or on the nitrocellulose membranes.

The heat-modified HMP was a major immunogen and variation in the molecular weight of this protein formed the basis of an OMP-immunotyping scheme. This scheme had some epizootiological significance since it was able to differentiate between strains of the same O-serotype. For example, the U.K. serotype O2 isolate RD34 was shown to be different from the West German serotype O2 isolate RD64. Although this scheme provided less information than the OMP-typing scheme, it was not necessary to heat the OM samples at two temperatures in order to differentiate molecular weight variation in the HMP. Whereas the scheme could differentiate OMP-types 1, 3 and 4 from OMP-types 2 and 5 it could not distinguish between OMP-types 1, 3 and 4 or between OMP-types 2 and 5, since these OMP-types are differentiated on the basis of variation in the PAPs.

In work on H. influenzae type b, absorption of serum with heterologous bacteria removed antibodies to nearly all proteins, confirming the extensive cross-reactivity among isolates (Erwin and Kenny, 1984). Absorption of antisera with whole cells will also establish whether OMPs are surface exposed or not (Loeb, 1984). Although immune-absorption of homologous antisera was not carried out in this study, such a procedure would have confirmed whether the OMPs of Y. ruckeri were surface exposed or not. Cross-absorption of antisera with heterologous whole cells would have demonstrated whether cross-reactivity was due to common surface-exposed epitopes or to the recognition of internal epitopes exposed by denaturation of proteins.

The PAPs (porin proteins) of Y. ruckeri, which were shown to be major OMPs by Coomassie blue staining, appeared not to be strongly immunogenic. Staining of gels after transfer indicated that the proteins had been transferred to nitrocellulose although this was not confirmed by staining of the nitrocellulose with Coomassie blue due to technical problems. Although not

attempted, amido black staining of nitrocellulose may have been a better alternative. The problem of poor (or non-existent) transfer of certain OMPs to nitrocellulose could have been overcome by incorporating 0.1% SDS in the transfer buffer (Erickson et al., 1982; Sutton et al., 1982b). The Omp A protein of S.marcescens (equivalent to the HMP of Y. ruckeri) was also shown to be the major immunogen of this organism whereas the porins reacted only weakly with antibodies (Jessop and Lambert, 1985). Antibodies to major OMPs have also not been detected in H. influenzae type b (Loeb and Smith, 1982), H. pleuropneumoniae (Rapp and Ross, 1986) and Bordetella pertussis (Redhead, 1984). This lack of recognition may be due to denaturing of antigenic epitopes during SDS-PAGE (Poolman et al., 1983; Loeb, 1984; Jessop and Lambert, 1985). The incorporation of zwitterionic detergents (e.g. Empigen BB) in either the transfer buffer or in the primary antobody buffer has been demonstrated to re-nature class 1 and 2 OMPs of N. meningitidis and allow antibody binding (Mandrell and Zollinger, 1984; Wedege et al., 1988). Alternatively, OM porin proteins may be shielded from antibody by O-antigenic chains of lipopolysaccharide (Van der Ley et al., 1986). Further investigation into the antigenic nature of the porin proteins of Y. ruckeri is required.

Flagellin and pilin antibodies have been detected by Western-blotting against some bacteria including C. jejuni (Nachamkin and Hart, 1985) and Pseudomonas aeruginosa (Hedstrom et al., 1984) even though these proteins were not recognized by Coomassie blue staining. Flagellin protein of Y. ruckeri was not detected in Coomassie blue-stained gels (by comparing OMP-profiles of motile and non-motile isolates) and it was not possible to identify flagellin in Western blots since the molecular weight of the protein was not known. Much of the flagellar protein was probably lost during preparation of the OMs as a result of sonication (Logan and Trust, 1982). The antigenic nature of the flagellin of Y. ruckeri could be investigated further.

Rainbow trout produced antibodies against LPS as well as against various proteins, including the HMP. Cross-reactivity of some proteins was also demonstrated. Rainbow trout did not, however, produce a strong antibody response against type O1 LPS (alternatively binding of antibody to type O1 LPS may have been poor) in comparison to the other LPS types. In addition, there was a stronger reaction against the O1 LPS of isolates RD20 and RD154 (avirulent isolates) than against the O1 LPS of isolates RD6, RD124 and RD170 (virulent isolates). The poor recognition of the LPS of virulent serotype O1 isolates could perhaps explain the virulence of Y. ruckeri and needs to be investigated further. There did not appear to be any significant differences between the OMPs recognized in virulent isolates (RD6, RD124, RD170) and the OMPs recognized in avirulent isolates. The fact that the HMP is a major antigen and exhibits cross-reactivity suggests that this protein might be an important protective antigen and might provide cross-protection in ERM vaccines. The porin protein F of P.aeruginosa has been demonstrated to provide cross-protection against six heterologous LPS immunotypes (Gilleland et al., 1984; Matthews-Greer and Gileland, 1987). This protein requires further investigation; in particular, the bactericidal effect of antibodies raised against the HMP needs to be investigated. Further Western-blot analyses could also be carried out with sera obtained from naturally infected fish. In particular, Western-blot analyses could be carried out using OMPs of isolates grown under iron-limiting conditions (see Section 7).

Section 7

Acquisition of Iron by Yersinia ruckeri

Acquisition of Iron by Yersinia ruckeri

7.1. INTRODUCTION

Iron is an essential requirement for bacterial growth and virulence (Bullen et al., 1978; Weinberg, 1978; Griffiths, 1987). Although the total iron concentration in the body fluid of vertebrate hosts is high, the amount of free iron which might be readily available to bacteria is extremely small. This is due to the fact that iron is mostly located intracellularly, in ferritin, haemosiderin or haem, and that which is extracellular is attached to the high affinity iron-binding glycoproteins transferrin, in serum and lymph, and lactoferrin, in external secretions. The concentration of unbound iron in plasma has been estimated to be approximately 10^{-15} M which is well below the 0.4 to 4.0 μM required for the growth of most microbes (Weinberg, 1978). During infection the host further reduces the total amount of iron bound to serum transferrin in a condition known as the hypoferraemia of infection (Cartwright et al., 1946).

To overcome these iron-deficient conditions pathogenic bacteria have evolved efficient iron-sequestration systems. Two major iron-uptake mechanisms occur in pathogenic bacteria. In the first mechanism bacteria produce and secrete siderophores (Neilands, 1981). Siderophores have extremely high affinities for ferric iron and are able to remove iron from the host Fe^{3+}-glycoprotein complex and deliver it, via specific OM receptor proteins, to the bacterial cell. Examples of bacterial genera that secrete siderophores include Escherichia, Klebsiella, Salmonella and Shigella (Griffiths, 1987). There are two major chemical types of siderophores, namely the hydroxamate type and the phenolate type, although siderophores of other chemical structure have been identified (Neilands, 1981). Enterobactin is a common phenolate siderophore and aerobactin is a common hydroxamate siderophore. Iron-regulated OM

receptor proteins and enzymes are involved in the uptake and release of iron from the iron chelator (Neilands, 1982). In the enteric bacteria these iron-regulated OMPs have apparent molecular weights in the range 74-84 KDa (Griffiths, 1987).

In the second mechanism there is a direct interaction between protein receptors on the bacterial cell surface and the Fe^{3+}-glycoprotein complex. Neisseria gonorrhoeae and N. meningitidis obtain their iron in this way and these bacteria do not produce siderophores (Norrod and Williams, 1978; Archibald and De Voe, 1979; Mickelson and Sparling, 1981; McKenna et al., 1988). Haemophilus influenzae (Herrington and Sparling, 1985) as well as Yersinia pestis, Yersinia pseudotuberculosis and Y. enterocolitica (Perry and Brubaker, 1979) also do not produce siderophores and these bacteria utilize haem and hemin as iron sources.

The ability of an invading pathogen to compete successfully for iron has been considered an important virulence factor. The fish pathogen V.anguillarum possesses a plasmid-mediated iron uptake system that correlates strongly with virulence (Crosa, 1980; Crosa et al., 1980). Enterobactin has been associated with virulence in S. typhimurium (Yancey et al., 1979) and the genes for aerobactin have been shown to be associated with a chromosomal segment associated with virulence in Shigella flexneri (Griffiths et al., 1985). In addition, aerobactin has been shown to be an essential factor of pathogenicity in Klebsiella pneumoniae (Nassif and Sansonetti, 1986). The relationship between siderophore production and virulence is not, however, absolute. Whereas aerobactin production by certain E.coli ColV strains is associated with increased virulence in an animal model (Williams, 1979), other pathogens such as Vibrio cholerae (Sigel et al., 1985), Y. pestis (Perry and Brubaker, 1979) and S. typhimurium (Benjamin et al., 1985) have been shown to retain virulence for the mouse in the absence of a siderophore-mediated iron

transport system. Escherichia coli ColV strains are septicaemic whereas V. cholerae is noninvasive and Y. pestis and S. typhimurium are intracellular pathogens. Siderophore synthesis may, therefore, be more important for highly invasive, extracellular pathogens (Lawlor et al., 1987).

Acquisition of iron by Y. ruckeri has not been investigated; iron-regulated OMPs and siderophores have not been described in this organism. The objective of this part of the study, therefore, was to investigate the production of iron-regulated OMPs and siderophores in Y. ruckeri, and to correlate the findings with the virulence of this organism.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial isolates

The effect of iron-limitation on the expression of OMPs and on the production of siderophores was examined in 36 isolates of Y. ruckeri. The isolates examined are shown in Table 16. In siderophore assays two laboratory strains of V. anguillarum and an E.coli strain were also investigated.

7.2.2 Growth conditions

7.2.2.1. Treatment of glassware. All glassware was rinsed with 0.5% EDTA and with deionized water prior to use to remove trace amounts of iron.

7.2.2.2. Production of iron-limited growth conditions. Iron-limited growth conditions were achieved by growing bacteria in TSB supplemented with 3 μM transferrin (Sigma), 200 μM 2,2'-dipyridyl (Sigma) or 280 μM ethylenediaminedihydroxyphenylacetic acid (EDDA)(Sigma). These concentrations of chelating agents (with the exception of transferrin) were

chosen because they were shown to produce iron-limited conditions in TSB for E.coli (Chart et al., 1986). EDDA was made iron-free by the method of Rogers (1973) (Appendix 2). In some experiments TSB was deferrated by the method of Donald et al. (1952) (Appendix 2). For the production of siderophores the Tris-buffered medium of Simon and Tessman (1963) without added iron was used (Appendix 2). The high pH (7.4) and relatively low levels of iron contamination make Tris medium an iron-poor medium suitable for production of siderophores without further deferration. Chrome azurol S (CAS) agar plates were also used for the detection of siderophores (Schwyn and Neilands, 1987) (Appendix 2).

7.2.3 Studies on the growth kinetics of Y. ruckeri under various conditions of iron-limitation

Twenty millilitre volumes of TSB with or without 3 μM transferrin, 200 μM 2,2'-dipyridyl and 280 μM EDDA were inoculated with isolates RD34 and RD88 and incubated at 22°C. Isolate RD88 was chosen because it is a virulent serotype O1 isolate; isolate RD34 was chosen because it is an avirulent serotype O2 isolate. Each medium was inoculated with sufficient bacteria to give an initial bacterial density of about 10^3 viable organisms per ml and growth was monitored by viable counts. Samples of each culture (0.1 ml) were diluted appropriately in saline and were plated out, in triplicate, on TSA.

Bacteria of isolates RD34 and RD88 were grown in 20 ml deferrated TSB supplemented with 0, 5, 10, 25 and 50 μM $FeCl_3$. Growth was monitored by measuring changes in optical density at 660 nm.

7.2.4 Analysis of iron-regulated outer membrane proteins

Bacteria were grown in TSB supplemented with 3 μM transferrin, 200 μM 2,2'-dipyridyl or 280 μM EDDA. Fifty millilitres of medium were inoculated with 0.1 ml of a light saline suspension of each isolate and incubated for 18-24 h at 22^{o}C Rate of growth dictated the time of incubation since slower growing isolates required a longer period of incubation to provide sufficient cells for OM preparation. To demonstrate that iron-deficiency was responsible for induction of iron-regulated proteins, bacteria were grown in 50 ml TSB containing 200 μM 2,2' -dipyridyl supplemented with 0, 50 or 100 μM $FeCl_3$. Outer membrane proteins were isolated for SDS-PAGE as described in Section 3.

7.2.5 SDS-PAGE

SDS-PAGE was carried out as described in Section 3 and proteins were visualized with Coomassie blue as described in Section 4.

7.2.6 Assays for the production of siderophores

Ten millilitres of Tris-buffered medium were inoculated with 0.1 ml of a light saline suspension of each isolate and incubated for 24 h at 22^{o}C. After sufficient growth, 0.1 ml of this culture was transferred into 10 ml of the same medium and incubated for a further 48 h. Cells were removed by centrifugation at 1000 x g for 10 min and the supernatants were assayed for hydroxamate-type and phenolate-type siderophores by the methods of Csaky (1948) and Arnow (1937) respectively (Appendix 2).

In addition, the universal chemical assay described by Schwyn and Neilands (1987) was used to screen the isolates for the production of siderophores.

Bacteria were grown on chrome azurol S (CAS) agar plates (Appendix 2) for up to seven days at 22^{o}C. The plates were examined daily for zones of colour-change (blue to orange) indicative of siderophore production.

7.3 RESULTS

7.3.1 Growth kinetics of Y. ruckeri isolates under various conditions of iron-limitation

Whereas 200 μM 2,2$^{'}$ -dipyridyl and 280 μM EDDA both retarded the growth rate of isolates RD34 and RD88 in TSB (and to the same extent) 3 μM transferrin had no effect on the growth rate. The final bacterial density was approximately the same in all cases although stationary phase was reached almost 10 h sooner in TSB alone and in TSB supplemented with 3 μM transferrin than in TSB supplemented with 200 μM 2,2$^{'}$ - dipyridyl and 280 μM EDDA (Figure 22).

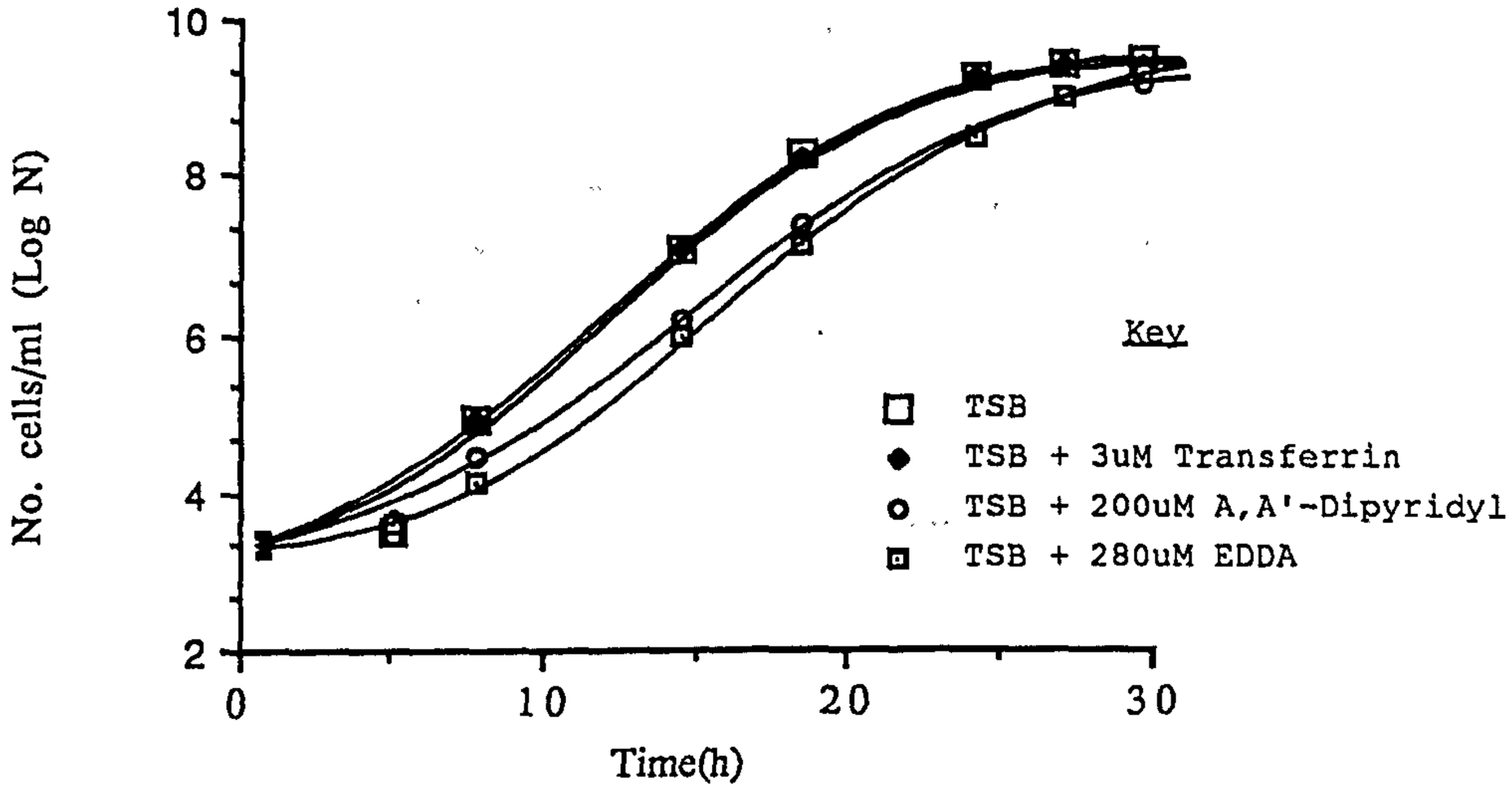
Iron-limitation was shown to be responsible for retarding the growth rate of isolates RD34 and RD88 in deferrated medium, since addition of $FeCl_3$ was shown to increase the rate of growth. In both isolates, however, the final optical density reading was highest in the medium not supplemented with $FeCl_3$ (Figure 23).

7.3.2 Expression of iron-regulated outer membrane proteins under conditions of iron-limitation

7.3.2.1 Comparison of transferrin, 2,2' -dipyridyl and EDDA as iron-chelators.

To ascertain whether there were any differences in the expression of the iron-regulated OMPs due to the nature of the chelating agent, the expression of these proteins was examined in a number of isolates after chelation with 3 μM

(a)



(b)

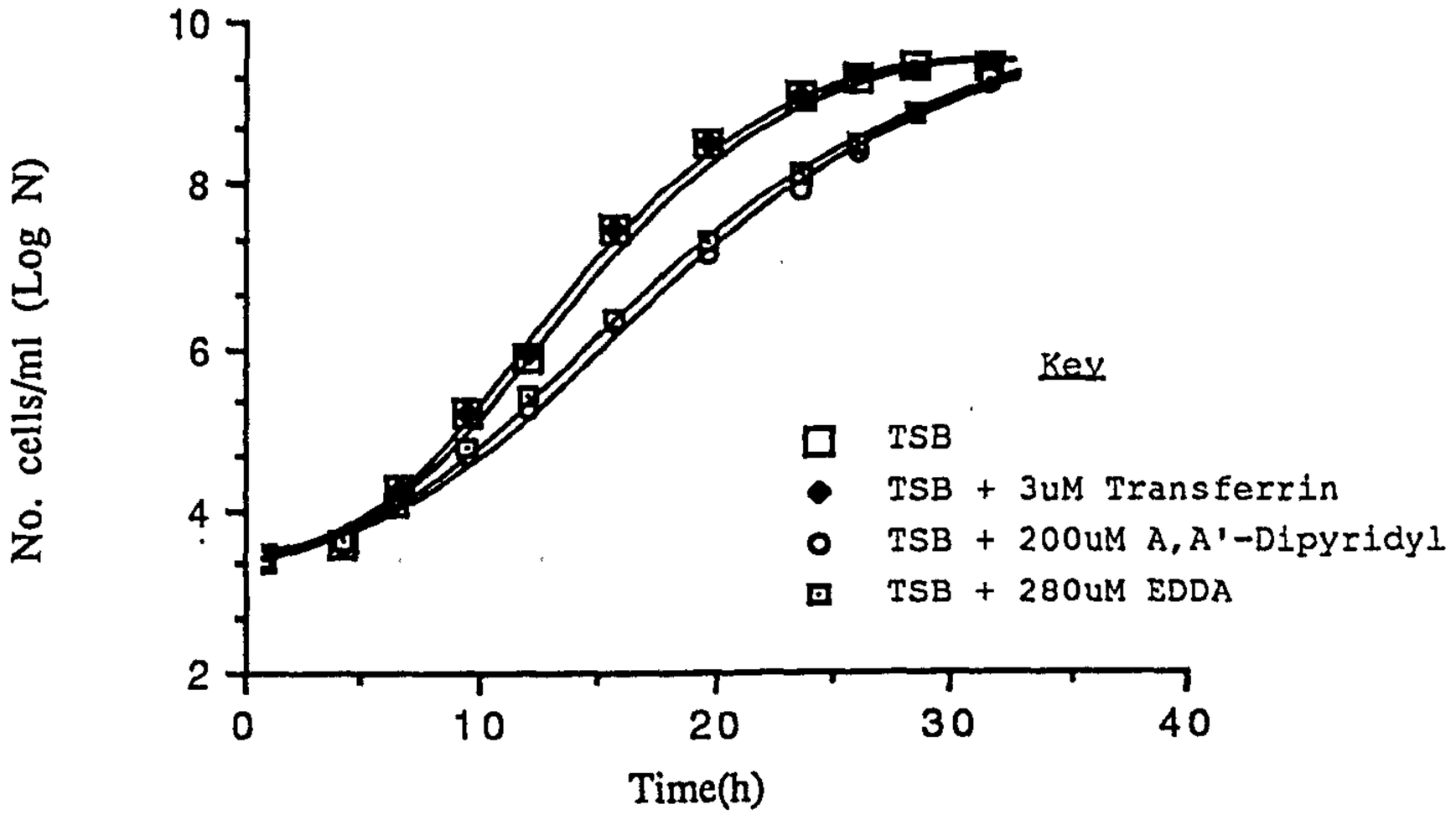
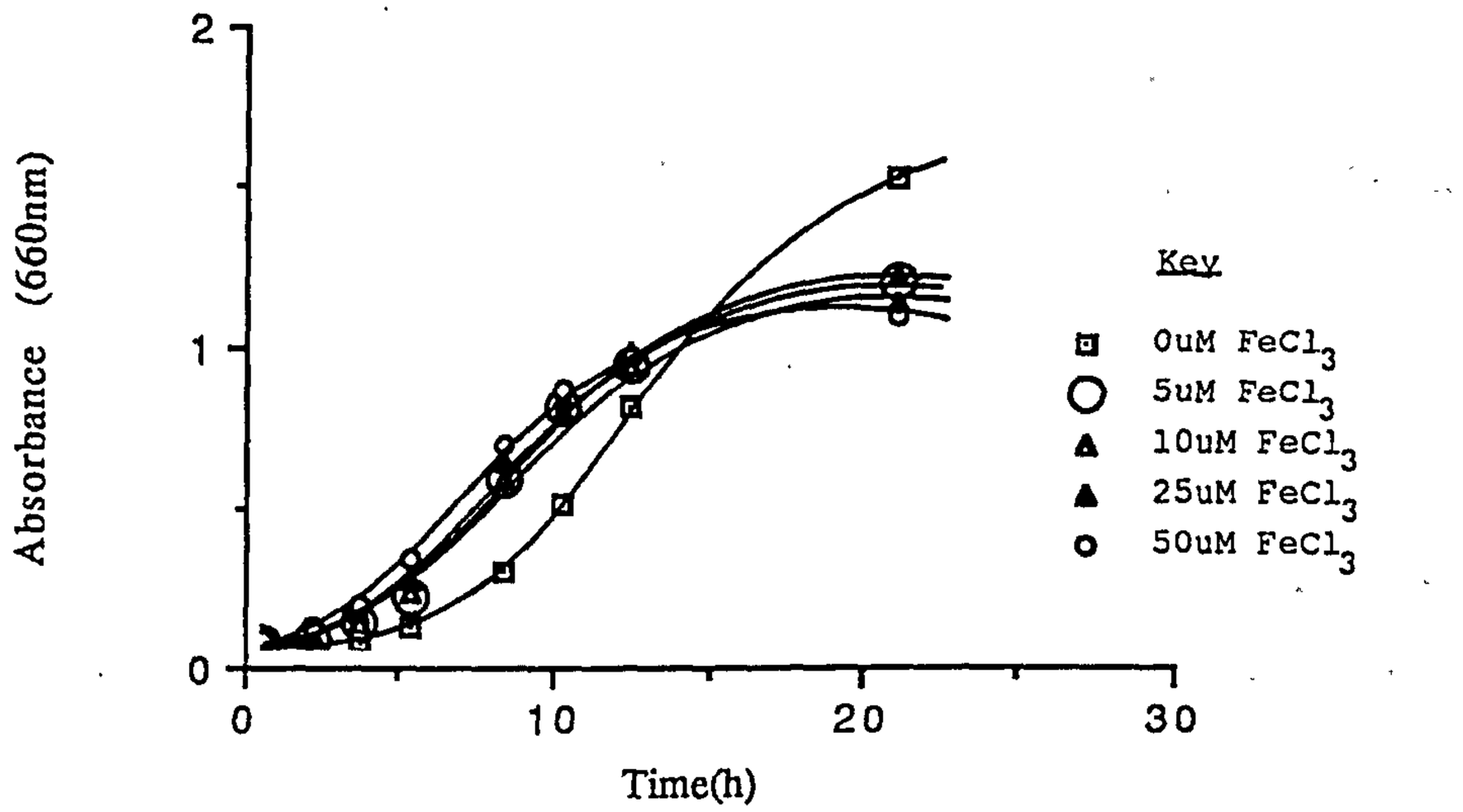


Figure 22. Growth curves of isolates RD34 (a) and RD88 (b) showing effects of iron limitation. Bacteria were grown in TSB, and in TSB supplemented with 3μM transferrin, 200μM 2,2'-dipyridyl and 280μM EDDA.

(a)



(b)

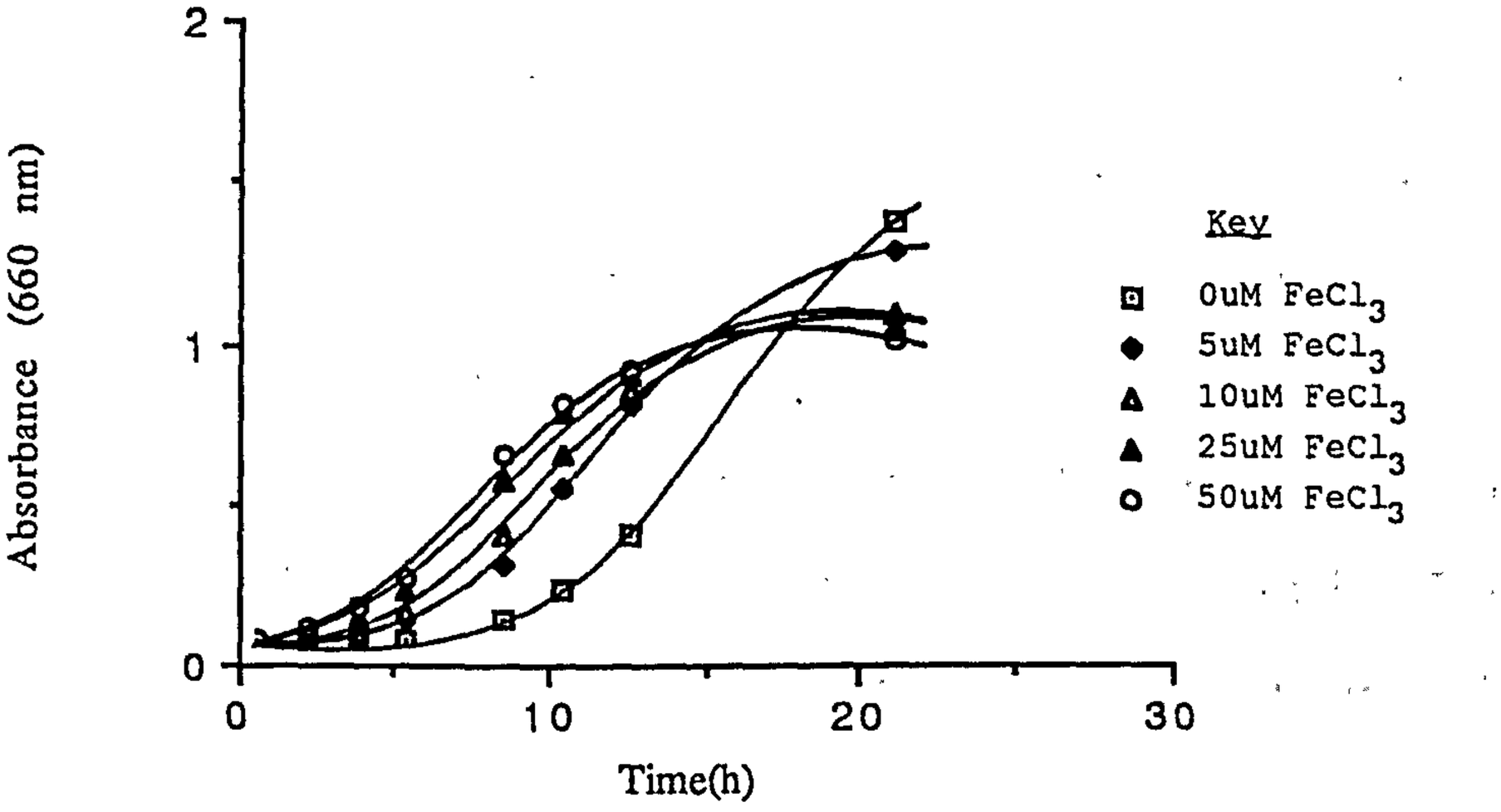


Figure 23. Growth curves of isolates RD34 (a) and RD88 (b) grown under various conditions of iron limitation. Cells were grown in deferrated TSB supplemented with 0, 5, 10, 25 and 50 μM $FeCl_3$.

transferrin, 200 μM 2,2' -dipyridyl and 280 μM EDDA. The nature of the chelating agent was shown not to affect the expression of the iron-regulated OMPs. Thus, in the presence of 200 μM 2,2' -dipyridyl and 280 μM EDDA four proteins of molecular weights 72, 69.5, 68 and 66 KDa were produced. The same four proteins were expressed, approximately to the same extent, in isolates RD6, RD28, RD34 and RD62 (Figure 24A) as well as in isolates RD66, RD124, RD150 and RD154 (results not shown). These isolates were chosen because they represented a selection of biotypes, serotypes and OMP-types. The 69.5 and 66 KDa proteins were expressed in greater abundance than the 72 and 68 KDa proteins. In the case of transferrin, iron-regulated OMPs were not induced at a concentration of 3 μM (note that this concentration did not retard growth). However, at a concentration of 25 μM small amounts of the same four proteins were induced and the growth rate was slightly retarded (results not shown). Due to the expense of transferrin this experiment was carried out only on one isolate - RD6. Due to the identical effects of the three chelators on the expression of iron-regulated OMPs all subsequent work was carried out with 2,2' -dipyridyl.

7.3.2.2 Confirmation that proteins are iron-regulated. To confirm that the 72, 69.5, 68 and 66 KDa proteins are, in fact, iron-regulated the effect of adding 0, 50 and 100 μM $FeCl_3$ to iron-limited medium (TSB supplemented with 200 μM 2,2' -dipyridyl) was examined in isolates RD6, RD28, RD34 and RD88. It was found that 50 μM $FeCl_3$ had very little effect on the expression of these proteins but 100 μM $FeCl_3$ repressed the production of the proteins (Figure 24B). These results confirmed that the proteins described above are, in fact, iron-regulated.

7.3.2.3 Comparison of iron-regulated outer membrane proteins in selected isolates. To compare the expression of iron-regulated OMPs in isolates of Y. ruckeri, 36 selected isolates were grown under iron-limiting conditions in the

Figure 24. Effect of iron limitation on the expression of iron-regulated outer membrane proteins in *Y. ruckeri*.

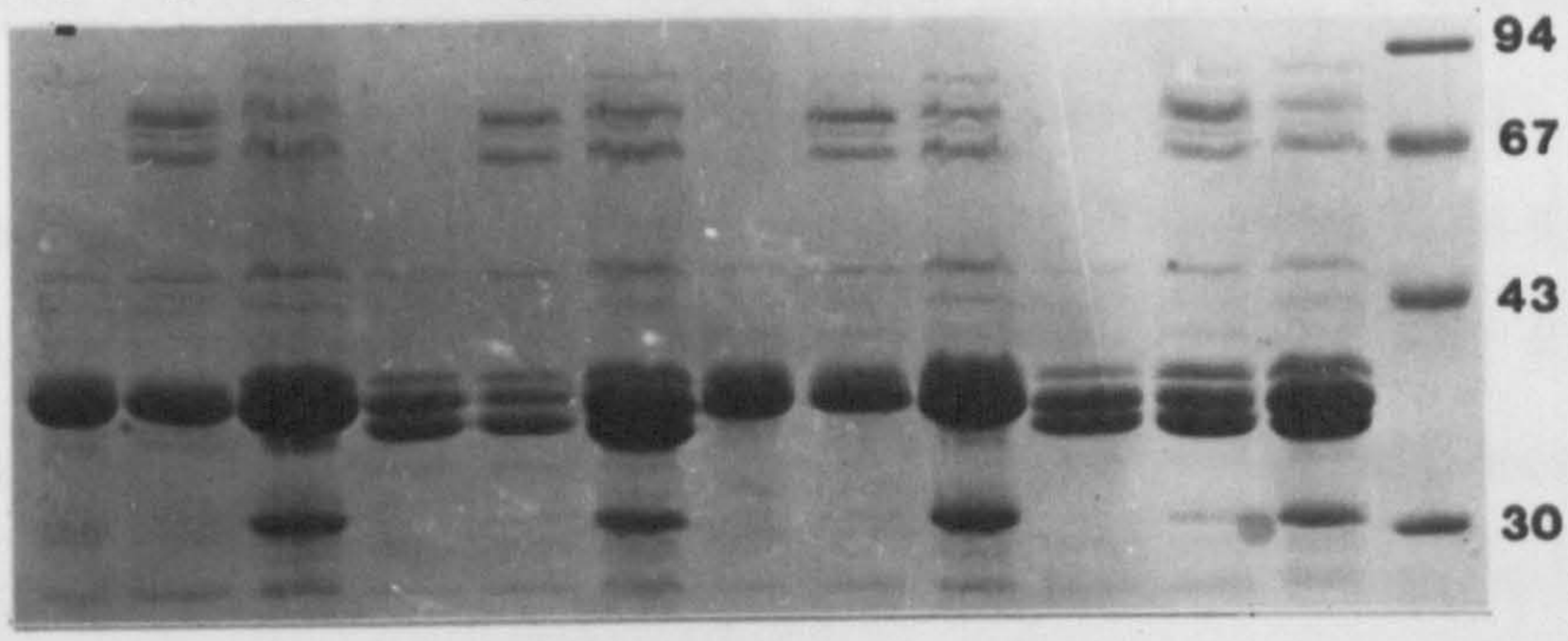
(A). Expression of iron-regulated OMPs in TSB (lanes 1, 4, 7 and 10), in TSB supplemented with 200 μM 2,2'-dipyridyl (lanes 2, 5, 8 and 11) and in TSB supplemented with 280 μM EDDA (lanes 3, 6, 9 and 12), in isolates RD6 (lanes 1 - 3), RD28 (lanes 4 - 6), RD34 (lanes 7 - 9) and RD62 (lanes 10 - 12). Molecular weight standards (KDa) are shown in lane 13.

(B). Expression of iron-regulated OMPs in iron-deficient TSB (i.e. TSB supplemented with 200 μM 2,2'-dipyridyl) supplemented with 0 μM (lanes 1, 4, 7 and 10), 50 μM (lanes 2, 5, 8 and 11) and 100 μM (lanes 3, 6, 9 and 12) $FeCl_3$, in isolates RD6 (lanes 1 - 3), RD28 (lanes 4 - 6), RD34 (lanes 7 - 9) and RD88 (lanes 10 - 12). Molecular weight standards (KDa) are shown in lane 13.

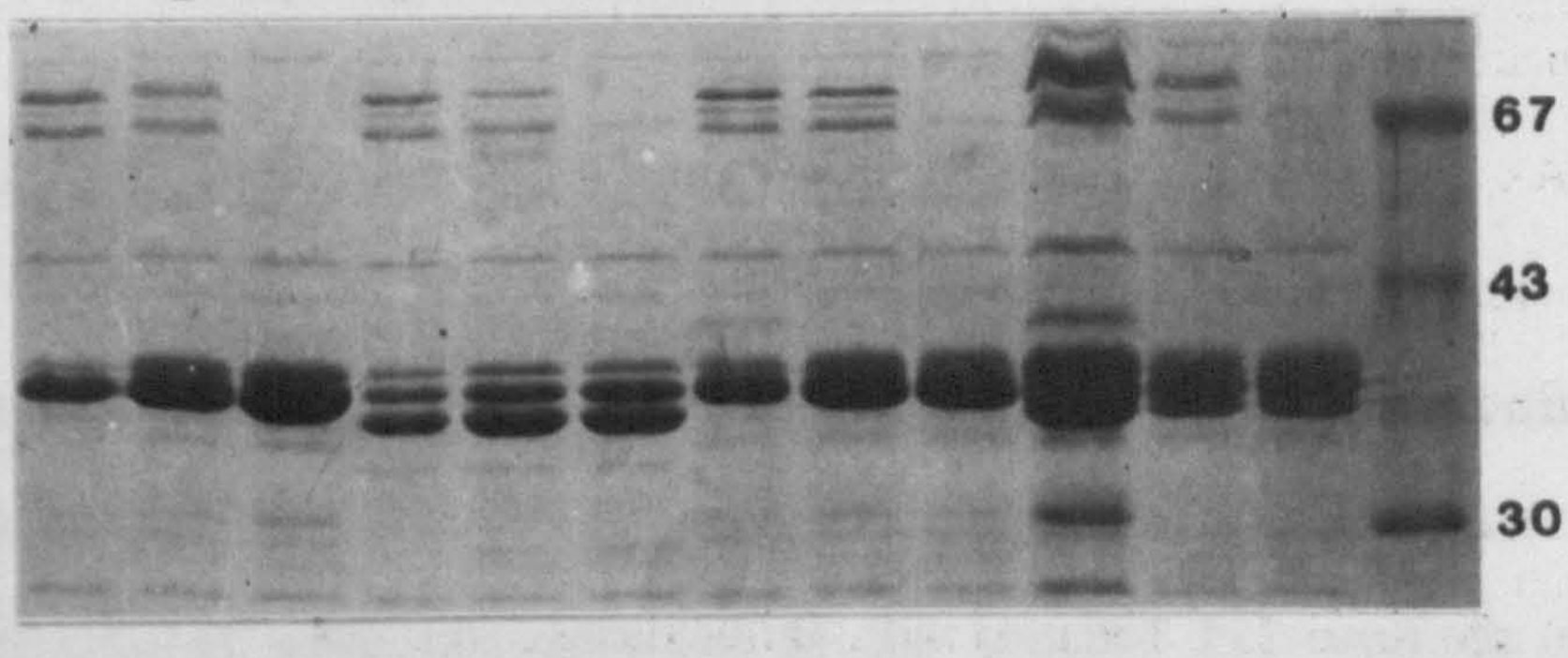
(C). Expression of iron-regulated OMPs in TSB supplemented with 200 μM 2,2'-dipyridyl in isolates RD6, RD20, RD22, RD34, RD36, RD38, RD40, RD42, RD48, RD52, RD58, RD60, RD62 and RD64 (lanes 1 - 15, respectively).

(D). Expression of iron-regulated OMPs in TSB supplemented with 200 μM 2,2'-dipyridyl in isolates RD66, RD70, RD86, RD88, RD124, RD126, RD136, RD138, RD140, RD150, RD152, RD154, RD156, RD168 and RD170 (lanes 1 - 15, respectively).

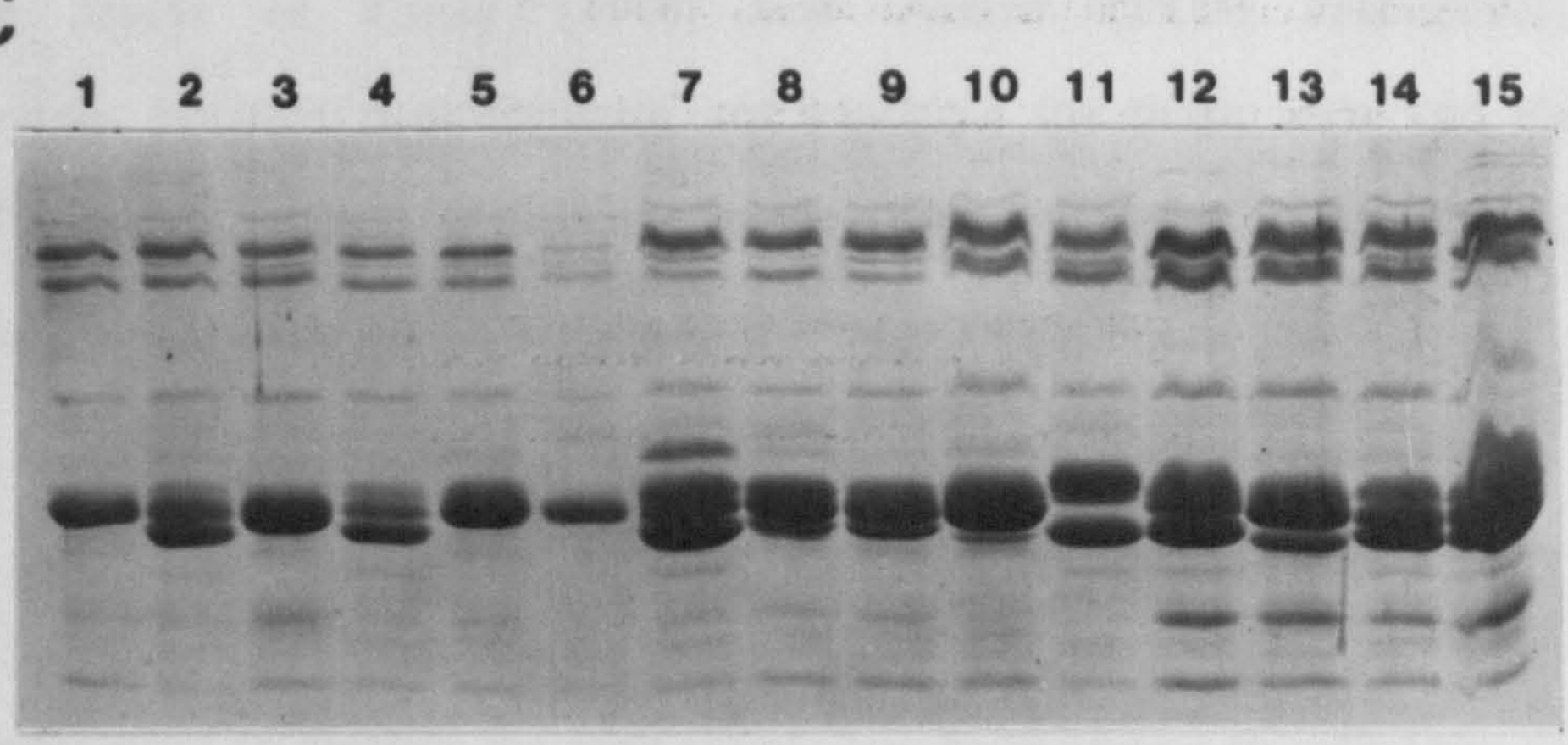
A


1 2 3 4 5 6 7 8 9 10 11 12 13
94
67
43
30

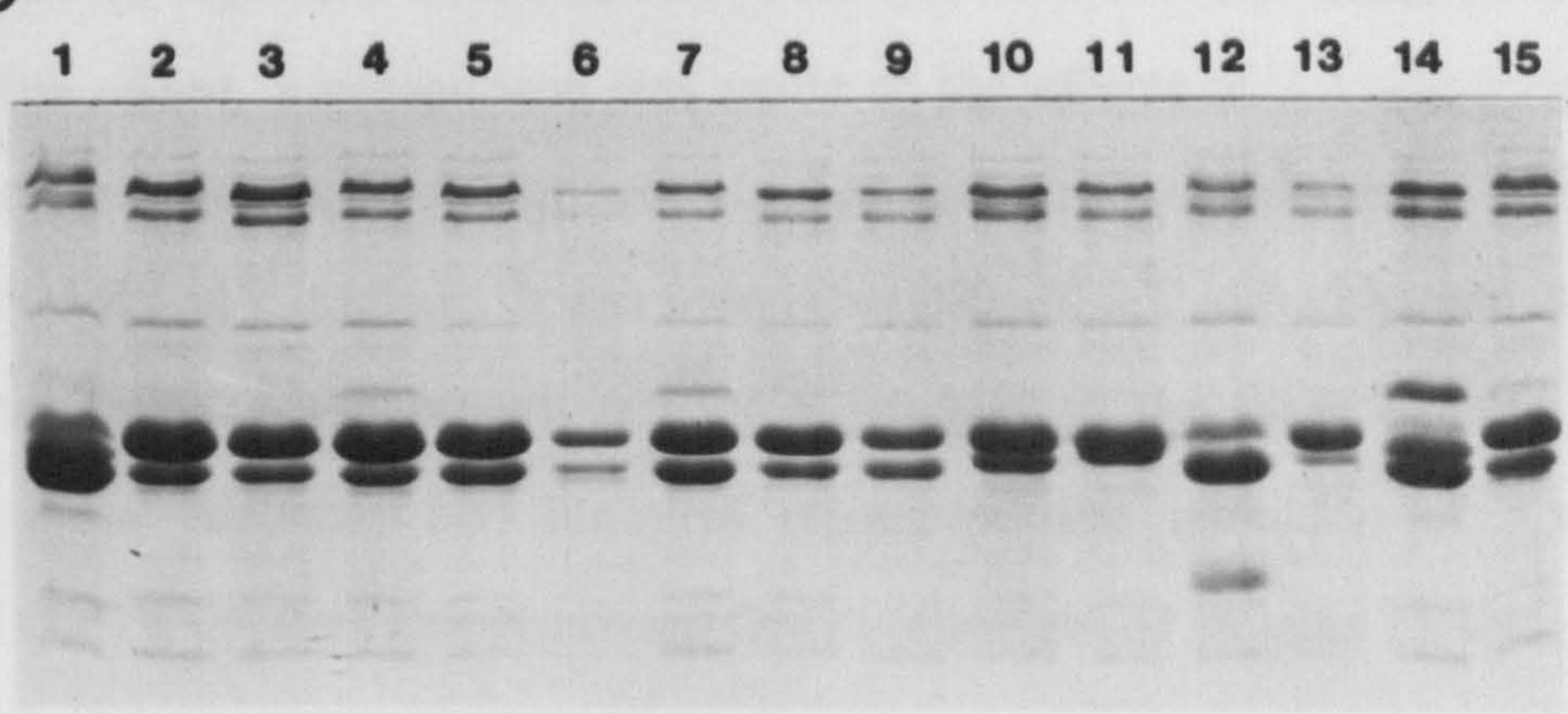
B


1 2 3 4 5 6 7 8 9 10 11 12 13
67
43
30

C


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

D


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

presence of 200 μM 2,2' -dipyridyl. The isolates were selected to include a range of biotypes, serotypes and OMP-types as well as differences in virulence (Section 9). All 36 isolates expressed four iron-regulated OMPs of approximate molecular weights 72, 69.5, 68 and 66 KDa. The 69.5 and 66 KDa proteins were expressed in greater abundance than the 72 and 68 KDa proteins. Allowing for slight differences due to the amount of protein loaded all 36 isolates were identical in their expression of these proteins (Figures 24C and D). These results are summarised in Table 16 in relation to the biotypes, serotypes and OMP-types of the isolates.

7.3.3 Production of siderophores under conditions of iron-limitation

The production of siderophores was not detected in the universal chemical assay of Schwyn and Neilands (1987) in any of the isolates examined. In addition, siderophores were not detected in the specific hydroxamate and phenolate assays of Csaky (1948) and Arnow (1937) respectively. Unfortunately, positive controls were not available for these tests and the results should be treated with some reservation. Results with two V. anguillarum isolates and an E.coli isolate were also negative.

7.4 DISCUSSION

The chelator used to restrict the iron supply has been shown to influence the synthesis of iron-regulated OMPs as well as the growth kinetics of E.coli (Chart et al., 1986). Thus, a comparison was made of the effects of 2,2' -dipyridyl, EDDA and transferrin on the growth kinetics and on the induction of iron-regulated OMPs of Y. ruckeri. It was found that 200 μM 2,2' -dipyridyl and 280 μM EDDA retarded the growth rate of Y. ruckeri to the same extent; in addition, these chelators had identical effects on the induction of iron-regulated OMPs, i.e. four iron-regulated OMPs of molecular weights 72, 69.5,

Table 16. Expression of iron-regulated outer membrane proteins in isolates of Y.ruckeri and their relation to other characteristics.

Isolate	Biotype	Serotype	OMP-type	Iron-regulated OMPs[a]
RD156	1	01	1	4
RD184	1	01	1	4
RD6	2	01	1	4
RD152	2	01	1	4
RD20	1	01	2	4
RD38	1	01	2	4
RD62	1	01	2	4
RD154	2	01	2	4
RD40	1	01	3	4
RD42	1	01	3	4
RD70	1	01	3	4
RD86	1	01	3	4
RD88	1	01	3	4
RD124	1	01	3	4
RD126	1	01	3	4
RD136	1	01	3	4
RD138	1	01	3	4
RD140	1	01	3	4
RD170	1	01	3	4
RD178	1	01	3	4
RD180	1	01	3	4
RD288	1	01	3	4
RD22	1	01	3	4
RD34	1	02	1	4
RD174	2	02	1	4
RD58	1	02	2	4
RD64	1	02	2	4
RD168	1	02	2	4
RD28	1	05	2	4
RD48	1	06	1	4
RD66	1	06	2	4
RD36	1	07	1	4
RD60	1	07	1	4
RD150	1	07	1	4
RD52	1	07	5	4

a = OMPs of 72, 69.5, 68 and 66 KDa

68 and 66 KDa were induced. Transferrin, however, at a concentration of 3 μM, did not retard the growth rate of Y. ruckeri and did not induce expression of iron-regulated OMPs. Even at the higher concentration of 25 μM , the retardation of the growth rate and induction of iron-regulated OMPs was not as great as it was with 200 μM 2,2' -dipyridyl and 280 μM EDDA. Nevertheless, transferrin appeared to induce expression of the same four iron-regulated OMPs that were induced by 2,2' -dipyridyl and EDDA. The results obtained with transferrin suggested, therefore, that either the transferrin was already partially saturated with iron or that Y. ruckeri has an extremely effective mechanism for acquiring iron from this glycoprotein.

That the expression of iron-regulated OMPs was due to iron-limitation, and not to chelation of some other metal or to the chelator itself, was confirmed by the addition of $FeCl_3$ to medium chelated with 2,2' -dipyridyl. Similarly, addition of $FeCl_3$ to deferrated medium demonstrated that retardation of growth was due to iron-limitation and not to some other factor.

The expression of iron-regulated OMPs was examined in 36 isolates of Y. ruckeri by chelation with 2,2' -dipyridyl. Iron-regulated OMPs of molecular weights 72, 69.5, 68 and 66 KDa were produced in all 36 isolates examined. Proteins of the same molecular weight in different isolates were assumed to be identical though this would need to be confirmed by immunological methods. The expression of iron-regulated OMPs did not exhibit variation according to the biotype, serotype or OMP-type of the isolates (Table 16). Furthermore, there was no variation in the expression of iron-regulated OMPs which could be related to virulence (Section 9). It was probably correct to assume, therefore, that the expression of iron-regulated OMPs is not an important virulence determinant in Y. ruckeri. This is not to say, however, that iron-regulated OMPs are not required for virulence in Y. ruckeri since a means of obtaining iron for growth is essential for pathogenic bacteria. This hypothesis

would need to be tested by producing mutants, lacking iron-regulated OMPs, from virulent isolates and by re-examining the virulence of the mutants. By contrast, in other Yersinia spp. the degree of virulence correlates with the presence of two high-molecular-weight iron-regulated OMPs (Carniel et al., 1987).

Although siderophore activity was not detected in Y. ruckeri isolates grown under iron-limiting conditions, the lack of a positive control questioned these results. Furthermore, the precise iron-concentration of the Tris medium used for the siderophore assays was not determined and there may have been sufficient iron to allow for growth without the production of siderophores. However, the experiment was repeated for some isolates incorporating EDDA in the medium; again, siderophores were not detected. Perhaps siderophores not detectable by chemical assay could have been detected by bioassay, as has been shown for Klebsiella spp. (Williams et al., 1987). If Y. ruckeri does not produce siderophores, this will perhaps not be surprising, since other Yersinia spp. also do not possess siderophore activity (Perry and Brubaker, 1979). Yersinia ruckeri may, therefore, possess a high-affinity iron-uptake mechanism that relies on direct contact between membrane protein receptors and Fe^{3+}-glycoprotein complexes as has been described in N. gonorrhoeae (Mickelson and Sparling, 1981), N. meningitidis (Archibald and De Voe, 1979) as well as in the fish pathogen A. salmonicida (Chart and Trust, 1983). Alternatively, Y. ruckeri may utilize other iron compounds such as haem and haemin as do H. influenzae (Pidcock et al., 1988) and other Yersinia spp. (Perry and Brubaker, 1979). It has been suggested (Lawlor et al., 1987) that siderophores may be more important in bacteria which cause bacteraemia, as opposed to intracellular pathogens. If this is the case it may be expected that Y. ruckeri, which causes a bacteraemia, would require the production of siderophores. Further experiments are needed to confirm that siderophores are not produced by Y. ruckeri and to examine in further detail the mechanism of iron-uptake.

The fact that all of the Y. ruckeri isolates examined possessed the same four iron-regulated OMPs indicated that the genes coding for these proteins are probably located on the chromosome. The plasmid content of Y. ruckeri isolates of different serotypes varies (De Grandis and Stevenson, 1982; Toranzo et al., 1983) and so it is unlikely that the genes coding for these proteins are located on plasmids. The plasmids of Y. ruckeri are therefore probably not involved in iron uptake as they are, for example, in V. anguillarum (Crosa, 1980) and other bacterial species (Crosa, 1984). This could be confirmed by examining the expression of iron-regulated OMPs in plasmid-cured derivatives. It is notable that the genes encoding the iron-regulated OMPs of other Yersinia spp. are also not located on the virulence plasmid (Carniel et al., 1987).

Chart et al. (1988) examined the iron-regulated OMPs from 70 strains of E.coli and showed that there were considerable differences between the patterns of different strains, with three distinct profiles predominating. Variation in the iron-regulated OMPs of Y. ruckeri did not occur and probably reflects the greater homogeneity of this organism, as demonstrated by biochemical tests, compared to E.coli. Iron-regulated OMPs could not, therefore, be used as epizootiological or virulence markers for Y. ruckeri as has been suggested for E.coli (Chart et al., 1988).

Pathogenic bacteria obtained direct from infected animals, or from patients, without subculture have been shown to express iron-regulated OMPs (Griffiths et al., 1983; Brown et al., 1984; Anwar et al., 1984; Lam et al., 1984). Such a study for Y. ruckeri would demonstrate further the role of these proteins in the pathogenesis of ERM. In particular, the antibody-response to these proteins in naturally-infected fish would reveal useful information about the importance of these proteins in natural infections.

Section 8

Susceptibility of Yersinia ruckeri to Complement-Mediated Bactericidal Activity of Rainbow Trout Serum

Susceptibility of Yersinia ruckeri to Complement-Mediated Bactericidal Activity of Rainbow Trout Serum

8.1 INTRODUCTION

The bactericidal effect of non-immune serum plays an important role in host defence against bacterial infection. It has been recognized since 1895 (Bordet, 1895) that some Gram-negative bacteria are sensitive to the lytic action of fresh serum, whereas others are highly serum-resistant. In general, serum-resistant organisms are more pathogenic than serum-sensitive bacteria in animal models of infection, and serum-resistant organisms are more commonly isolated from the bloodstream of patients with Gram-negative bacteraemia (Roantree and Rantz, 1960). The bactericidal effect of serum is due principally to the complement system and the subject has been reviewed by Taylor (1983).

Complement activation by Gram-negative bacteria can occur via the classical or the alternative pathways. The classical pathway can be activated, independent of antibody, by the lipid A region of LPS whereas the alternative pathway is activated, also independent of antibody, by the O-antigen polysaccharide part of LPS (Morrison and Kline, 1977). Thus, LPS from smooth strains of bacteria can initiate complement activation both by an interaction of lipid A with classical components and by an interaction of the polysaccharide with alternative pathway components. Vukajlovich et al. (1987) presented evidence for two independent modes of polysaccharide-dependent activation of the alternative pathway of complement by LPS. One mechanism was dependent upon specific O-antigen polysaccharides and the second was

defined by a specific L-glycero-D-mannoheptose/glucose region of the oligosaccharide core. Classical pathway activation may also be antibody-mediated, by antibodies against any of the surface antigens including LPS and OMPs.

Although serum resistance of Gram-negative bacteria probably has a multifactoral basis in vivo the OM, as the most peripheral component of the bacterial cell envelope (except when a capsule is present), is clearly involved. Outer membrane proteins have been implicated in the resistance to the bactericidal activity of serum in a number of bacterial species including E.coli (Taylor and Parton, 1977; Moll et al., 1980), N. gonorrhoeae (Hildebrandt et al., 1978), A. salmonicida (Munn et al., 1982), C. fetus (Blaser et al., 1987) and S. typhimurium (Hackett et al., 1987). In those bacteria possessing polysaccharide capsules external to the OM, this structure has been implicated in providing resistance to the bactericidal activity of serum, e.g. in E.coli (Glynn and Howard, 1970) and in Klebsiella aerogenes (Williams et al., 1983).

The LPS component of the OM, however, is of extreme importance in providing resistance to the bactericidal effects of serum. It has been recognized that smooth strains of Gram-negative bacteria, synthesizing LPS with a high degree of substitution of core units by O-specific side chain moieties, are more resistant to serum than rough isolates or mutants that have lost the ability to either synthesize or attach the O-antigen component of LPS (Wardlaw, 1963; Nelson and Roantree, 1967; Rowley, 1968; Taylor, 1975). However, whereas Wardlaw (1963) suggested that rough strains of E.coli were more sensitive than smooth strains because the former contain less polysaccharide, and Feingold (1969) suggested that resistance was attributed to the amount of O-specific side chain material compared with core material in the LPS, the work of Luderitz et al. (1966) suggested that the terminal sugars of the LPS are more important than the overall amount.

The complement system of rainbow trout is very similar to, and probably shares a common evolutionary origin with, that of higher vertebrates (Nonaka et al., 1981). Both classical and alternative complement pathways occur in rainbow trout serum (Nonaka et al., 1981; Trust et al., 1981; Sakai, 1983). In the channel catfish (Ictalurus punctatus) the alternative rather than the classical pathway of complement was shown to be important in bactericidal activity against Salmonella paratyphi and other Gram-negative bacteria (Ourth and Wilson, 1982a; 1982b). The alternative complement pathway in poikilotherms probably has advantages over the classical pathway, since conditions may not be favourable for the protein synthesis required for immunoglobulin production. Resistance to the bactericidal mechanisms of normal serum is an important contributor to the virulence of fish-pathogenic vibros (Trust et al., 1981) and is also important in the virulence of A. salmonicida (Munn et al., 1982).

A comparative study of the susceptibility of isolates of Y. ruckeri to rainbow trout serum has not been undertaken. The objective of this study, therefore, was primarily to compare the susceptibility of a large number of isolates of Y. ruckeri, with a wide range of differing surface characteristics, to the bactericidal effect of rainbow trout serum in order to identify surface components which may be important in providing resistance to complement-mediated killing and which may also be important as virulence determinants. Additional objectives were to identify the complement pathway(s) involved in bactericidal activity and to examine the role of antibody.

8.2 MATERIALS AND METHODS

8.2.1 Bacterial isolates and growth conditions

The susceptibility to pooled normal rainbow trout serum (PNRTS) of 36 isolates of Y. ruckeri was examined. The isolates were selected to include various combinations of biotype, serotype, OMP-type and virulence. In particular, a wide range of serotype O1 isolates was selected for study because LPS analysis (Section 3), OMP-typing (Section 4) and virulence studies (Section 9) had demonstrated variation within this group. Details of the isolates including their biotypes, serotypes and OMP-types are given in Table 17.

Cells used in serum-killing assays were, without exception, in the logarithmic phase of growth. Bacteria were grown overnight in 10 ml TSB at 22^{o}C and 0.2 ml of this culture was used to inoculate 20 ml TSB which was incubated at 22^{o}C for 3-4 h. Growth was followed spectrophotometrically and the cells were harvested only when logarithmic growth was achieved.

8.2.2 Collection of rainbow trout serum

Normal serum was obtained from twenty rainbow trout of approximate weight 1 Kg. Blood was collected from the caudal vein of anaesthetized fish (Section 6) and allowed to clot at room temperature for 1 h. After centrifugation at 1000 x g for 5 min the serum was separated, pooled and filter-sterilized through a 0.45 μm Millepore filter. The serum was tested for the presence of agglutinating antibodies against each of the five O-serotypes by slide-agglutination tests (Section 2) and was subsequently divided into 0.4 ml aliquots and stored at -70^{o}C until required. To check that storage at -70^{o}C did not reduce complement activity, the bactericidal effect of fresh serum was

compared with serum that had been stored at -70^{o}C for one month; there was no significant difference.

8.2.3 Serum-killing assays

Logarithmic-phase cells were harvested by centrifugation at 1000 x g for 15 min and resuspended in Dulbecco's phosphate buffered saline without added Ca^{2+} and Mg^{2+} (PBS) (Flow Laboratories, Hertfordshire, England) to an optical density of 0.425 ± 0.010 at 610 nm. This was equivalent to a cell density of approximately 5 x 10^{8} colony forming units (c.f.u.'s) per ml as determined from a standard curve. To 0.4 ml PNRTS was added 0.1 ml of bacterial cell suspension (final serum concentration of 80%) and viable counts were carried out by the Miles and Misra (1938) method, after 0, 1.5 and 3 h of incubation at 22^{o}C. Five serial ten-fold dilutions of 0.1 ml of the bacteria-serum mixture were made in PBS and 20 μl of each dilution were dropped onto a dried (37^{o}C for 1 h) TSA plate from a height of approx 3-4 cm. All plate counts were made in triplicate. The plates were incubated at 22^{o}C and the colonies counted after 24 h. Results were expressed as percentage survivors of the inoculum and, where possible, were based on colony counts of the same dilutions. All isolates were also tested in PNRTS previously heated at 46^{o}C for 20 min to destroy the complement activity (Sakai, 1981).

8.2.4 Effect of antibody on the bactericidal effect of serum

Logarithmic-phase cells were harvested and resuspended in PBS to an optical density of 0.425 ± 0.010 at 610 nm as described above. Cells from three 1.0 ml aliquots were harvested by centrifugation at 11,500 x g for 5 min in a microcentrifuge. The cells were resuspended in 200 μl decomplemented (46^{o}C for 20 min) types 1, 2 and 5 rainbow trout antisera and incubated for 30 min at 22^{o}C with occasional mixing. The cells were harvested, washed and

resuspended in 1.0 ml PBS. To 0.4 ml PNRTS was added 0.1 ml of antibody-treated bacterial suspension which was incubated at 22°C. Viable counts were carried out after 0, 1.5 and 3 h as described above. In control experiments, decomplemented PNRTS was used instead of PNRTS.

8.2.5 Effect of chelation with EDTA and EGTA, and of treatment with inulin, on the bactericidal effect of serum

To 1.08 ml PNRTS was added 0.12 ml of either 100 mM ethylenediaminetetraacetate (EDTA) (Sigma), or 100 mM ethylene glycol-bis (β-aminoethyl ether) -N,N,N',N'-tetraacetic acid (EGTA) (Sigma) plus 100 mM $MgCl_2$, or 20 mg/ml inulin. Thus, final concentrations were 10 mM EDTA, 10 mM EGTA + 10 mM $MgCl_2$ and 2 mg/ml inulin. In some experiments 100 mM $CaCl_2$, $MgCl_2$ or both were added with EDTA. To 0.4 ml aliquots of each of these was added 0.1 ml of bacterial suspension in PBS (final serum concentration of 72%) as described above. Viable counts were carried out after 0, 1.5 and 3 h incubation at 22°C also as described above. In controls, serum was replaced with PBS in order to determine the effect of the chelating agent or inulin alone.

8.3 RESULTS

8.3.1 Definitions of serum-resistant and serum-sensitive isolates

Serum-resistant isolates were defined as those which increased in number after 3 h, i.e. greater than 100% survivors. Serum-sensitive isolates were defined as those which decreased in number after 3 h, i.e. less than 100% survivors. To differentiate between isolates which showed total killing after 3 h and isolates which showed only partial killing after 3 h, the former isolates were defined as

showing complete-sensitivity whereas the latter isolates were defined as showing intermediate-sensitivity.

8.3.2 Sensitivity of isolates to the bactericidal effects of normal rainbow trout serum

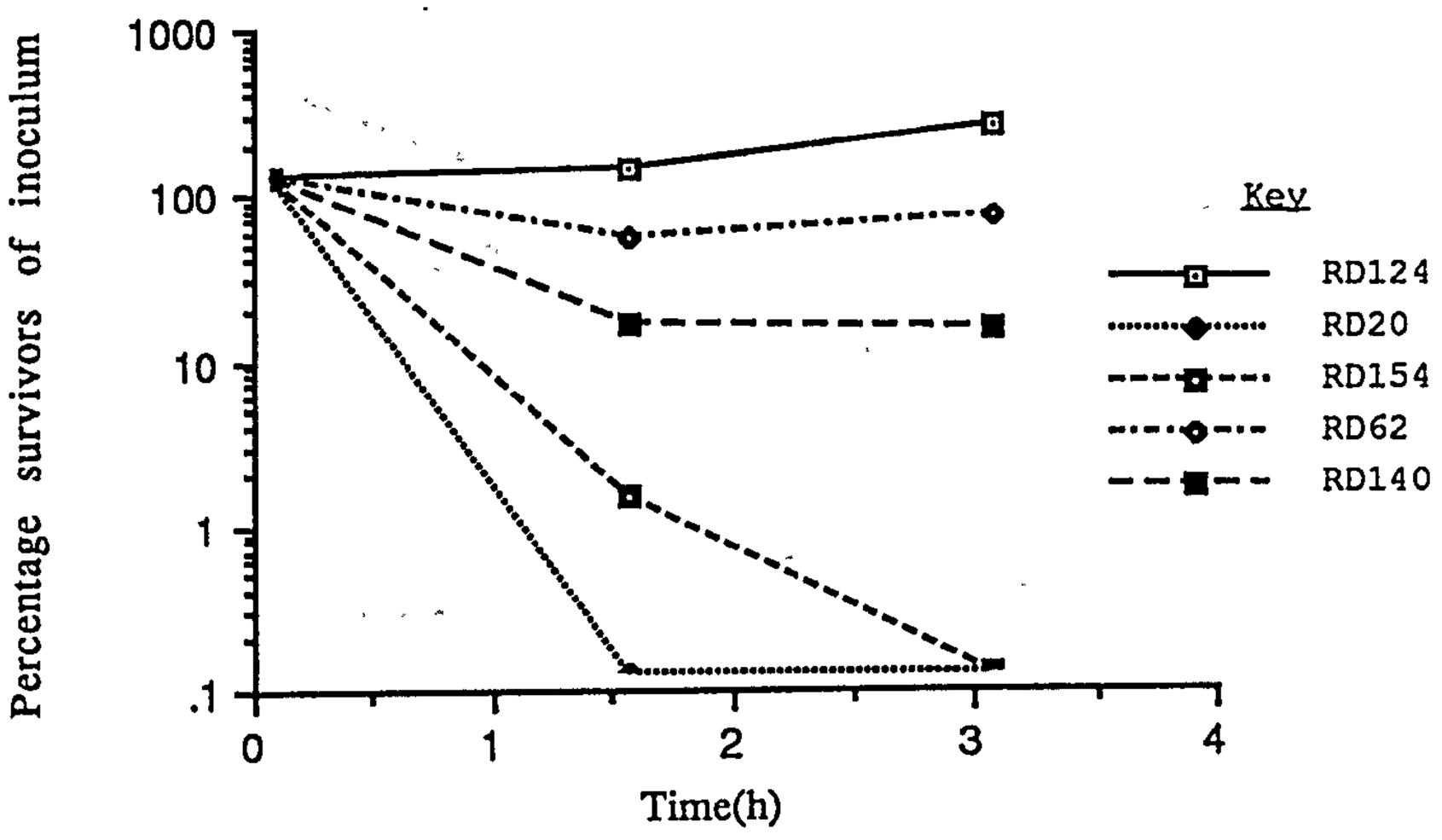
The sensitivities to the bactericidal effect of PNRTS, of the 36 isolates of Y. ruckeri tested, are shown in Figure 25 and in Table 17. Of the serotype O1 isolates tested, six were serum-resistant (isolates RD6, RD38, RD40, RD42, RD86, RD124) and 12 were serum-sensitive (isolates RD156, RD184, RD186, RD20, RD62, RD158, RD160, RD154, RD140, RD22, RD190, RD280). Of the serum-sensitive isolates, two (isolates RD62 and RD140) were of intermediate-sensitivity whereas the remainder were completely sensitive (Figure 25a). It should be noted that with the exception of isolate RD38 (clone 3) all isolates of clones 1, 3, 4 and 6 were serum-sensitive and that serum-resistant isolates belonged to clones 2 and 5 only (Table 17).

The serotype O2 isolates also showed a range of serum-sensitivities (Figure 25b) although only one isolate (RD294) was serum-resistant. Two isolates (RD34 and RD168) were of intermediate-sensitivity and two isolates (RD58 and RD64) were completely sensitive.

All of the serotype O5 isolates tested were serum-sensitive, two isolates (RD28 and RD188) being of intermediate-sensitivity and two isolates (RD192 and RD290) showing complete-sensitivity (Figure 25c).

The serotype O6 isolates also were all serum-sensitive; three isolates (RD66, RD162, RD194) were completely sensitive and a single isolate (RD48) was of intermediate-sensitivity (Figure 25d).

(a)



(b)

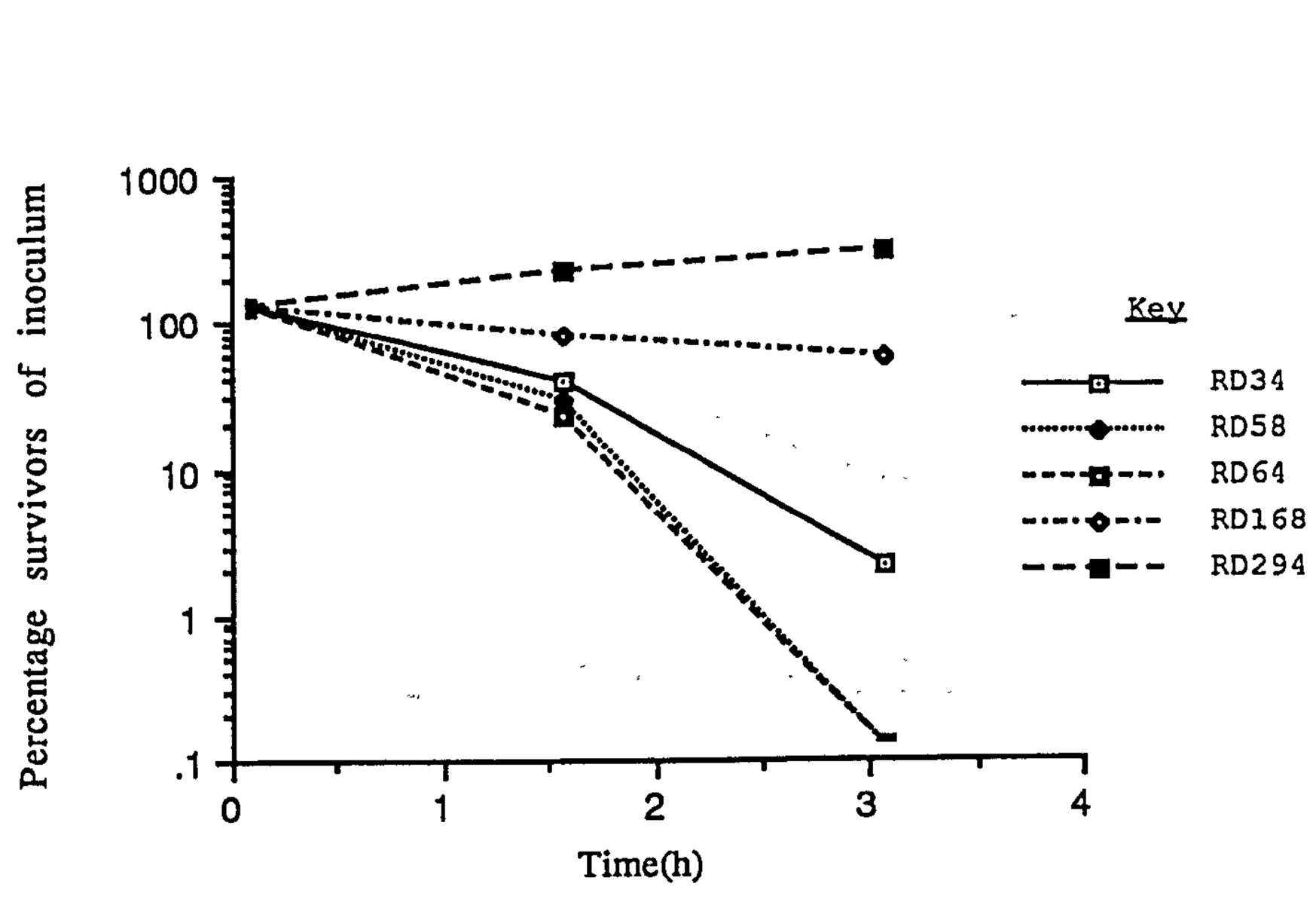
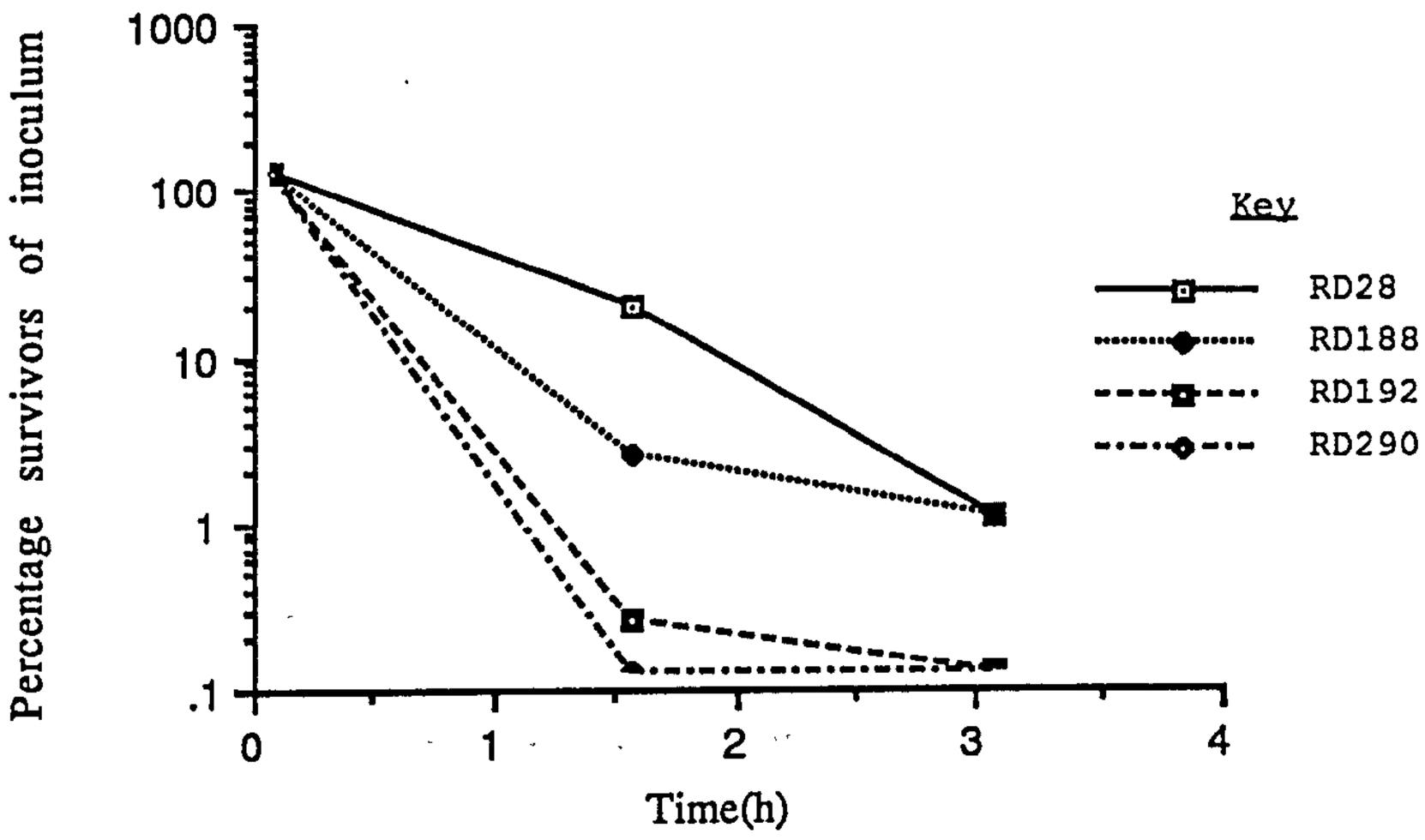


Figure 25. Kinetics of killing of *Y. ruckeri* isolates by 80% normal rainbow trout serum: (a) serotype 01 isolates; (b) serotype 02 isolates. For clarity, control results are not shown but in all cases there was growth in 80% serum preheated at 46°C for 20 min.

(c)



(d)

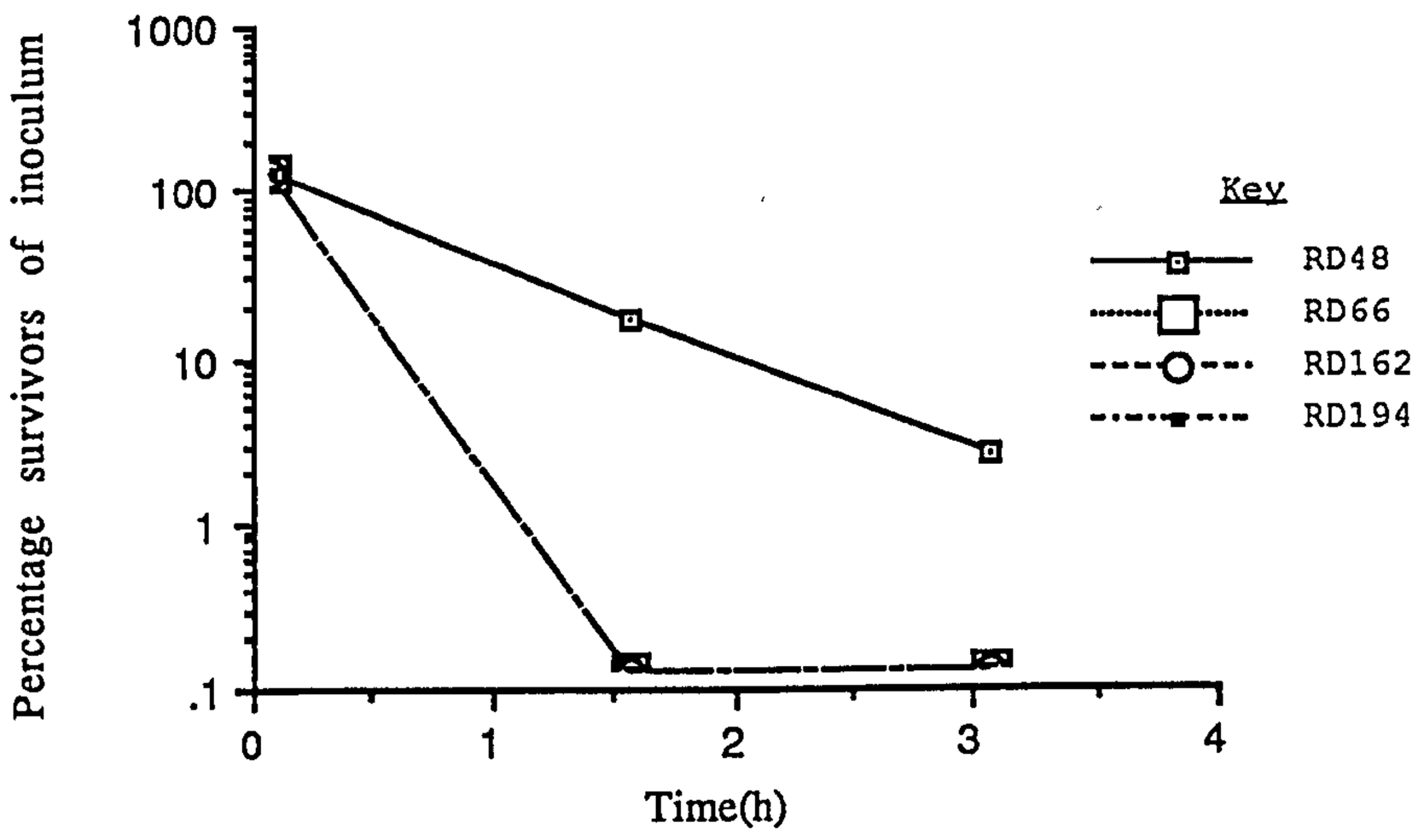


Figure 25 (continued). Kinetics of killing of *Y. ruckeri* isolates by 80% normal rainbow trout serum: (c) serotype 05 isolates ; (d) serotype 06 isolates. For clarity, control results are not shown but in all cases there was growth in 80% serum preheated at 46°C for 20 min.

(e)

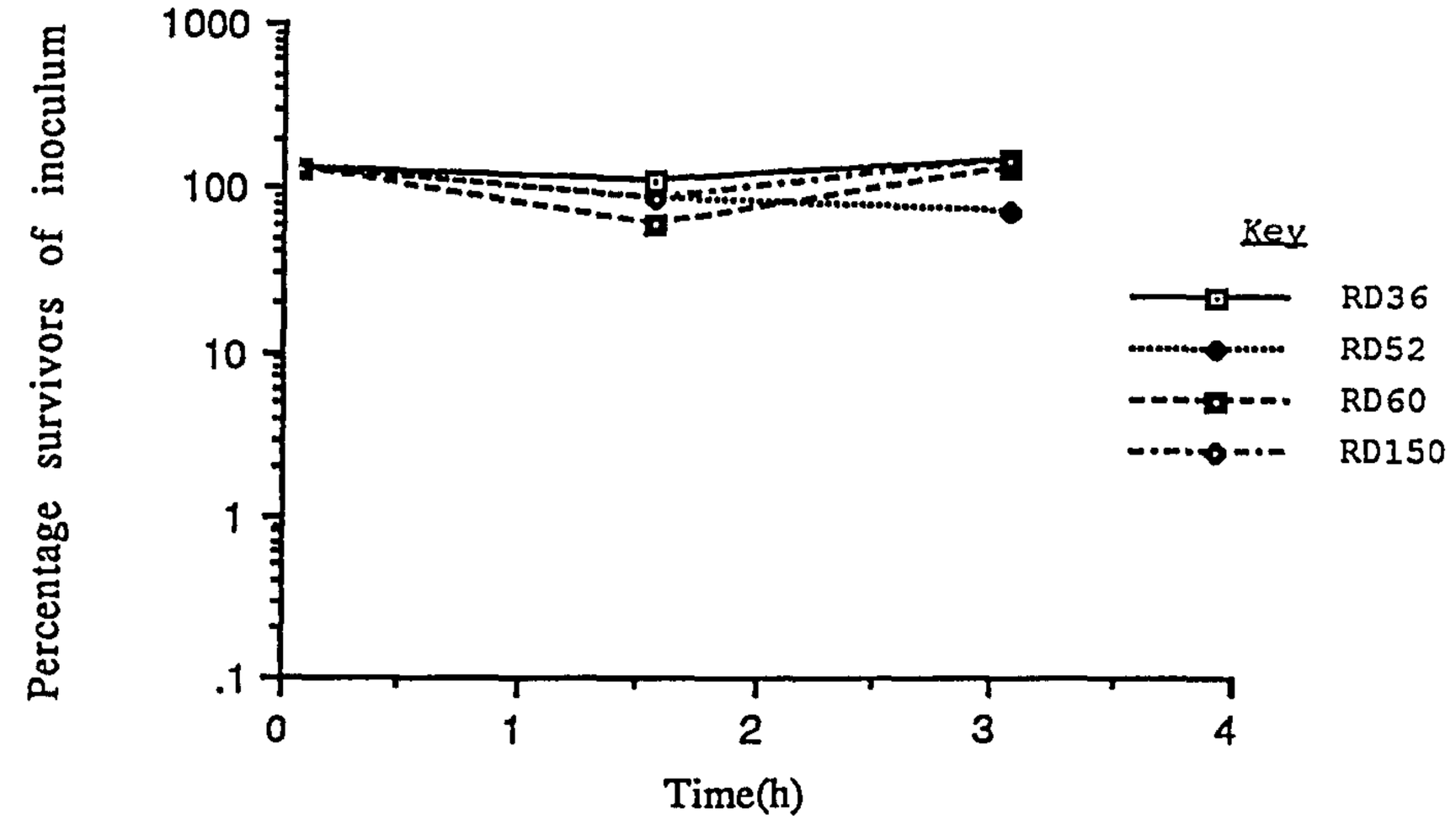


Figure 25 (continued). Kinetics of killing of *Y. ruckeri* isolates by 80% normal rainbow trout serum: (e) serotype 07 isolates. For clarity, control results are not shown but in all cases there was growth in 80% serum preheated at 46°C for 20 min.

Table 17. Relation between biotype, serotype, OMP-type and serum-resistance of isolates of Y. ruckeri.

Isolate	Biotype	Serotype	OMP-type	Serum resistance[a]
RD156	1	01	1	-
RD184	1	01	1	-
RD186	1	01	1	-
RD6	2	01	1	+
RD20	1	01	2	-
RD38	1	01	2	+
RD62	1	01	2	±
RD158	1	01	2	-
RD160	1	01	2	-
RD154	2	01	2	-
RD40	1	01	3	+
RD42	1	01	3	+
RD86	1	01	3	+
RD124	1	01	3	+
RD140	1	01	3	±
RD22	1	01	4	-
RD190	1	01	4	-
RD280	1	01	4	-
RD34	1	02	1	±
RD58	1	02	2	-
RD64	1	02	2	-
RD168	1	02	2	±
RD294	1	02	2	+
RD192	1	05	1	-
RD28	1	05	2	±
RD188	1	05	2	±
RD290	1	05	2	-
RD48	1	06	1	±
RD66	1	06	2	-
RD162	1	06	2	-
RD194	1	06	2	-
RD36	1	07	1	+
RD60	1	07	1	+
RD150	1	07	1	+

RD52	1	07	S	±
RD46	1	UT	1	-

[a] = +, serum-resistant,
±, intermediate sensitivity,
-, completely sensitive.

UT = untypable

Of the serotype O7 isolates three were serum-resistant (RD36, RD60, RD150) whereas one isolate (RD52) showed intermediate-sensitivity (Figure 25e). In the case of the serum-resistant isolates, however, the percentage survivors after 1.5 h was less than 100% (indicating serum sensitivity) but after 3 h the percentage survivors was 100% or just over. The survival rate of these isolates in PNRTS was not as high as the survival rate in decomplemented PNRTS (results not shown).

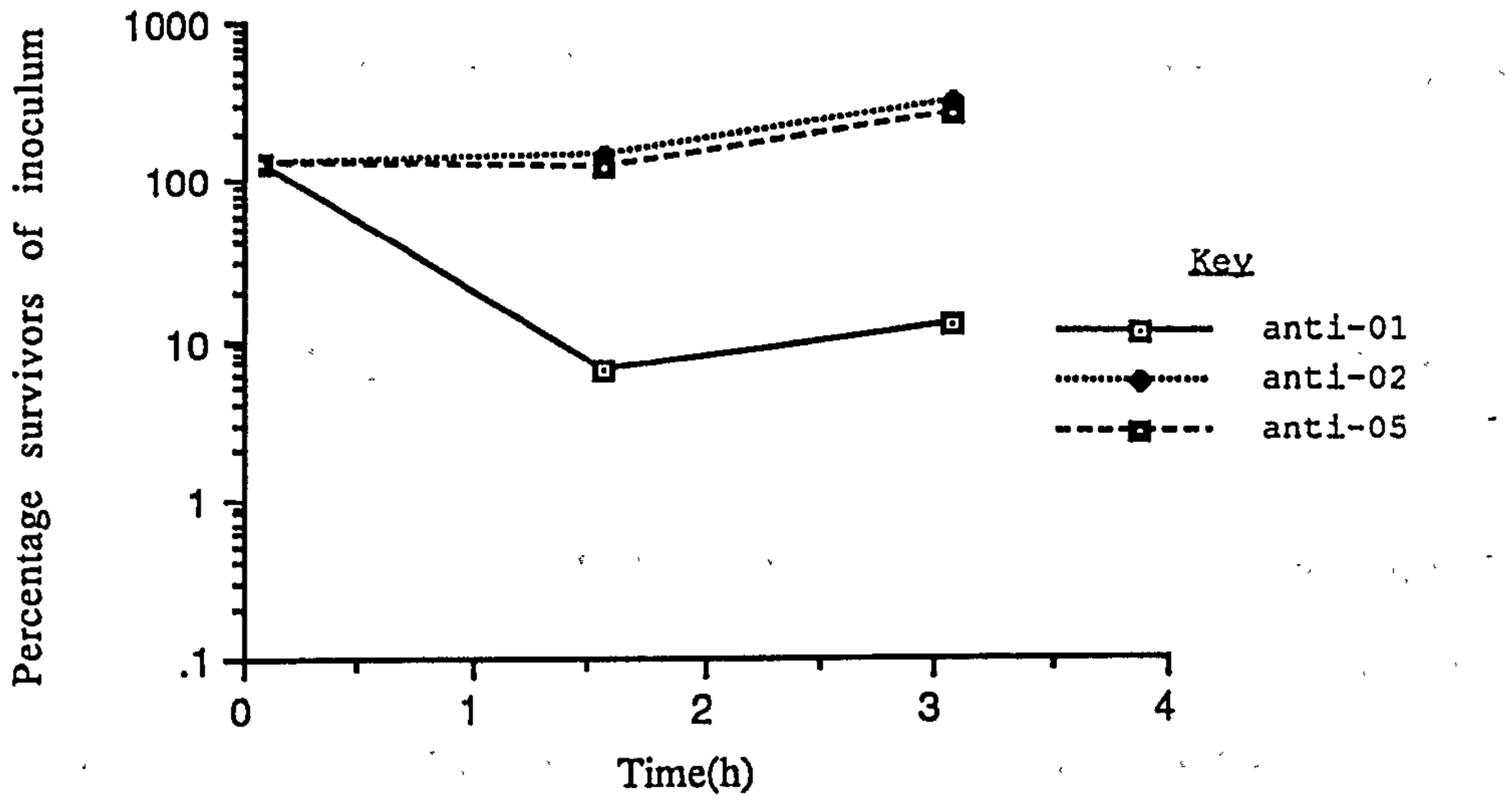
Decomplementation of serum by heating at 46^{o}C for 20 min eliminated the bactericidal effect of serum, thus confirming that killing was in fact due to the complement system. There was substantial growth of all isolates which usually, but not always, exceeded the growth rate of serum-resistant isolates in PNRTS (results not shown).

The relation between biotype, serotype, OMP-type and serum-resistance of the 36 isolates examined is shown in Table 17.

8.3.3 Effect of antibody on the bactericidal effect of normal rainbow trout serum

When homologous and heterologous antibodies were incubated with isolate RD124 (serotype O1) and isolate RD168 (serotype O2) prior to incubation with PNRTS, it was shown that the homologous antiserum caused serum-killing whereas the heterologous antisera did not (Figures 26a and b). Isolate RD124 was serum-resistant in PNRTS alone (Figure 25a); after incubation with type 1 antiserum isolate RD124 was serum-sensitive (intermediate sensitivity) but after incubation with type 2 and 5 antisera isolate RD124 was serum-resistant (Figure 26a). Identical results were obtained with isolate RD6 (serotype O1) (results not shown). Isolate RD168 was of intermediate-sensitivity after incubation with PNRTS alone (Figure 25b); after incubation with type 2

(a)



(b)

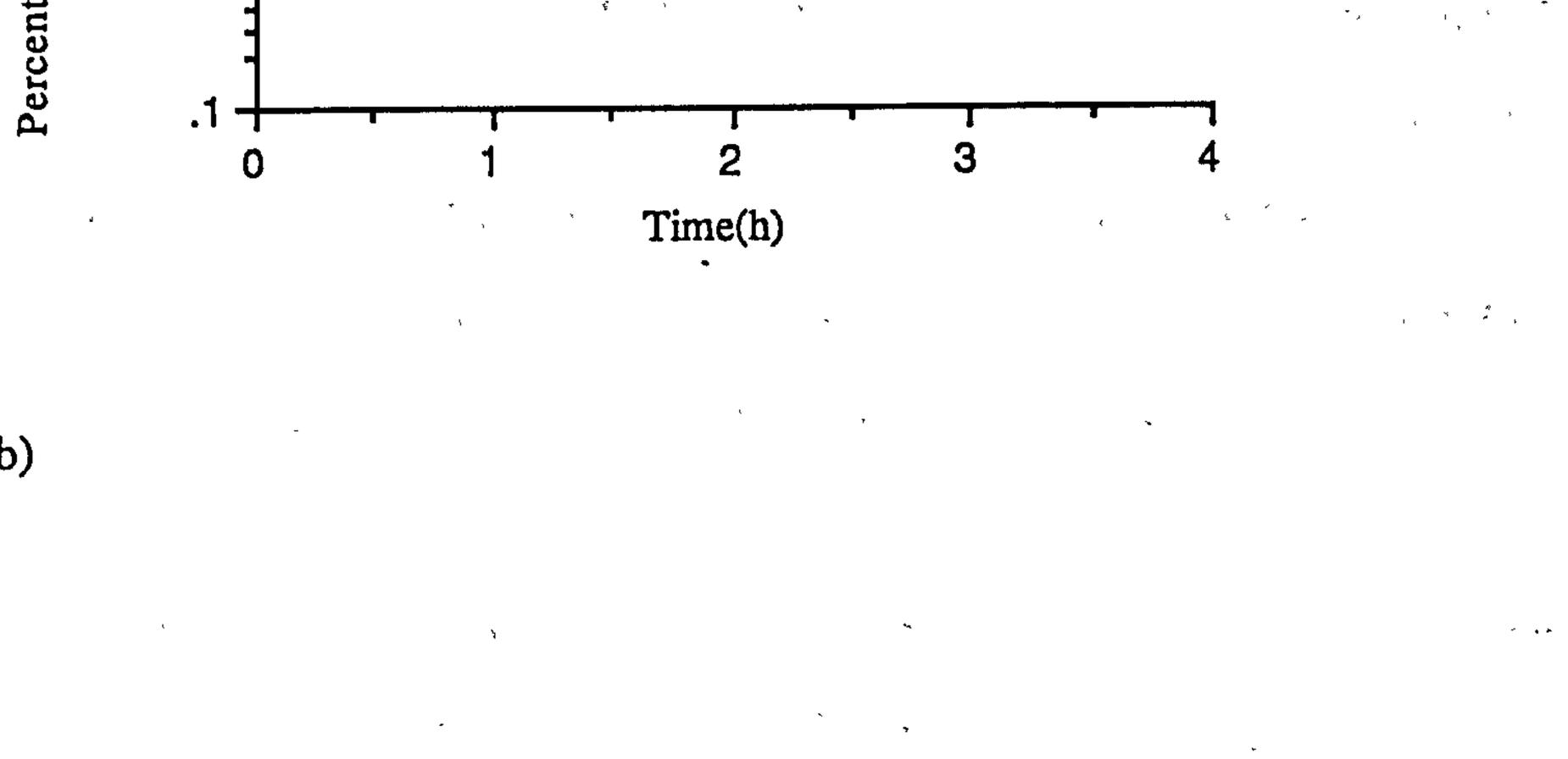


Figure 26. Demonstration of antibody-mediated complement killing of *Y. ruckeri*. Cells of isolates RD124 (serotype 01) (a) and RD168 (serotype 02) (b) were incubated for 30 min at 22°C with decomplemented types 1, 2 and 5 antisera before being incubated at 22°C with 80% normal rainbow trout serum.

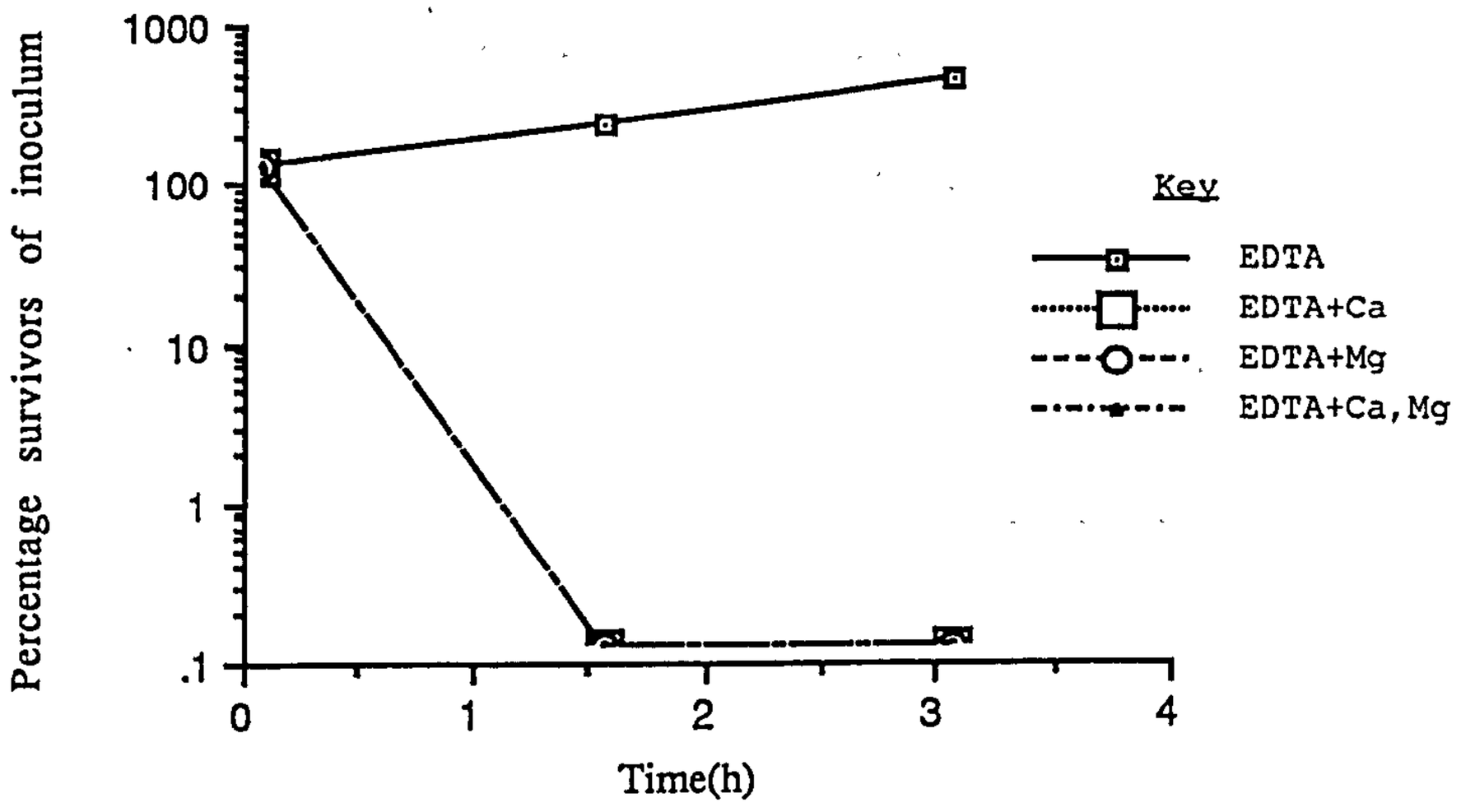
antiserum isolate RD168 was again of intermediate-sensitivity but after incubation with type 1 and 5 antisera isolate RD168 was serum-resistant (Figure 26b).

8.3.4 Effect of chelation with EDTA and EGTA, and of treatment with inulin, on the bactericidal effect of serum

The effect of EDTA, EGTA and inulin on serum-killing was studied only on those isolates exhibiting complete-sensitivity to untreated serum. Chelation with EDTA, which inhibits both the alternative and the classical complement pathways, completely inhibited complement-mediated killing of isolates RD20 (serotype O1), RD290 (serotype O5) and RD66 (serotype O6). However, in the presence of Ca^{2+} or Mg^{2+} ions, or both, killing of isolate RD20 was restored but the killing of isolates RD66 and RD290 was not (Figure 27).

Chelation with EGTA (plus Mg^{2+}), which specifically inhibits the classical complement pathway, had variable results on the bactericidal effect of serum on the isolates examined. Whereas isolates RD58 (serotype O2), RD290 (serotype O5), RD66 (serotype O6) and RD162 (serotype O6) were resistant to serum-killing in the presence of EGTA, isolate RD20 (serotype O1) was completely sensitive and isolates RD154 (serotype O1) and RD192 (serotype O5) showed intermediate-sensitivity (Figure 28). These results suggested that classical complement activation was responsible for killing isolates RD58, RD290, RD66 and RD162 in untreated normal serum and that alternative complement activation was responsible for killing isolates RD20, RD154 and RD192. However, killing of isolates RD154 and RD192 was not as extensive as it was with untreated serum; this suggested that the classical pathway may also have been involved in the killing of these isolates.

(a)



(b)

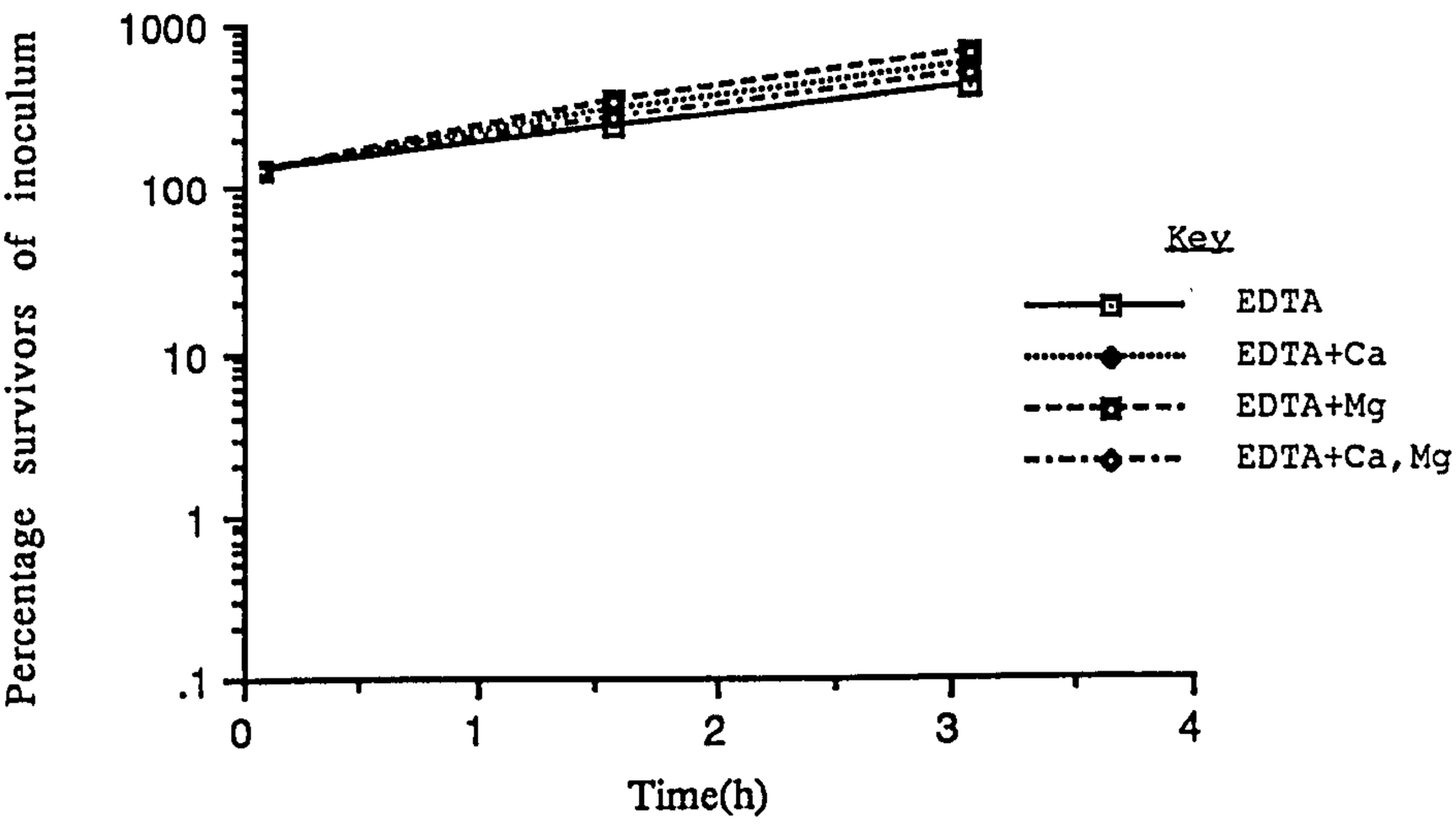


Figure 27. Effect of chelation with 10 mM EDTA on the bactericidal effect of 80% normal rainbow trout serum on isolates RD20 (serotype 01) (a) and RD66 (serotype 06) (b). The effect of adding 10 mM $CaCl_2$, 10 mM $MgCl_2$, and both, is also shown.

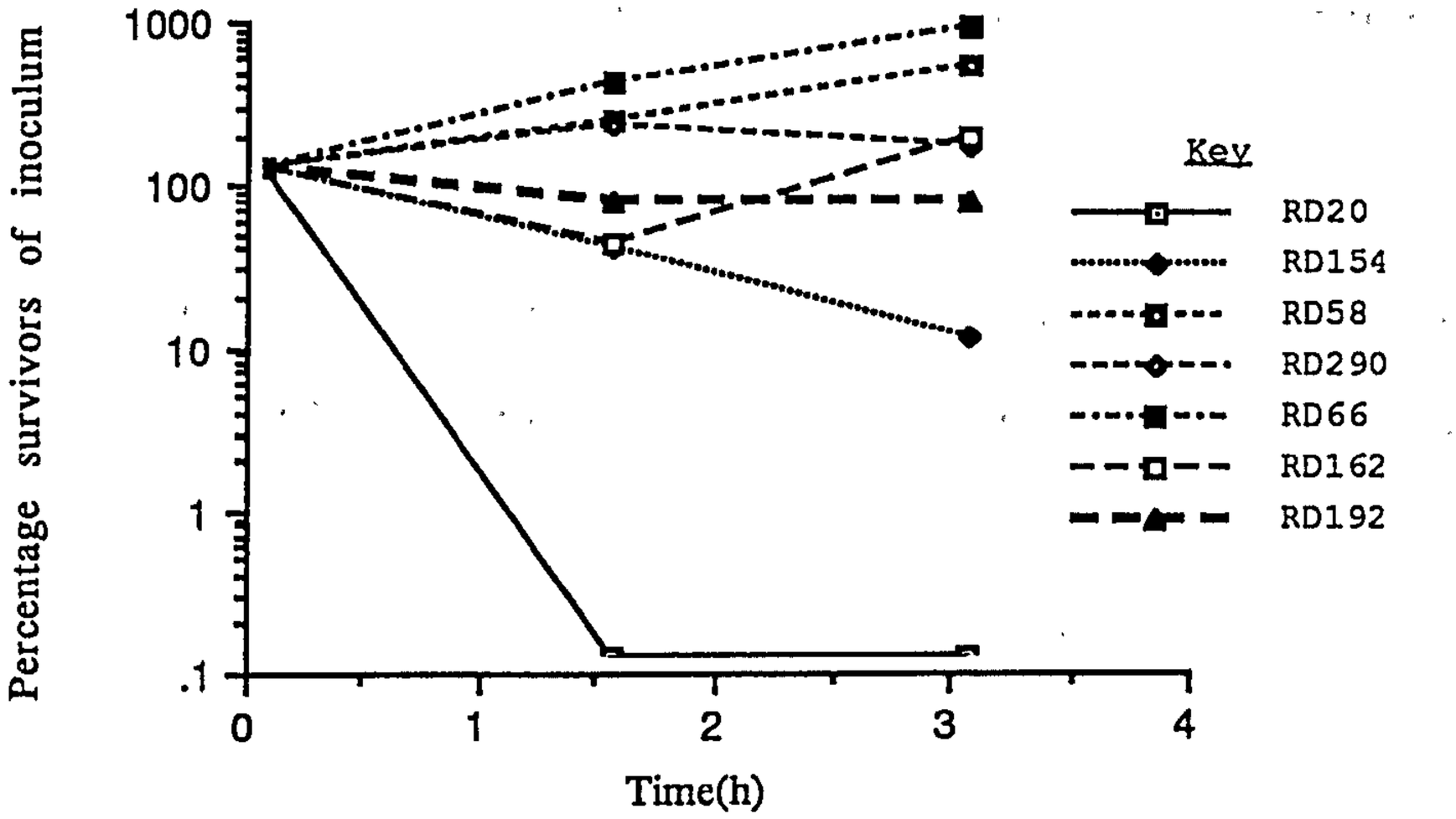


Figure 28. Effect of chelation with 10 mM EGTA + 10 mM $MgCl_2$ on the bactericidal effect of 80% normal rainbow trout serum on isolates RD20, RD154 (serotype 01), RD58 (serotype 02), RD192, RD290 (serotype 05), RD66 and RD162 (serotype 06).

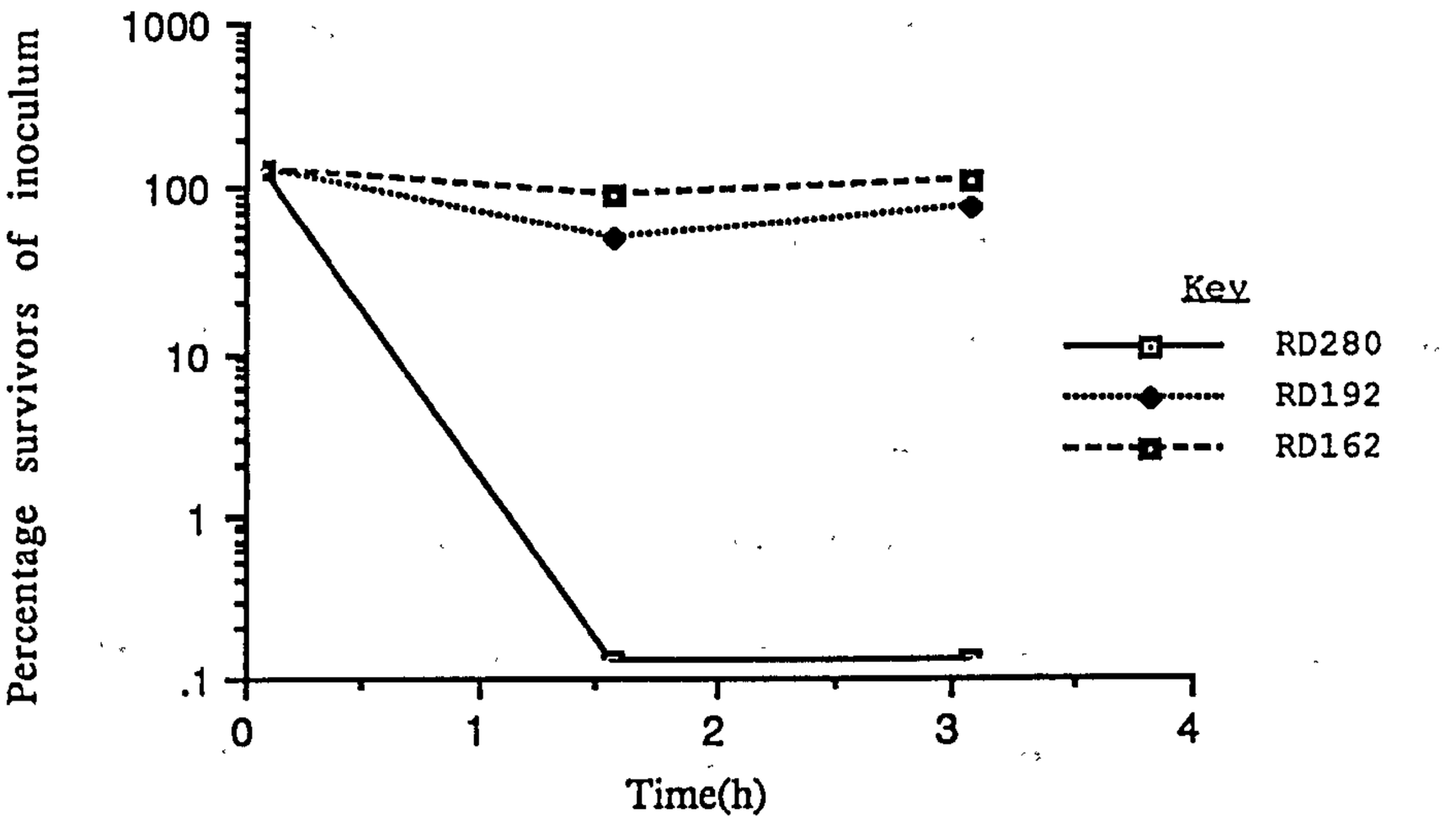


Figure 29. Effect of chelation with inulin (2mg/ml) on the bactericidal effect of 80% normal rainbow trout serum on isolates RD280 (serotype 01), RD192 (serotype 05) and RD162 (serotype 06).

In the presence of inulin, which specifically inhibits the alternative complement pathway, isolate RD280 (serotype O1) was completely sensitive to killing and isolates RD192 (serotype O5) and RD162 (serotype O6) showed intermediate-sensitivity (Figure 29). These results suggested that classical pathway activation was responsible for the killing of isolate RD280 and was also involved in the killing of RD192 and RD162. However, killing of isolates RD192 and RD162 was not extensive (compared to that observed in untreated serum) and indicated that the classical pathway alone was not entirely responsible for the killing observed in untreated serum.

EDTA, EGTA and inulin, alone, had no effect on the survival of isolates of Y. ruckeri in PBS.

8.4 DISCUSSION

The susceptibility of Y. ruckeri to the bactericidal effects of normal rainbow trout serum appeared to have a multifactorial basis and could not be explained solely in terms of possession of a certain LPS- or OMP-type. Some serotype O1 isolates, demonstrated to be serum-sensitive, also had slightly different LPS patterns compared to serum-resistant serotype O1 isolates (Section 3). For example, the LPS profiles of isolates RD20, RD62, RD154, RD140, RD22 and RD190 could be distinguished by the presence of doublet bands; isolate RD154 also had a unique LPS immunotype. In addition, the fact that serotype O2, O5 and O6 isolates (with the exception of a single serotype O2 isolate) were all serum-sensitive also suggested that LPS-type might be an important factor in serum-susceptibility. Numerous authors have demonstrated that resistance to the bactericidal effect of serum is provided by the LPS component of the OM. In particular, the chemical structure of LPS has been implicated in susceptibility to serum and in the virulence of a number of bacterial species including E.coli (Pluschke and Achtman, 1984), H. ducreyi (Odumeru et al.,

1985), K.pneumoniae (Ciurana and Tomas, 1987), Salmonella dublin (Terakado et al., 1988) and Shigella spp. (Okamuru et al., 1988). In addition, LPS molecules of high-molecular-weight have been shown to be important in S. typhimurium (Vandenbosch et al., 1987; Tomas et al., 1988) and in E.coli (Tomas et al., 1988).

The end result of complement activation, whether it be via the classical or the alternative pathway, is the formation of a membrane attack complex (MAC) involving the five terminal components C5 to C9 (C5b-9). For killing to occur the MAC must insert into the hydrophobic OM where it is responsible for irreversible damage to the cytoplasmic membrane that leads to cell death (Taylor, 1983). It has been shown in Salmonella montevideo that long-chain LPS molecules sterically hinder access of large molecules such as the MAC to the OM. Thus, the MAC is unable to insert into the OM and the complex is released without sustaining lethal membrane damage (Joiner et al., 1982a; 1982b; 1982c; 1986). To provide protection a critical density of long O-antigen polysaccharides is necessary (Grossman et al., 1987). It has also been shown that Salmonella strains having different O-antigen structures activate C3 at different rates (Liang-Takasaki et al., 1983) and that C3 activation by Salmonella LPS is sensitive to slight variations in the chemical structure but not to large variations in the length of the O-antigen side chains of LPS (Grossman and Leive, 1984). Thus, two distinct LPS-dependent mechanisms can account for resistance to complement-mediated killing: steric hindrance by long-chain LPS molecules and variation in the ability to activate the complement system which is dependent on the chemical structure of the LPS. A membrane attack complex has been demonstrated in rainbow trout which is very similar to the MAC of human complement (Nonaka et al., 1981) and the same principles probably apply to Y. ruckeri.

The five LPS types of Y. ruckeri each possessed a heterogeneous range of LPS molecules, including molecules of high-molecular-weight (Section 3). It would appear from this evidence, therefore, that steric hindrance may not be an important factor in this case, since serotypes with an apparent abundance of high-molecular-weight LPS were serum-sensitive. However, a serum-sensitive serotype O1, OMP-type 3 isolate with weakly staining LPS (isolate RD140) indicated that the abundance of LPS might be important, suggesting that steric hindrance might play a role in serum-susceptibility of Y. ruckeri. The fact that a serum-sensitive serotype O1 isolate (isolate RD154) appeared to have a different LPS structure (as evidenced by LPS profile analysis and Western-blotting) compared to serum-resistant serotype O1 isolates, and yet had abundant high-molecular-weight molecules, suggested that the chemical structure of LPS might be important in providing serum-resistance in Y. ruckeri.

There was also evidence, however, to suggest that OMPs were involved in serum-susceptibility. For example, serotype O1 isolates comprised serum-resistant isolates belonging to clones 2 and 5, and serum-sensitive isolates belonging to clones 1, 3, 4 and 6. Although there were slight differences in the LPS profiles of serotype O1 isolates (Section 3), these clonal groups differed principally in their OMP profiles, thus suggesting a protein involvement in serum-resistance. However, isolates belonging to clonal groups 1 and 2, which were serum-sensitive and serum-resistant respectively, had identical OMP profiles and differed only in their biotype. Isolates of serotypes O2, O5 and O6 were serum-sensitive (with the exception of a single serotype O2 isolate) and had OMP profiles identical to serum-sensitive isolates of clones 1 and 3, i.e. OMP-types 1 and 2. In addition, serum-resistant serotype O7 isolates could be differentiated from a serum-sensitive serotype O7 isolate on the basis of having a different OMP-type. The fact that biotype 2, OMP-type 1 and OMP-type 3 were associated only with type O1 LPS indicated that there is, perhaps, a direct

association of certain proteins with this LPS type which can account for serum-resistace. The absence of these proteins from other serotype O1 isolates and from isolates of other serotypes could account for the serum-susceptibility of these isolates.

The difference in serum-susceptibility between biotype 1, serotype O1, OMP-type 1 (clone 1) and biotype 2, serotype O1, OMP-type 1 (clone 2) isolates is difficult to explain. A similar problem was addressed by Blaser et al. (1987) in the case of C. fetus. These authors postulated that intrinsic differences in a protein associated with serum-susceptibility could explain why both serum-resistant and serum-sensitive isolates existed which possessed this protein. These authors also concluded that for a C. fetus strain to be fully serum-resistant, both the typical type A LPS and the protein associated with serum-resistance, must be present. This protein causes serum-resistance by making the cell-surface hydrophobic (Rosenberg et al., 1980). Similarly, the serum-resistance of isolates RD38 (serotype O1, OMP-type 2) and RD294 (serotype O2) was exceptional and is difficult to explain.

Antibody-mediated killing of serotype O1 isolates was demonstrated in isolates resistant to the bactericidal effects of normal serum. Presumably, antibody-mediated classical complement activation was responsible for killing in this case. The results demonstrated that the protective antibodies appear to be directed against the sero-specific LPS components of the OM and not against cross-reacting protein antigens, since antibody-mediated serum-killing occurred only in the presence of homologous antibody. This provided further evidence for the role of LPS in providing resistance to serum-killing. The presence of homologous antibodies did not increase the sensitivity of isolate RD168 to serum-killing, although the presence of heterologous antibodies increased the resistance of this isolate to serum-killing. The reason for this was not known. The demonstration of antibody-mediated complement-killing of serotype O1

isolates, which are resistant to normal serum, is able to explain the basis of successful vaccination against this organism.

In bacteria such as E.coli, Salmonella spp., H. influenzae, Campylobacter spp. and Shigella spp. both complement pathways are involved in bactericidal activity whereas N. gonorrhoeae, P. aeruginosa and H. ducreyi activate mainly the classical pathway (Odumeru et al., 1985; Okamuru et al., 1988). Trust et al. (1981) implicated a mechanism similar to the alternative pathway in mammalian serum in the bactericidal effect of normal trout sera against low-virulence marine vibrios and Ourth and Wilson (1982a) suggested that the alternative rather than the classical complement pathway was important in the bactericidal activity of channel catfish serum against S. paratyphi. The work described here indicated that although a complement-mediated bactericidal effect is responsible for the killing of serum-resistant isolates, there appear to be at least two distinct non-specific mechanisms responsible for the killing of different serotypes. Thus, both classical and alternative complement pathways appear to be involved in the killing of serum-sensitive serotype O1 isolates, whereas the killing of serotype O2, O5 and O6 isolates was difficult to explain since killing was significantly reduced when each pathway was inhibited in turn. The most notable difference between these two groups was the fact that addition of either Ca^{2+} or Mg^{2+} to serum containing EDTA neutralized the effect of the EDTA towards the serotype O1 isolate but had no effect on the serotype O5 and O6 isolates. This latter observation requires further investigation because the phenomenon has not been described before.

Plasmids have been shown to carry genes coding for serum resistance in a number of bacterial species including S. typhimurium (Hackett et al., 1987; Vandenbosch et al., 1987) and S. dublin (Terakado et al., 1988). In the case of S. dublin a 50 MDa plasmid was associated with changes in the neutral sugar composition of the LPS (Terakado et al., 1988) whereas in S. typhimurium a

cryptic plasmid was associated with a 11 KDa protein which mediates serum resistance (Hackett et al., 1987). Differences in the plasmid content of serotype O1 and O2 isolates of Y. ruckeri have been described by De Grandis and Stevenson (1982) and Stave et al. (1987). Furthermore, Toranzo et al. (1983) demonstrated variation in the plasmid content of virulent and avirulent isolates of Y. ruckeri. It would be interesting to speculate that plasmids possible play a role in mediating serum-resistance in Y. ruckeri, perhaps by modifying the OMP or LPS composition of the OM.

Further work to determine the role of OM components in the serum-susceptibility of isolates of Y. ruckeri could include a chemical analysis of the different LPS-types and, in particular, of different O1 LPS types, comparison of the effectiveness of high-molecular-weight and low-molecular-weight LPS molecules of the different serotypes in the activation of complement, comparison of the hydrophobicity of the cell surfaces of serum-resistant and serum-sensitive isolates with emphasis on the hydrophobicity of different protein species, and further investigation of the possible role of OMPs of biotype 2, OMP-type 1 and OMP-type 3 isolates in providing resistance to the bactericidal effects of serum. In addition, the role of plasmids (if any) in serum-resistance could be investigated.

The relationship between serum-susceptibility and virulence is discussed in Section 9.

Section 9

Virulence of Yersinia ruckeri : Relation of Biotype, Serotype and OMP-type to Virulence and Serum-Resistance as a Major Virulence Determinant

Virulence of Yersinia ruckeri : Relation of

Biotype, Serotype and OMP-type to Virulence

and Serum-Resistance as a Major Virulence Determinant

9.1 INTRODUCTION.

Serotype O1 isolates of Y. ruckeri are responsible for the majority of disease outbreaks in North America (Busch, 1982) as well as in Europe. The serotype O1 organism is associated with both frank clinical cases of ERM as well as with subclinical asymptomatic infections. With the exception of an epizootic in chinook salmon (Cipriano et al., 1986) the serotype O2 organism has not been associated with any significant mortalities and has been isolated mainly from asymptomatic and subclinically infected carrier fish (Busch, 1982). The recognition of additional serotypes (Stevenson and Airdrie, 1984a; Pyle and Schill, 1985; Daly et al., 1986) raises further questions regarding the virulence of different serotypes and the relative capacity of these other serotypes to produce disease in different species of fish.

Bullock et al. (1981) demonstrated that serotype 1 isolates of Y. ruckeri were significantly more virulent in rainbow trout than serotype 2 isolates; the Australian serotype 3 isolate was shown to be avirulent. McCarthy and Johnson (1982) also described significant differences between the virulence of serotype 1 and 2 isolates in rainbow trout. Cipriano et al. (1987), however, demonstrated that in brook trout serotype 2 isolates of Y. ruckeri were potentially as virulent as serotype 1 isolates. The serotype 2 isolates described by these authors actually comprised isolates of serotypes O2, O5, O6 and O7.

Although very little is known about the virulence determinants of Y. ruckeri, variation in the possession of plasmids between serotype 1 and 2 isolates has been described by De Grandis and Stevenson (1982), Cook and Gemski (1982), Toranzo et al. (1983) and Stave et al. (1987). Virulent serotype 1 isolates differed from avirulent serotype 2 isolates in that the former possessed a 70 Mdal plasmid whereas the latter did not (Cook and Gemski, 1982). Stave et al. (1987) demonstrated variation in the phagocytic chemiluminescence response of serotype 1 and 2 isolates of Y. ruckeri; this variation was correlated with the possession of a 70 Mdal plasmid. Plasmids and plasmid-mediated factors are known to be important in the virulence and pathogenesis of bacteria and bacterial infections (Elwell and Shipley, 1980) and are of particular importance, for example, in the virulence of Yersinia species (Gemski et al., 1980; Portnoy and Falkow, 1981).

The objective of this part of the study was to investigate the virulence, in rainbow trout, of isolates of Y. ruckeri having different surface characteristics and to identify those surface characteristics associated with virulence. The identification of virulent clones of Y. ruckeri would, in addition, facilitate investigations into the virulence mechanisms of this organism and would also be useful in explaining the findings of the epizootiological study. Finally, the importance of serum-resistance as a virulence determinant could be ascertained by comparing the virulence of serum-resistant and serum-sensitive isolates.

9.2 MATERIALS AND METHODS

9.2.1 Bacterial isolates

The virulence of 32 isolates of Y. ruckeri was investigated. The isolates examined in the study were selected to represent a wide range of biotypes, serotypes and OMP-types (Table 20).

9.2.2 Virulence tests

Rainbow trout, of average weight 7.7 g, were obtained from Clone hatchery, Scotland, maintained in flowing, dechlorinated mains water at ambient temperature and fed twice daily. The same initial batch of fish was used in all of the virulence tests. For virulence tests groups of 30 fish were transferred to 50 litre tanks, also containing flowing water, and the water temperature was increased to 14-15^{o}C, with aquarium heaters, over a period of 5 days. According to the ambient water temperature the water flow was adjusted to maintain the desired temperature, and the water was aerated with airstones.

Bacteria were grown overnight at 22^{o}C (to stationary phase) in 500 ml TSB and were harvested by centrifugation at 5,000 x g for 30 min. The bacteria were resuspended in 0.85% saline to an optical density of 2.0 ± 0.10 at 610nm (equivalent to approximately 5 x 10^{9} c.f.u.'s per ml). Viable counts were carried out on each bacterial suspension to confirm the cell density. Bacteria were diluted in 100- and 10-fold serial dilutions in 0.85% saline and 0.1 ml aliquots were plated out in triplicate on TSA and incubated at 22^{o}C for 24 h.

For each virulence test 4950 ml of water were removed from the appropriate tank and transferred to a clean bucket. The fish were netted from the tank, excess water was allowed to drain away, and the fish were transferred to the

bucket. During the course of the experiment the water was vigorously aerated with an airstone. Fifty millilitres of the bacterial suspension were added to the bucket to give a final cell density of approximately 5×10^7 c.f.u.'s per ml. The fish were exposed to the bacteria for 1 h after which time they were returned to their original tank.

Challenged fish were fed and observed two or three times a day and dead and moribund fish were removed immediately. The intestines and kidneys of dead and moribund fish were sampled and streaked on TSA plates which were incubated at 22°C. Bacterial growth was visually inspected and tested by rapid slide-agglutination with anti- Y. ruckeri antiserum for confirmation of the presence of Y. ruckeri. The experiment was terminated after 21 days when all surviving fish were sampled in the same way. Fish were anaesthetized (Section 6) and despatched by severing the spinal cord behind the head.

Isolates were tested in groups of eight, i.e. eight groups of 30 fish were challenged. For each series of tests, a control challenge was carried out using a known virulent isolate (RD6). This isolate had been isolated from a disease outbreak and had been subcultured only twice prior to use in the virulence tests.

9.2.3 Effect of in vivo passage on virulence

To study the effect of in vivo passage on virulence eight isolates were passaged four times through rainbow trout. Bacteria were grown overnight in 10 ml TSB, washed once in 0.85% saline and resuspended in 10 ml 0.85% saline to give an approximate cell density of 10^9 c.f.u.'s per ml. Five rainbow trout were anaesthetized (Section 6) and injected intraperitoneally with 0.1 ml of the bacterial suspension. Bacteria were recovered from the kidneys of dead, moribund and surviving fish and subcultured on TSA for repeated passage.

Where possible, bacteria recovered from dead fish were used in the subsequent passage. In the case of some isolates, however, fish were not killed by the challenge and recovery of isolates was difficult. Virulence tests were carried out on the eight isolates before the first passage and after the last passage.

9.3 RESULTS

9.3.1 Definition of virulence

Virulent isolates were defined as those isolates which caused mortality and which also gave rise to persistent infection of fish after three weeks. Mortalities were only accounted as being due to Y. ruckeri infection if the organism was detected in the kidney. Low levels of mortalities within the first few days of the challenge were observed in some cases, without infection of surviving fish being detected. These mortalities were usually in small debilitated fish and were ignored.

9.3.2 Effect of in vivo passage on virulence

To examine the effect of in vivo passage on the virulence of Y. ruckeri, eight isolates (RD6, RD20, RD22, RD28, RD126, RD152, RD168 and RD170) were passaged four times through rainbow trout. Virulence was tested before the first and after the last passage. No difference was observed in the virulence of the isolates before and after in vivo passage (Table 18). Isolates RD6, RD152 and RD170 were virulent both before and after passage, whereas isolates RD20, RD22, RD28, RD126 and RD168 were avirulent both before and after passage.

Table 18. Results of virulence tests showing the effect of in vivo passage on the virulence of eight isolates of Y. ruckeri.

Isolate	Before in vivo passage		
	Challenge dose c.f.u.'s[a]/ml	%Mortality	%Infected survivors
RD6	6.7×10^7	60	46
RD20	5.7×10^7	11	0
RD22	5.8×10^7	0	0
RD28	6.8×10^7	0	4
RD126	6.1×10^7	0	0
RD152	5.1×10^7	63	50
RD168	7.7×10^7	0	0
RD170	5.5×10^7	24	53

Isolate	After in vivo passage		
	Challenge dose c.f.u.'s[a]/ml	%Mortality	%Infected survivors
RD6	7.3×10^7	76	29
RD20	5.6×10^7	0	8
RD22	5.2×10^7	0	0
RD28	5.7×10^7	0	0
RD126	5.9×10^7	0	0
RD152	6.6×10^7	67	20
RD168	6.8×10^7	5	0
RD170	5.8×10^7	5	84

a = colony forming units

9.3.3 Virulence of isolates of Y. ruckeri

Of the 32 isolates tested 11 were virulent and 21 were avirulent (Table 19). Virulent isolates were easily differentiated from avirulent isolates since the former caused both mortality and persistent infection after 21 days whereas the latter did not. In each series of tests the control challenge (RD6) gave approximately the same level of mortality (results not shown).

9.3.4 Relation of virulence to biotype, serotype, OMP-type and serum-resistance

The relationship between biotype, serotype, OMP-type, serum resistance and virulence of the 32 isolates examined is shown in Table 20. The most notable feature was the fact that virulent isolates consisted of serotype O1 isolates of biotype 2, OMP-type 1 (i.e. clone 2) and OMP-type 3 (i.e. clone 5) only. Three isolates of clone 5 (RD126, RD138 and RD140) were, however, avirulent. Isolates of the other serotype O1 clonal groups (i.e. clones 1, 3, 4 and 6) were avirulent, as were all of the isolates belonging to the other serotypes (i.e. serotypes O2, O5, O6 and O7).

When serum resistance was examined in conjunction with these other characteristics, it became apparent that all of the virulent isolates were resistant to the bactericidal effect of serum. Conversely, with the exception of four isolates, all serum-sensitive isolates were avirulent. The exceptions were isolates RD38, RD36, RD60 and RD150 which were serum-resistant and avirulent. Of these, however, the latter three isolates were not as resistant to the bactericidal effects of serum as were virulent serum-resistant isolates. In serum-killing assays these isolates were serum-sensitive after 1.5 h and after 3 h only just showed 100% survival (Section 8). The only serum-resistant serotype O2 isolate (RD294) was not, unfortunately, tested for virulence.

Table 19. Results of virulence tests on isolates of Y. ruckeri.

Isolate	Challenge dose c.f.u.'s[a]/ml	%Mortality	%Infected survivors
RD6	6.7×10^7	60	46
RD20	5.7×10^7	11	0
RD22	5.8×10^7	0	0
RD28	6.8×10^7	0	4
RD34	6.9×10^7	3	0
RD36[b]	5.8×10^7	0	0
RD38	6.7×10^7	0	0
RD40	2.3×10^7	28	48
RD42[b]	3.4×10^7	7	63
RD46	5.5×10^7	0	0
RD48[b]	4.9×10^7	0	0
RD52[b]	4.5×10^7	7	0
RD58[b]	3.6×10^7	0	0
RD60	3.4×10^7	3	0
RD62	7.1×10^7	7	0
RD64	6.5×10^7	3	0
RD66	6.7×10^7	0	0
RD70	3.9×10^7	33	63
RD86	6.3×10^7	66	80
RD88	7.0×10^7	53	100
RD124	6.4×10^7	83	100
RD126	6.1×10^7	0	0
RD136	2.9×10^7	4	30
RD138	3.8×10^7	0	0
RD140	3.6×10^7	0	0
RD150	5.8×10^7	0	0
RD152	5.1×10^7	63	50
RD154	6.0×10^7	0	0
RD156	4.9×10^7	3	0
RD168	7.7×10^7	0	0
RD170	5.5×10^7	24	53
RD288	5.9×10^7	21	50

[a] = colony forming units.

[b] = isolates shown to be virulent in brook trout by Cipriano et al. (1987).

Table 20. Relation between biotype, serotype, OMP-type, serum-resistance and virulence of isolates of Y. ruckeri.

Isolates	Biotype	Serotype	OMP-type	Serum-resistance[a]	Virulence
RD156	1	01	1 (1)[b]	-	-
RD6	2	01	1 (2)	+	+
RD152	2	01	1 (2)	NT	+
RD20	1	01	2 (3)	-	-
RD38	1	01	2 (3)	+	-
RD62	1	01	2 (3)	±	-
RD154	2	01	2 (4)	-	-
RD40	1	01	3 (5)	+	+
RD42	1	01	3 (5)	+	+
RD70	1	01	3 (5)	NT	+
RD86	1	01	3 (5)	+	+
RD88	1	01	3 (5)	NT	+
RD124	1	01	3 (5)	+	+
RD126	1	01	3 (5)	NT	-
RD136	1	01	3 (5)	NT	+
RD138	1	01	3 (5)	NT	-
RD140	1	01	3 (5)	±	-
RD170	1	01	3 (5)	NT	+
RD288	1	01	3 (5)	NT	+
RD22	1	01	4 (6)	-	-
RD34	1	02	1	±	-
RD58	1	02	2	-	-
RD64	1	02	2	-	-
RD168	1	02	2	±	-
RD28	1	05	2	±	-
RD48	1	06	1	±	-
RD66	1	06	2	-	-
RD36	1	07	1	+	-
RD60	1	07	1	+	-
RD150	1	07	1	+	-
RD52	1	07	5	±	-
RD46	1	UT	1	-	-

[a] = +, serum-resistant;
±, intermediate sensitivity;
-, completely sensitive.

[b] = clonal groups

NT = not tested

UT = untypable

9.4 DISCUSSION

In Section 5 evidence was presented to suggest that serotype O1 isolates of Y. ruckeri could be divided into six clonal groups based on biotype and OMP-type. An analysis of serum-susceptibility demonstrated that two of these clonal groups (clones 2 and 5) were serum-resistant whereas the other four were serum-sensitive (Section 8). An investigation into the virulence of these clonal groups has now revealed that serum-resistant isolates belonging to clones 2 and 5 are also virulent whereas serum-sensitive isolates belonging to clones 1, 3, 4 and 6 are avirulent. Isolates of serotypes O2, O5, O6 and O7, which were shown to be serum-sensitive, were also shown to be avirulent. Three serotype O7 isolates as well as a single serotype O1, clone 3 isolate were exceptions, however, in that they were serum-resistant but were avirulent (although the serotype O7 isolates were not as serum-resistant as were virulent isolates). In addition three serotype O1, clone 5 isolates were avirulent; one of these was also serum-sensitive.

There are two important conclusions pertaining to these results. First, the recognition of distinct differences in the serum-susceptibility and virulence of the serotype O1 clonal groups confirms the existence of these clonal groups and demonstrates that biotype/serotype/OMP-type analysis is an extremely useful tool in epizootiological and virulence studies of Y. ruckeri. Second, the results demonstrated that serum-resistance is a major virulence determinant of Y. ruckeri.

Evidence was presented in Section 8 to account for the differences in serum-susceptibility of the serotype O1 isolates (as well as the other serotypes) in terms of differences in OMP profiles and differences in LPS profiles. These explanations may similarly be used to account for differences in the virulence

of these isolates. However, the facts that the LPS profiles of the serotype O1, clone 5 avirulent isolates RD138 and RD140 (Figure 2B, lanes 9 and 10), as well as the LPS profile of isolate RD126 (not shown), were different from the LPS profiles of typical virulent serotype O1, clone 5 isolates (compare with Figure 2B, lanes 1-5), implicates LPS as the factor responsible for differences in serum-resistance and virulence. Slight variations in the LPS profiles of virulent and avirulent isolates of H. ducreyi have been described by Odumeru et al. (1987) whereas differences in the chemical structure of the O-antigens of E.coli are responsible for differences in complement sensitivity and virulence (Pluschke and Achtman, 1984). Alternatively, however, serum-resistance and virulence of Y. enterocolitica has been associated with altered outer membrane proteins (Pai and DeStephano, 1982).

Resistance to the bactericidal effects of normal serum has been shown to be a major factor in the virulence of numerous bacteria including A. salmonicida (Munn et al., 1982) E.coli (Pluschke and Achtman, 1984), H. ducreyi (Odumeru et al., 1984), H. influenzae type b (Sutton et al., 1982a), Y. enterocolicitica (Pai and DeStephano, 1982) and fish pathogenic vibrios (Trust et al., 1981). Resistance to the bactericidal effect of rainbow trout serum is also an important determinant in the virulence of Y. ruckeri. However, the fact that serotype O7 isolates as well as a serotype O1, clone 3 isolate were serum-resistant but avirulent suggests that other factors, besides serum-resistance, may be important in the pathogenesis of Y. ruckeri infections. This is analogous to the situation in A. salmonicida where loss of virulence does not coincide with loss of serum-resistance (Munn et al., 1982).

Plasmid-associated virulence has been described in various bacteria including E.coli (Binns et al., 1979), S. flexneri (Sansonetti et al., 1982), Shigella sonnei (Sansonetti et al.,1981), Y. pestis and Y. enterocolitica (Portnoy and Falkow, 1981), Y. pseudotuberculosis (Gemski et al., 1980) and V. anguillarum (Crosa,

1980). Furthermore, the presence of plasmids in Salmonella serotypes has been correlated with resistance to the bactericidal activity of normal human serum (Helmuth et al., 1985; Hackett et al., 1986). Plasmid-mediated LPS O-antigen expression has been demonstrated in some Shigella species (Hale et al., 1984; Kopecko et al., 1980; Sansonetti et al., 1983), Salmonella serotypes (Popoff and Le Minor, 1985) and E.coli (Riley et al., 1987), whereas plasmids are involved in the expression of outer membrane proteins in S. flexneri, S. sonnei and E.coli (Hale et al., 1983) as well as in Yersinia species (Portnoy et al., 1984). In E.coli a plasmid-specified major OMP is responsible for serum resistance (Moll et al., 1980).

Differences in the plasmid content of serotype 1 and 2 isolates of Y. ruckeri have been described (De Grandis and Stevenson, 1982; Cook and Gemski, 1982; Toranzo et al., 1983; Stave et al., 1987) and it is possible that serum-resistance and virulence in Y. ruckeri is plasmid-mediated and is associated either with the chemical composition and/or structure of the LPS or with the OMP composition of the OM or both. Variation in the plasmid content of serotype O1 isolates of Y. ruckeri was described by De Grandis and Stevenson (1982) and it would be interesting to speculate that these differences are associated with the differences observed in the serum-resistance and virulence of the six serotype O1 clonal groups described in this study. Further work is required to determine the role (if any) of the serotype O1 plasmid in virulence and to determine the structure(s) responsible for serum-resistance.

In this study, two virulent serotype O1 clonal groups were identified whereas other serotype O1 clonal groups, as well as other serotypes, were shown to be avirulent. These observations contradict those of Cipriano et al. (1987) who demonstrated that serotype 2 isolates (actually serotypes O2, O5, O6 and O7) were as virulent as serotype 1 isolates. It should be noted that some of the isolates used by these authors were also used in this study (Table 19).

However, the study by Cipriano et al. (1987) was carried out on brook trout whereas the study described here was carried out on rainbow trout. These differences imply, therefore, that the virulence of Y. ruckeri varies according to the host species. Such host species variation in susceptibility to pathogenic bacteria is not surprising. For example, rainbow trout are resistant to furunculosis (A. salmonicida) whereas salmon are highly susceptible. These observations may also account for the fact that serotype 2 isolates have in the past been recovered in North America mainly from Pacific salmon (McCarthy and Johnson, 1982). In addition, serotype O2 isolates of Y. ruckeri have been responsible for disease outbreaks in Atlantic salmon in Norway. It was also notable in this study that the virulent serotype O1, clone 2 and 5 isolates were recovered primarily from diseased rainbow trout whereas the avirulent serotype O1, clone 1, 3, 4 and 6 isolates were recovered from other species including Atlantic salmon, whitefish, brook trout and minnows (Tables 12 and 13, and Appendix 1). These observations indicate, therefore, that there might be host species variation in susceptibility to different clonal groups and/or serotypes of Y. ruckeri.

In different host species different virulence mechanisms may be involved and detailed investigation of the host-pathogen relationship in different salmonid species may reveal further information not only about the virulence determinants of Y. ruckeri but also about differences in the immune responses of different fish species. Such an investigation may lead to a better understanding into how the immune response in different fish species can be enhanced to respond to different pathogens.

Conclusions

Isolates of Y. ruckeri from wide geographic areas including Europe, North America, Australia and South Africa had extremely uniform biochemical characteristics, thus confirming the results obtained by other authors. However, motile, Tween-positive isolates were distinguished from non-motile, Tween-negative isolates; these were designated biotypes 1 and 2 respectively. With the exception of two isolates, all of the biotype 2 isolates originated from the U.K. These isolates formed a geographically isolated clonal group which could be differentiated from the two other biotype 2 isolates in terms of serotype and OMP-type respectively.

An O-serotyping scheme is proposed incorporating the numerical designations of Stevenson and Airdrie (1984a) and Daly et al. (1986) but based on O-antigens. The scheme is superior to that of these authors and can also be related to schemes published by Pyle and Schill (1985) and Pyle et al. (1987). To avoid further confusion it is suggested that this O-serotyping scheme is followed in serological characterisations of Y. ruckeri. Five O-serotypes (O1, O2, O5, O6 and O7) were identified among both European and North American isolates suggesting a movement of the organism from one continent to the other. This supports evidence that suggests that Y. ruckeri was introduced into Europe from North America through the importation of infected carrier fish. Conversley, isolates from Australia and South Africa were of serotype O1 only, suggesting that Australia and South Africa are more isolated from the dissemination of Y. ruckeri. The Australian isolate previously recognized as serotype 3 (or III) is a rough-type mutant and does not constitute a separate O-serotype.

Lipopolysaccharide analysis confirmed earlier work (Pyle and Schill, 1985) which demonstrated that LPS patterns in SDS-PAGE gels can be used to

predict O-serotype. Unlike these authors, however, variation of the LPS profiles of individual serotypes was demonstrated; this was particularly the case for serotype O1 isolates. In the case of serotype O6 and O7 isolates variation in LPS profiles could be used to demonstrate isolate relatedness. Variation in the LPS profiles of type O1 LPS may explain variation in serum resistance and virulence (see below). Lipopolysaccharide was demonstrated, by Western-blotting, to be the sero-specific antigen responsible for the O-serotyping scheme, confirming earlier observations by Pyle and Schill (1985). Unlike these authors, however, cross-reactions were demonstrated, some of which could account for cross-agglutination reactions observed in microplate agglutination assays and in slide agglutination tests. Two O1 LPS immunotypes were recognized, further confirming that variation in O1 LPS might occur which could account for differences observed in serum resistance and virulence.

An analysis of OMPs revealed inter-strain variation in the molecular weight of a heat-modifiable protein and in the molecular weight of peptidoglycan-associated proteins. Based on these variations an OMP-typing scheme was developed and five OMP-types were recognized; 95% of the isolates belonged to one of the three major OMP-types. Outer membrane protein analysis was able to distinguish between isolates of the same serotype and proved to be an extremely useful epizootiological tool. Whereas major proteins exhibited inter-strain variation minor proteins were extremely uniform. Analysis of minor proteins enabled Y. ruckeri isolates to be distinguished from other species and has taxanomic significance.

When biotype, serotype and OMP-type were analyzed together, serotype O1 isolates could be divided into six clonal groups. Of these, three were major and three were minor clones. The geographic distribution of these serotype O1 clonal groups differed and the results demonstrated that

biotype/serotype/OMP-type analysis is extremely effective for population studies of, and as an epizootiological tool for; Y. ruckeri.

Western-blot analysis of OMPs using rabbit antisera demonstrated a greater range of reactions with homologous antisera. In particular, type 2 antiserum recognized very little in heterologous isolates. The heat-modifiable protein was recognized in heterologous isolates by antisera other than type 2 and isolates were designated as OMP-immunotypes 1 or 2 based on variation in the molecular weight of this protein. Peptidoglycan-associated proteins appeared not to be strongly immunogenic in most cases. Rainbow trout antisera recognized a wide range of OMPs as well as LPS. Cross-reactions with OMPs occurred but not with LPS. The LPS of virulent serotype O1 isolates did not give such a strong reaction with homologous antisera as did the LPS of avirulent serotype O1 isolates and the LPS of serotype O2, O5 and O6 isolates. There did not appear to be any association of specific OMPs with virulent isolates. The weak antibody response to the LPS of virulent serotype O1 isolates may, to some extent, account for the virulence of these isolates. The HMP was strongly immunogenic in both rabbit and rainbow trout and was cross-reactive; this protein may be important in cross protection. By comparison, the sero-specific LPS will not provide cross-protection.

Under conditions of iron-limitation four iron-regulated OMPs were produced in the isolates examined. These proteins were produced in both virulent and avirulent isolates, and in isolates of various combinations of biotype, serotype and OMP-type. Production of these proteins did not, therefore, appear to be a virulence determinant and was not associated with any particular surface characteristics. Since plasmid variation has been described in different serotypes of Y. ruckeri it is probable that production of iron-regulated OMPs is not plasmid-mediated but is chromosomally-mediated. Production of siderophores was not detected in any of the isolates examined under iron-

limited growth conditions, thus indicating a direct iron-uptake mechanism between iron-regulated OMPs and Fe^{3+}-glycoprotein complexes.

Resistance to bactericidal killing in normal rainbow trout serum was associated principally with serotype O1 isolates of clones 3 and 5; isolates belonging to other serotype O1 clones and to other serotypes were, with some exceptions, serum-sensitive. Serum-killing was due to complement activation and at least two distinct mechanisms of killing appeared to exist. The mechanism of killing of serum-sensitive serotype O1 isolates was different to that of other serotypes. Isolates resistant to killing in normal rainbow trout serum were sensitive to killing in the presence of homologous antibodies but not in the presence of heterologous antibodies. This suggested that LPS plays an important role in resistance to serum-killing because LPS antibodies are sero-specific whereas OMP antibodies are cross-reactive. Although there was some indirect evidence to suggest that OMPs were involved in serum-resistance, there was also evidence to suggest that LPS was the factor responsible.

Virulence was associated with the two serum-resistant serotype O1 clonal groups, i.e. clones 3 and 5. Serum-sensitive isolates, including isolates of other clonal groups and other serotypes, were avirulent. Thus, serum-resistance is a major virulence determinant in Y. ruckeri. The existence of serum-resistant avirulent isolates, however, suggested that other factors besides serum-resistance are important in the pathogenesis of ERM. The fact that the LPS profiles of avirulent serotype O1 clone 5 isolates were different to the profiles of virulent isolates of the same clonal group suggested that LPS is possibly an important virulence determinant. However, a combination of LPS and protein may be required for full virulence.

The virulence of the two serotype O1 clones 3 and 5 was able to account for the relative abundance of these isolates in Europe as well as the rapid spread of

ERM in the U.K. and Europe respectively. The avirulence of isolates of other serotype O1 clones and other serotypes accounted for their relative infrequent occurrence.

Future work should investigate in greater detail the role of LPS and OMPs in complement activation and in providing protection against the bactericidal effect of complement. The molecular basis of serum resistance should be investigated with emphasis placed, perhaps, on the role of the 70 MDa plasmid. Additional studies could include an investigation into phagocytosis and the role of LPS and OMPs in resistance to phagocytic uptake and phagocytic killing. Attention should also be given to differences in the immune response of different salmonid species to various surface antigens and to the efficacy of cross-protecting OMP antigens as vaccine components.

Appendix 1

Information about isolates used in this study including laboratory codes, previous designations, geographic origins, species of origin and the senders.

Lab code	Previous designations	Geographic origin	Species of origin	Source (see below)
RD2	NCMB 1315	U.S.A.	Salmo gairdneri	-
RD4	NCMB 1316	U.S.A.	" "	-
RD6	-	Scotland	" "	-
RD10	-	"	" "	-
RD14	-	"	" "	-
RD16	-	Unknown,U.K.?	Unknown	-
RD20	Bacter 1	Finland	Coregenus peled	PR
RD22	Bacter 3	"	Salmo salar	"
RD24	Bacter 4	"	Coregenus peled	"
RD26	Bacter 7	"	Salmo salar	"
RD28	BA2	U.K.	Salmo gairdneri	BA
RD30	BA3	U.K.	" "	"
RD32	BA13;NCTC10477	U.S.A.	" "	"
RD34	BA24	U.K.	Salmo trutta	"
RD36=(RD60)	11.31(11.66)	U.S.A.	Salmo gairdneri	WBS
RD38	11.38	"	Micropterus salmoides	"
RD40	11.39	"	Salmo gairdneri	"
RD42	11.40	"	" "	"
RD44	11.43	Australia	" "	"
RD46	11.44	"	" "	"
RD48	11.48	U.S.A.	" "	"
RD50	11.49	"	" "	"
RD52=(RD166)	11.59;ONT258	Canada	" "	"
RD54=(RD176)	11.60;ONT288	Canada	Coregenus artedii	"
RD56	11.62	U.S.A.	Salmo gairdneri	"
RD58	11.63	"	" "	"
RD60=(RD36)	11.66(11.31)	"	" "	"
RD62	F111/81	West Germany	" "	KHB
RD64	F53.1/82	" "	" "	"
RD66	F84.5/82	" "	Cyprinus carpio	"
RD68	F159.1/83	" "	Salmo gairdneri	"
RD70	F13II/84	" "	" "	"
RD72	F232.1/84	" "	" "	"
RD74	4130/83	Italy	" "	GG
RD76	4241/83	"	" "	"
RD78	4263/83	"	" "	"
RD80	4307/83	"	" "	"
RD82	5361/83	"	" "	"
RD84	5710/83	"	" "	"
RD86	5730/83	"	" "	"
RD88	6856/83	"	" "	"
RD90	6891/83	"	" "	"
RD92	2069/84	"	" "	"
RD94	2812/84	"	" "	"
RD96	3081/84	"	" "	"
RD98	3703/84	"	" "	"
RD100	3737/84	"	" "	"
RD102	3831/84	"	" "	"

RD104	3946/84	"	" "	"
RD106	4048/84	"	" "	"
RD108	3661/84	"	" "	"
RD110	4194/84	"	" "	"
RD112	840727	Denmark	" "	ID
RD114	840905	"	" "	"
RD116	840918	"	" "	"
RD118	841110	"	" "	"
RD120	850419	"	" "	"
RD122	850724	"	" "	"
RD124	851014	"	" "	"
RD126	CN8161	Unknown,USA?	Unknown	-
RD128	-	England	"	GW
RD130	-	"	"	"
RD132	-	"	"	"
RD136	MLSSP.05	France	"	RL
RD138	MLSP.06	"	"	"
RD140	MLSP.31	"	"	"
RD142	MLSP.43	"	"	"
RD144	MLSP.46	"	"	"
RD146	18/83	Italy	Salmo gairdneri	CR
RD148	17/85	England	Pike	"
RD150	38/85	Denmark	Anguilla anguilla	"
RD152	LT 11/4	England	Salmo gairdneri	"
RD154	85/09/1578	Norway	Salmo salar	TH
RD156	84.12235	Australia	Salvelinus fontinalis	JDH
RD158	85.9919	"	Salmo salar	"
RD160	-	Finland	" "	PR
RD162	-	"	" "	"
RD164	RS1;ONT270	Canada	" "	RMWS
RD166=(RD52)	RS2;ONT258	"	Salmo gairdneri	"
RD168	RS3;BC74	U.S.A.	O.tschawaytscha	"
RD170	RS4;;FHL11.4	"	Salmo gairdneri	"
RD172	RS5;ERMII	Unknown	Unknown	"
RD174	RS6;ONT193	Canada	Salvelinus fontinalis	"
RD176=(RD54)	RS8;ONT288	Canada	Coregonus artedii	"
RD178	RS9;9-4-5	"	Salmo gairdneri	"
RD180	RS11;ATCC29473	U.S.A.	" "	"
RD182	RS13;C-75-199	Canada	" "	"
RD184	RS20;0634	Australia	Salvelinus fontinalis	"
RD186	RS21;0589	Australia	Salmo salar	"
RD188	RSSS24;2.87	U.S.A.	Salmo gairdneri	"
RD190	RS54;YR-40	Canada	Salvelinus malma	"
RD192	RS62;83-206-1	"	Coregenus clupeaformis	"
RD194	RS80;16A	"	Salmo gairdneri	"
RD196	4321/84	Italy	" "	GG
RD198	4829/84	"	" "	"
RD200	4838/84	"	" "	"
RD202	5032/84	"	" "	"
RD204	5379/84	"	" "	"
RD206	5497/84	"	Thymallus thymallus	"
RD208	5557/84	"	Salmo gairdneri	"
RD210	5787/84	"	" "	"
RD212	6925/84	"	" "	"
RD214	7260/84	"	" "	"
RD216	7271/84	"	" "	"
RD218	7364/84	"	" "	"
RD220	7383/84	"	" "	"
RD222	7587/84	"	" "	"
RD224	7704/84	"	" "	"

RD226	7906/84	"	" "	"
RD228	7914/84	"	" "	"
RD230	8439/84	"	" "	"
RD232	8602/84	"	" "	"
RD234	70/86	"	" "	"
RD236	125/86	"	" "	"
RD246	MT252	Scotland	" "	TSH
RD248	MT260	"	" "	"
RD250	24/86	Wales	" "	CR
RD252	29/86	England	River water	"
RD254	36/86	"	Salmo gairdneri	"
RD256	46/86	"	" "	"
RD258	48/86	"	" "	"
RD260	53/86	"	" "	"
RD264	62/86	"	Thymallus thymallus	"
RD266	63/86	"	" "	"
RD268	64/86	"	" "	"
RD270	65/86	"	" "	"
RD272	66/86	"	" "	"
RD274	67/86	"	" "	"
RD278	16/86	France	Pimephales promelas	CM
RD280	19/86	"	" "	"
RD282	20/86	"	Rudd	"
RD284	S556	"	Salmo gairdneri	"
RD286	8406	South Africa	" "	RB
RD288	2/1-87	" "	" "	"
RD290	-	Scotland	Salmo salar	-
RD292	115/87	Norway	" "	TTP
RD294	302/87	"	" "	"
RD296	Ye1	Bulgaria	Unknown	IG
RD298	Ye2	"	"	"
RD300	Ye3	"	"	"
RD302	Ye4	"	"	"
RD304	Ye5	"	"	"
RD306	Ye6	"	"	"
RD308	Ye7	"	"	"
RD310	Ye8	"	"	"
RD312	Ye9	"	"	"
RD314	Ye10	"	"	"
RD316	Ye11	"	"	"
RD318	Ye12	"	"	"
RD320	Ye13	"	"	"
RD322	Fi247/85	Switzerland	"	WM
RD324	Fi109/87	"	"	"
RD326	Fi128/87	"	"	"
RD328	Fi14/88	"	"	"

Source of isolates

BA, Dr. B. Austin, Heriot-Watt University, Edinburgh, Scotland; **KHB**, Dr. K.H. Bohm, Institut fur Microbiologie und Tierseuchen der Tierarztlichen Hochschule, Hannover, West Germany; **RB**, R. Bragg, Department of Agriculture and Water Supply, Onderstepoort, South Africa; **ID**, Dr. Inge Dalsgaard, Danmarks Fiskeri-Og Havundersogelser, Frederiksberg, Denmark; **IG**, Dr. I. Gelev, Veterinary Institute, 4006 Plovdiv, Bulgaria; **GG**, Dr. G. Giorgetti, Istituto Zooprofilattico Sperimentale Delle Venezie, 33030 Basaldella Di Campoformido, Italy; **TH**, Dr. T. Hastein, National Veterinary Institute, Oslo, Norway; **TSH**, T.S. Hastings, Department of Agriculture and Fisheries for Scotland, Aberdeen, Scotland; **JDH**, J.D. Humphrey, Australian Fish Health Reference Laboratory, Benalla, Australia; **RL**, Dr. R. Lesel, Institut National de la Recherche Agronomique, Nivelle, France; **WM**, Dr. W. Meier, Institut fur Tierpathologie, Bern, Switzerland; **CM**, Dr. C. Michel, Institut National de la Recherche Agronomique, Thiverval-Grignon, France; **TTP**, Dr. T.T. Poppe, National Veterinary Institute, Oslo, Norway; **PR**, Dr. P. Rintamaki, University of Oulu, Oulu, Finland; **CR**, C. Rodgers, Ministry of Agriculture, Fisheries and Food, Weymouth, England; **WBS**, Dr. W.B. Schill, U.S. Fish and Wildlife Service, Kearneysville, West Virginia 25430, U.S.A.; **RMWS**, Dr. R.M.W. Stevenson, University of Guelph, Guelph, Ontario, Canada; **GW**, G. Withnell, BP Nutrition, Witham, England.

Appendix 2

Composition of buffers and media and details of assays and procedures

1. Components for SDS-PAGE

(a) Stacking gel preparation

(4.0% gel, 0.125 M Tris, pH 6.8)

Distilled water	12.2	ml
0.5 M Tris-HCl, pH 6.8	5.0	ml
10% (w/v) SDS	0.2	ml
Acrylamide:BIS (30% T, 2.67% C)	2.6	ml
10% ammonium persulphate	0.1	ml
TEMED	0.02	ml

(b) Separating gel preparation

(12.0% gel, 0.375 M Tris, pH 8.8)

Distilled water	20	ml
1.5 M Tris-HCl, pH 8.8	15	ml
10% (w/v) SDS	0.6	ml
Acrylamide:BIS (30% T, 2.67% C)	24	ml
10% ammonium persulphate	0.3	ml
TEMED	0.03	ml

(c) Gradient gel preparation (8-20%)

(1) 8% acrylamide mixture

Distilled water	14	ml
1.5 M Tris-HCl, pH 8.8	7.5	ml
10% (w/v) SDS	0.3	ml
Acrylamide:BIS (30% T, 2.67% C)	8.0	ml
10% ammonium persulphate	0.15	ml
TEMED	0.015	ml

(2) 20% acrylamide mixture

Distilled water	2.0	ml
1.5 M Tris-HCl, pH 8.8	7.5	ml
10% (w/v) SDS	0.3	ml
Acrylamide:BIS (30% T, 2.67% C)	20	ml
10% ammonium persulphate	0.15	ml
TEMED	0.015	ml

(d) Electrode buffer, pH 8.3

Tris base	3.0	g
Glycine	14.4	g
SDS	1.0	g
Distilled Water	to 1000	ml

(e) Sample buffer

Distilled water	8.0	ml
0.5 M Tris-HCl, pH 6.8	2.0	ml
Glycerol	1.6	ml
10% (w/v) SDS	3.2	ml
2-Mercaptoethanol	0.8	ml
0.05% (w/v) Bromophenol blue	0.4	ml

(f) Coomassie blue (0.2%) staining solution

Coomassie brilliant blue	2.0	g
Methanol	450	ml
Acetic acid	100	ml
Distilled Water	450	ml

(g) Destaining solution

Methanol	300	ml
Acetic Acid	100	ml
Distilled Water	600	ml

(h) Molecular weight markers

(1) Sigma Dalton Mark V11-L

Bovine albumin	66,000	Da
Ovalbumin	45,000	Da
Glyceraldehyde-3-phosphate dehydrogenase	36,000	Da
Carbonic anhydrase	29,000	Da
Trypsinogen	24,000	Da
Trypsin inhibitor	20,100	Da
α-Lactalbumin	14,200	Da

(2) Pharmacia LMW Calibration Kit

Phosphorylase b	94,000	Da
Albumin	67,000	Da
Ovalbumin	43,000	Da
Carbonic anhydrase	30,000	Da
Trypsin inhibitor	20,100	Da
α-Lactalbumin	14,400	Da

(i) Silver-stain (Wray et al., 1981)

Basic staining procedure was as follows

(1) The gel was soaked in 50% reagent grade methanol for a least 1 h.

(2) The stain, solution C, was prepared as follows.

Solution A. Dissolved 0.8 g silver nitrate in 4 ml distilled water.

Solution B. Mixed 21 ml of 0.36% sodium hydroxide and 1.4 ml of 14.8 M ammonium hydroxide.

Solution C. Added solution A dropwise into solution B with constant stirring, and then increased to 100 ml with water. Solution C was used within 5 min.

(3) The gel was stained in solution C for 15 min with constant gentle agitation.

(4) The gel was washed in deionized water with gentle agitation for 5 min.

(5) The developer, solution D, was prepared as follows.

Solution D. Mixed 2.5 ml of 1% citric acid and 0.25 ml of 38% formaldehyde, and increased to 500 ml with water. The solution must be used fresh.

(6) The silver-stain was developed by soaking the gel in solution D until the bands appeared.

(7) The gel was washed with water and placed in 50% methanol to stop the stain development.

2. Western-Blotting

(a) Transfer buffer

(25 mM Tris, 192 mM Glycine, 20% [v/v] methanol, pH 8.3)

Tris	3.03	g
Glycine	14.4	g
Methanol	200	ml
Distilled Water	to 1000	ml

(b) Tris-buffered saline (TBS)

(20 mM Tris, 500 mM NaCl, pH 7.5)

Tris	4.84	g
NaCl	58.48	g
Distilled Water	to 2000	ml
pH 7.5		

(c) Tween-20 wash solution and diluant (TTBS)

(20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5)

Tris-buffered saline	1000	ml
Tween-20	0.5	ml

(d) Horseradish peroxidase colour development solution

(1) Sixty milligrams of HRP colour development reagent (containing 4-chloro-1-naphthol) were added to 20 ml ice cold methanol. This solution was protected from light.

(2) Immediately prior to use 60 μl of ice cold 30% H_2O_2 (hydrogen peroxide, stabilized) were added to 100 ml room temperature TBS. This was mixed with (a) above and used immediately.

3. Deferration of Ethylenediaminedihydroxyphenylacetic Acid (EDDA) (Rogers, 1973)

A solution containing 1g EDDA in 19 ml of boiling 1N HCl was prepared; after being cooled and filtered it was diluted with 150 ml of acetone and the pH was raised to 6.0 by adding 1N NaOH. After standing overnight at $4^{o}C$, the precipitate was filtered off and washed with cold acetone.

4. Deferration of TSB (Donald et al., 1952)

One hundred millilitres of TSB were vigorously shaken (by hand) for 4 min in a 250 ml separating funnel with 5 ml of a solution of 8-hydroxyquinoline (0.5%, w/v) in CCl_4 at pH 9. The CCl_4 layer was run off, and the extraction was repeated three times. The medium was then washed at pH 9 and 7 with CCl_4 and boiled to remove CCl_4 , then autoclaved.

5. Simon and Tessman Low-Iron Medium (Simon and Tessman, 1963).

NaCl	5.8	g
KCl	3.7	g
$CaCl_2.2H_2O$	0.15	g
$MgCl_2.6H_2O$	0.10	g
NH_4Cl	1.1	g
Na_2SO_4	0.142	g
KH_2PO_4	0.272	g
Tris (hydroxymethyl) aminomethane	12.1	g
50% glucose (w/v)	10	ml
Distilled Water	to 1000	ml
pH 7.4		

The medium was modified by adding deferrated casamino acids (0.3%) and sodium succinate (0.2%), both filter-sterilized through a 0.45 μm Millipore filter.

6. Chrome Azurol S (CAS) Agar Plates (Schwyn and Neilands, 1987).

To prepare 1 litre of blue agar, 60.5 mg chrome azurol S (CAS) (Sigma) was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_20$, 10 mM HCl). Under stirring this solution was slowly added to 72.9 mg hexadecyltrimethylammonium bromide (HDTMA) (Sigma) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. Also autoclaved was a mixture of 750 ml H_2O, 100 ml 10 x MM9 salts (see below), 15 g agar, 30.24 g Pipes (1,4-piperazinediethanesulfonic acid) (Sigma) and 12.0 g of a 50% (w/w) NaOH solution to raise the pH to the pKa of Pipes (6.8). After cooling to 50^oC, 30 ml casamino acids (10%) and 10 ml glucose (20%) as a carbon source were added as sterile solutions. Each plate received 25 ml of blue agar.

x10 minimal salts :

NaCl	29	g
KCl	18.5	g
$CaCl_2.2H_2O$	0.75	g
$MgCl_2.6H_2O$	0.5	g
NH_4Cl	5.5	g
Na_2SO_4	0.71	g
KH_2PO_4	1.36	g
distilled water	to 500	ml

7. Csaky Assay for the Detection of Hydroxamate-Type Siderophores (Csaky, 1948)

<u>Reagents</u>

(1) Sulphanilic acid. Dissolved 1 g sulphanilic acid in 100 ml 30% acetic acid by heating on a water bath.

(2) Iodine solution. Dissolved 1.3 g iodine in 100 ml glacial acetic acid.

(3) Sodium arsenite solution. Dissolved 2 g sodium arsenite in 100 ml distilled water.

(4) α-Naphthylamine solution. Dissolved 0.3 g α-naphthylamine in 100 ml 30% acetic acid.

Procedure

Five millilitres of the test solution was placed in a test tube and 1 ml of sulphanilic acid and 0.5 ml iodine solution were added. After 3 to 5 minutes the excess of iodine was destroyed with 1 ml of sodium arsenite solution and 1 ml of α-naphthylamine solution was added. Development of a pink colour indicated a positive result. Acetohydroxamic and benzohydroxamic acids (1.5 mM) were used as controls.

8. Arnow Assay for the Detection of Phenolate-Type Siderophores (Arnow, 1937).

Reagents

(1) 0.5 N hydrochloric acid

(2) Nitrite-molybdate reagent. Dissolved 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml distilled water.

(3) 1 N sodium hydroxide.

(4) Standard solution. Dissolved 50 mg of 3,4-dihydroxyphenylalanine (Sigma) in 500 ml distilled water contained in a litre volumetric flask. Added 2 ml 0.1 N hydrochloric acid and made up to 1 litre. Preserved under toluene.

Procedure

One millilitre of unknown and standard solutions were placed in separate test tubes. The following reagents were added to the test tubes in the order given, mixing well after each addition : 1 ml of 0.5 N hydrochloric acid, 1 ml of nitrite-

molybdate reagent (a yellow colour resulted at this point), and 1 ml 1 N sodium hydroxide (a red colour resulted).

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