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Review

Protein mutations revealed by two-dimensional electrophoresis

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Abstract

High-resolution two-dimensional electrophoresis (2DE) can resolve many hundreds of proteins present in complex mixtures depending on the method of detection. These proteins can be characterised qualitatively, with respect to their electrophoretic mobilities (i.e. charge and apparent molecular mass) and quantitatively, using densitometry, to determine their amounts. There has been a widespread application of 2DE in the analysis and characterisation of protein mutations for a range of organisms. This review presents examples of the use of 2DE to study naturally occurring protein mutations and polymorphisms as well as the characterisation of induced protein mutations in prokaryotes and eukaryotes. Examples are presented to illustrate the use of 2DE to detect mutations affecting the electrophoretic mobility and biosynthesis of individual proteins as well as mutations leading to global alterations in cellular protein synthesis. The advantages and disadvantages of 2DE in the detection of protein mutations are discussed.

Contents

1. Introduction

Two-dimensional electrophoresis (2DE) on polyacrylamide gels is one of the most sensitive biochemical methods currently available for the separation of the individual components of complex protein mixtures. Although any two methods, which separate proteins by independent criteria, can be used, the most widely adopted techniques use isoelectric focusing (IEF) in the

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first dimension and discontinuous electrophoresis, in the presence of sodium dodecyl sulphate (SDS), for the second dimension. Proteins resolved by 2DE can be characterised on the basis of their native charge, in the presence of urea, and apparent molecular mass. The commonly used high-resolution 2DE methods have evolved from the initial descriptions of Klose [1] and O'Farrell [2] both of which proposed the use of 2DE for the detection and characterisation of protein mutations. A number of studies soon appeared to illustrate the method's applicability in detecting charge variants of proteins present in complex protein mixtures [3–5]. Suzuki [6] has recently reviewed the application of one-dimensional (1D) and two-dimensional (2D) electrophoretic methods to identify and characterise mutant proteins in human inherited diseases. The following review will consider specifically the application of 2DE using IEF and SDS–polyacrylamide gel electrophoresis (PAGE) in the analysis of protein mutations in prokaryotes and eukaryotes. A brief discussion of the methodology will be given followed by examples of the application of 2DE for detecting and characterising protein mutations. The examples presented will consider naturally occurring protein mutations as well as protein mutations induced by mutagens or other means.

2DE can detect mutations affecting a protein's electrophoretic mobility reflecting alterations in the protein's primary structure, i.e. the amino acid sequence. By the inclusion of densitometry and computer analysis of the 2D protein profiles to quantify the amounts of individual protein spots, mutations can be detected which affect the biosynthetic levels of the protein as well as detecting the mutation of individual alleles of genes in diploid organisms [7,8]. Inspection of the genetic code indicates that a third of base substitutions, occurring within the coding sequence of a protein, result in amino acid substitutions which affect the charge of a protein. Thus, these mutations could be detected by 2DE through an altered electrophoretic mobility generally in the first dimension separation. In most applications of 2DE, the mutations detected affect the protein's electrophoretic mobility and not necessarily the function of the protein [9]. However, as discussed in more detail below a number of laboratories have endeavoured to relate mutations detected by 2DE with an altered biological property.

The suitability of 2DE for detecting and characterising protein mutations must be considered in the context of other biochemical procedures for mutation detection. Extensive work has been carried out on nucleic acid analysis for mutation detection (see, for example, the review by Cotton [10] and Cortopassi and Arnheim [11]). Analysis of proteins by 2DE has a number of advantages over nucleic acid based methods. Current 2DE protocols can resolve many hundreds of proteins on a single analytical gel. Within the constraints discussed later, the resolved proteins can be characterised qualitatively, with respect to their electrophoretic mobility, as well as quantitatively to determine in vivo expression levels. Since non-specific methods of protein detection are normally employed with 2DE, no detailed information on the protein's function or structure (e.g. amino acid sequence) is required before mutations can be monitored. This contrasts with other strategies for the detection of mutations employing for example either the polymerase chain reaction (PCR) or enzyme electrophoresis. In these cases, either a partial nucleotide sequence or enzyme activity, respectively, must be determined before the start of the study. In addition, the latter methods examine only a limited number of proteins (or the gene encoding the protein) at one time. In studies defining the response of organisms to mutagen exposure, 2DE shows additional advantages over the analysis of nucleic acids since 2DE directly assesses damage to functional rather than total DNA [12]. In recent years, developments in PCR technology have made this a popular and powerful method to characterise mutations at the nucleotide sequence level. Its high sensitivity makes PCR suitable for detecting mutations in nucleic acid extracted from single human cells, although there are a number of technical problems at this detection level [11]. In addition, multiplex PCR has been developed to simultaneously locate multiple polymorphisms of a

single gene [13]. Nevertheless, at present no single method, whether it be 2DE or PCR, can detect all mutational lesions. A combined strategy to locate and characterise protein mutations incorporating the advantages of 2DE and PCR will be discussed below.

2. The methodology of two-dimensional electrophoresis for protein analysis

A requirement of any study applying 2DE to protein analysis is to achieve a high degree of reproducibility within and between analytical runs carried out over an extended period of time. Many of the applications discussed below compare 2D protein profiles from maybe hundreds of independent gels. Since the protein mutations of interest might affect the protein's electrophoretic mobility, the 2D protein profiles must be reproducible for accurate comparison; this also applies when quantitative data are extracted from the protein profiles. Considerations must be given to the method of sample preparation, the electrophoresis conditions, the method of protein detection and the subsequent analysis of the protein profiles. Protocols on sample preparation can be obtained from the papers describing specific 2DE applications. There are a number of excellent reviews which present detailed protocols for gel preparation and electrophoresis conditions and prospective users of 2DE are referred to these for further information [14–16]. The following briefly reviews the methodology of 2DE for protein analysis as an introduction to the applications described later.

An initial starting point to obtain reproducibility is at the choice of chemicals used for sample preparation and electrophoresis. Many companies now supply highly purified reagents developed specifically for 2DE and it is wise to identify a reliable source of reagents particularly in long-term investigations where batch-to-batch variation of chemicals might occur. Small-scale, or infrequent, users of 2DE can also achieve these objectives by the use of commercially produced gels for the first- and second-dimension separations. The successful application of 2DE requires accurate matching of 2D protein profiles from different gels. To achieve this, batch processing of the gels under reproducible conditions is important. Large-scale analysis of 2D gels on dedicated equipment has been described by Anderson and Anderson [17,18] and Patton et al. [19]; both of these systems are commercially available. The gel format may be a matter of personal preference, although tube gels for the first dimension combined with vertical slab gels for the second dimension are the commonly used formats [17–19]. Horizontal systems for both the first- and second-dimension gels have also been used to achieve high-resolution 2D protein profiles [20–22]. The dimensions of the gel clearly influences protein resolution. The majority of investigations employ slab gels on the order of 20×25 cm with 1 mm diameter tube gels in the first dimension. Large gels (32×36 cm) allow the detection of 3- to 4-fold more proteins in a single 2D protein profile than the "standard" gel format [23]. However, increasing the gel dimensions leads to increased complexity in profile analysis. At the opposite end of the scale, 2DE can be carried out on smaller gel systems where relatively simple 2D protein profiles are being analysed [24–26].

Proteins are separated in the first dimension on the basis of their net charge using a pH gradient established in low-concentration polyacrylamide gels. This separation is normally carried out in the presence of 9 *M* urea and either a non-ionic (e.g. NP40) or zwitterionic detergent {e.g. 3-[(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS)}. The protocol originally described by O'Farrell [2] used carrier ampholytes in the gel to establish the pH gradient and these are still widely used for 2DE. Commercial preparations of carrier ampholytes provide a variety of pre-defined pH intervals between approximately pH 3 and pH 10. The shape and range of the gradient can be further modified by mixing these ampholyte preparations in varying proportions. Carrier ampholytes show some limitations in the effective range of the pH gradient with only poor focusing of basic proteins. In addition, the pH gradient

exhibits instability during prolonged focusing times. The use of an immobilised pH gradient in the first-dimension gel overcomes many of the problems associated with carrier ampholytes [20,27]. Immobilised pH gradients are formed from buffering components covalently linked to the gel support media and the pH gradient is generated by casting a gradient gel using immobilines possessing the desired pH extremes. The gels prepared in this manner provide stable pH gradients capable of focusing both acidic and basic proteins on broad-range pH gradients [22,28]. The second-dimension separation is based on the protein's apparent molecular mass in the presence of SDS and is routinely carried out on either a vertical or a horizontal polyacrylamide slab gel. The slab gels can be prepared with either a gradient or single polyacrylamide concentration; this can be optimised to resolve proteins within a specific molecular mass range.

Following electrophoresis, the proteins are located using a variety of methods of different sensitivities. Coomassie Brilliant Blue (CBB) R250 used with methanol and acetic acid detects approximately 0.5 μg of protein. Improved sensitivity can be achieved using CBB G-250 as a colloid in the presence of phosphoric acid and ethanol [29]. The latter staining method also provides a convenient means of batch processing slab gels [30]. Highly sensitive silver stains can be used to increase the numbers of proteins detected in a sample. Although more "hands-on" processing of the gel is required with silver staining, the method improves protein detection by up to 40-fold compared to CBB staining. Densitometric analysis of the 2D protein profiles is frequently used to locate quantitative protein mutations. Both CBB and silver staining produce linear responses over a range of protein concentrations. CBB staining is linear over protein concentrations of 0.5 to 20 μg whereas silver staining is linear at protein concentrations of 0.02 ng/mm^2 to 0.8 ng/mm^2 [31]. A limitation to quantitative analysis using either CBB or silver staining is that the response slopes differ between proteins [8,32]. Radiolabelling proteins with radioactive amino acid precursors can be used as a high-sensitivity detection method for studies in which prokaryotic and eukaryotic cells can metabolise in vitro to incorporate amino acid tracers during protein synthesis [33–36]. For quantitative studies, radiolabelling is limited by the incorporation of the amino acid tracer and the poor linear response of the X-ray film. The latter can be overcome by replacing the film with a phosphor imaging system to detect radiolabelled proteins and extend the linearity for quantitation [37,38]. The aforementioned detection methods are non-specific and will detect almost all proteins resolved by 2DE. For the analysis of previously characterised proteins, immunoblotting with specific antibodies can be used to locate the protein after electrophoresis.

For small numbers of gels, the 2D protein profiles can be compared simply by overlaying the gels and manually inspecting the profiles for proteins with aberrant electrophoretic mobilities. As the number of proteins being screened increases, and the number of gels within the study grows, the use of dedicated computer programs for profile matching becomes necessary. In addition, the detection of protein mutations based on quantitative differences in spot intensity has an absolute requirement for computer-assisted densitometry. Specialised computer programs have been developed for the analysis of 2D protein profiles [39–42]. Due to the large amounts of data processed by these programs they generally require the use of high-specification computer workstations. Analytical programs running on personal desktop computers have been applied to small-scale studies of protein mutations [43]. Irrespective of the computer system applied to the analysis, the priority remains in producing high-quality, reproducible 2D protein profiles.

3. Application of two-dimensional electrophoresis to the detection of protein mutations

The following section describes some of the applications of 2DE for the detection of protein mutations. The emphasis of the discussion is placed on those studies in which 2DE has been

fundamental in the investigation of protein mutations. Specific examples will be drawn from the investigations of prokaryotes and eukaryotes. The widespread use of 2DE precludes a detailed description of all applications; thus this review will provide a summary of the technique for the characterisation of protein mutations.

3.1. Detection and characterisation of protein mutations in prokaryotes

From its earliest developments 2DE has been applied to the analysis of viruses and bacteria. Indeed, O'Farrell [2] developed his initial separation procedure by analysing *Escherichia coli* proteins. In the field of microbiology, 2DE has been used as a tool to characterise specific protein mutations and, along with other molecular methods, to type and identify microorganisms. Although the same basic approaches can and have been used for all microorganisms (i.e. from viruses to protozoa) the examples discussed will be drawn from studies of viruses and bacteria. In the analysis of microorganisms, the detection of naturally occurring protein mutations has been widely adopted as a means of typing for taxonomic and epidemiological investigations and these areas of study will be included with the review of protein mutations resolved by 2DE.

3.1.1. Characterisation of mutations in virus proteins

2DE has been applied to a number of areas of virology. The proteins of various viruses have been characterised by 2DE to identify the gene products encoded by virus genomes [44–47]. Another major application of 2DE to virology has been to determine the response of the host cell protein synthesis following virus infection —i.e. either during lytic virus infection or in virus-induced tumours. Although these latter investigations have primarily analysed virus infections of in vitro-grown cell lines [48–51], similar procedures can be used to examine the effect of virus infection on cellular protein synthesis in the intact animal [52,53]. The relative simplicity of the majority of virus genomes combined with rapid nucleic acid sequencing means that the direct detection of protein mutations at the nucleotide sequence level is a realistic experimental strategy. Nevertheless, 2DE can detect protein mutations and is useful as a screening procedure to identify regions of the virus genome deserving detailed analysis.

When analysed by 2DE, many virus proteins resolve as charge chains of polypeptides with the same molecular mass, even within a single virus isolate [24,54–56]. There are a number of reasons for the observed virus protein heterogeneity. Many virus proteins undergo post-translational modifications that may affect the mobility in 2DE, including phosphorylation [57], glycosylation [58] and myristylation [59]. The p30 protein of murine leukaemia virus has a characteristic isoelectric point (p*I*) depending on the virus strain analysed and the host cell in which the virus is grown. In addition, p30 shows both a major spot and up to three additional minor protein spots differing in their tryptic peptide digests [55]. Virus isolates are themselves highly heterogeneous populations and a single clinical virus isolate may contain members which differ in their biological and physical properties. This can be demonstrated by selecting cloned virus stocks from a single clinical isolate of coxsackie B virus (CBV). When the proteins of these sub-populations of CBV are analysed by 2DE, differences in the electrophoretic mobilities of the virus proteins can be resolved (Fig. 1) [24]. Table 1 shows the estimated p*I* values of the p39 and p33 virus proteins encoded by the original uncloned CBV isolate and five cloned virus stocks from this clinical virus isolate.

The application of 2DE to monitor natural variation among virus isolates has been fairly limited and most effort in this area has been placed on the use of nucleic acid based techniques to detect differences between virus isolates, e.g. RNase T1 oligonucleotide fingerprinting [60] and direct sequencing of the genomic nucleic acid [61]. 2DE has been used to compare African swine fever virus (ASFV) variants collected from geographically different areas of Spain over a period of years; no differences were observed for the 2D protein profiles of these

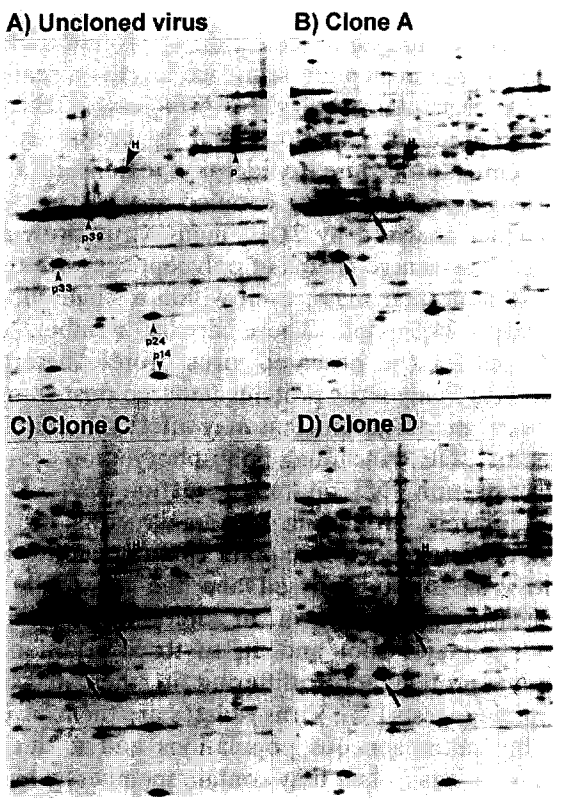

Fig. 1. Analysis of the 2D protein profiles of HEp-2 cells infected with cloned CBV virus stocks prepared from a single isolate of CBV serotype 5 as described elsewhere [24]. Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles. Five of the virus-induced proteins are indicated in (A). The locations of the virus proteins p39 and p33 are indicated by arrows in (B)–(D). A host protein (labelled H) is indicated in each panel to relate to the relative positions of p39 and p33 for each virus preparation. From Ref. [24].

Table 1
Estimated p*I* values of virus proteins for cloned virus derived from single clinical isolates of serotype 5 coxsackie B virus

Virus	Estimated p*I* values	
	p39	p33
Uncloned virus	6.29	6.12
Clone A	6.29	6.14
Clones C, B and E	6.46	6.36
Clone D	6.39	6.29

From Ref. [24].

virus isolates [62]. Naturally occurring protein variation among clinical isolates of CBV has been demonstrated by 2DE which discriminated between virus isolates [24,46]. Mutations were observed that affected either the apparent molecular mass or isoelectric point of the virus proteins. Fig. 2 shows an example of the heterogeneity of virus proteins from serotype 2 CBV collected from two geographic locations. This approach to the comparison of clinical isolates of viruses and bacteria (see below) is fairly labour intensive compared to other methods and only

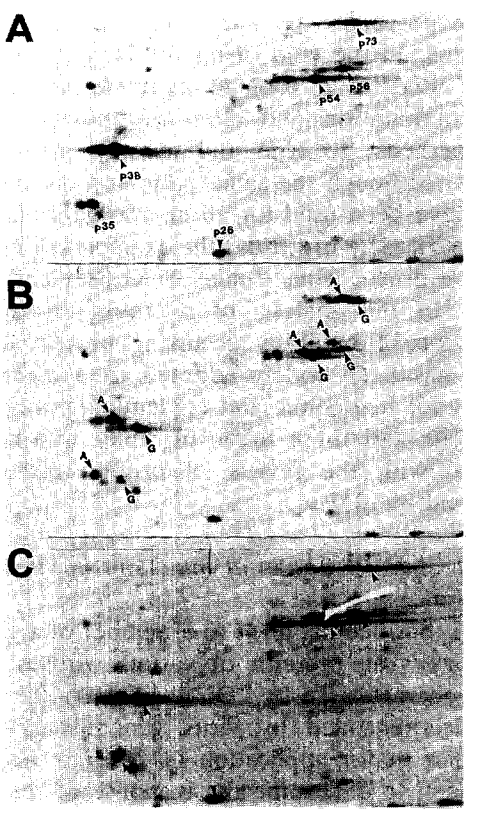

Fig. 2. Analysis of 2D protein profiles of HEp-2 cells infected with one of two isolates of serotype 2 CBV isolated from Aberdeen (A) or Glasgow (C). (B) Co-electrophoresis of the two protein preparations; the arrows indicate the origins of the proteins —i.e. A = Aberdeen CBV isolate and G = Glasgow CBV isolate. The intracellular proteins were prepared as described previously [24]. Six virus-induced proteins are indicated in (A) and indicated by arrows in (C). Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles. From Ref. [24].

suitable for relatively small defined collections of microbes.

The host cell or organism in which viruses are grown can lead to the selection of new virus variants. This can be demonstrated in vivo by the selection of myocarditic CBV variants from an amyocarditic parental virus following sequential passage of the virus in mice. 2DE has been used to examine in vitro the effect of the host cell on the virus proteins. Katoh et al. [55] reported that the charge of the major protein spot of p30 of murine leukaemia viruses (see above) correlated with their observed tissue tropisms. Changes in the 2D protein profiles occur during the adaptation of ASFV to growth in monkey stable (MS) cells in vitro [62]. During the adaptation of ASFV to MS cells, multiple forms of an M_r 25 000 protein, designated p54, were detected after 44 sequential virus passages in MS cells. The different forms of p54 were due to the selection of variant virus populations during adaptation of ASFV to in vitro cell culture. There was no correlation of the altered p54 protein with either virus virulence or virus infectivity for MS cells and pig macrophages, the latter being the natural cell type infected by ASFV [62]. The selection of virus variants during in vitro adaptation must be borne in mind when looking for protein markers of virus virulence as discussed later. We have used 2DE to examine the evolution CBV during persistent infection of rhabdomyosarcoma (RD) cells. CBV can establish persistent infections in RD cells and the cell population as a whole survives the virus infection and continues to release infectious virus during cell passage in culture [36,48]; one cell line (designated "piRD-3673" cells) has been maintained in culture for over 70 passages. The proteins of the virus released from piRD-3673 cells at different passage levels were compared by 2DE [36]. Two virus proteins (p33 and p39) changed their electrophoretic mobility (Fig. 3); mutated proteins were first observed by passage 10 of piRD-3673 cells. This contrasted with no detected alteration in the electrophoretic mobilities of p33 and p39 when the virus was passaged 23 times in HEp-2C cells; a cell line which supports a lytic CBV infection. The parallel mutation of p33 and p39 suggested that p33 might be derived from p39 by proteolytic cleavage [36]. This proposal was supported from the analysis of the polypeptides of purified CBV. On the basis of their molecular masses the identity of p33 was consistent with VP2 and p39 with VP0, which is the known precursor of VP2 in picorna viruses [63]. Similar observations of protein mutations during persistent infection have been made for Theiler's murine en-

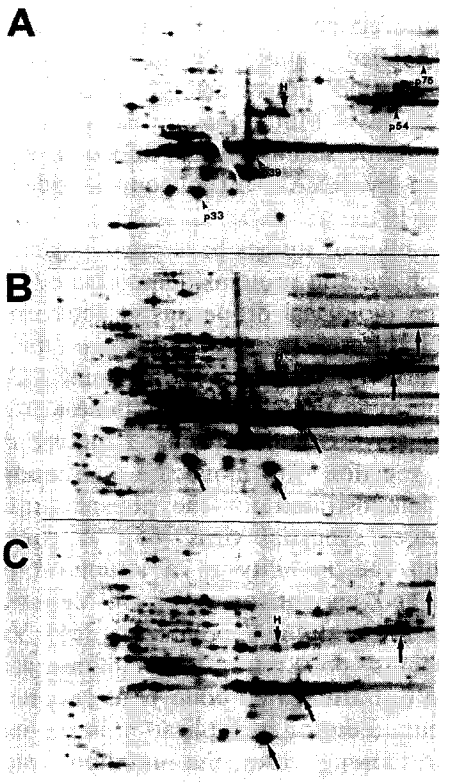

Fig. 3. Analysis of 2D protein profiles of HEp-2 cells infected with either parental CBV-3673 (A) or piCBV released from persistently infected RD cells at passage 10 of the cells (C). Intracellular proteins from virus-infected HEp-2C cells were prepared and analysed as described by McLaren et al. [36]. (B) Co-electrophoresis of the proteins preparations of (A) and (C). The virus proteins p75, p54, p39 and p33 are marked in (A) and indicated by arrows in (B) and (C). A host protein (labelled H) is indicated in each panel to relate to the relative positions of p36 and p33 for each virus preparation. Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles. From Ref. [36].

cephalomyelitis virus (TMEV), another picorna virus, unrelated to CBV. 2DE revealed the occurrence of mutations in many of the eleven structural and non-structural TMEV proteins detected [64]. Mutations in the virus genome during the persistent TMEV infection were also detected by RNAse T1 oligonucleotide fingerprinting [64].

A number of studies have been carried out to relate changes in proteins demonstrated by 2DE with the biological properties of the virus. Antigenic differences are probably the most straightforward property to examine. Correlations between protein mobility demonstrated by 1D SDS-PAGE and antigenic structure defined by the reaction with monoclonal antibodies has been demonstrated for the phosphoprotein of respiratory syncytial virus [65]. A correlation between the mobility of a structural protein (VP3) and an antigenic site on VP3 was described for serotype 1 polio virus [66]. Vaccination against polio virus is commonly achieved using a live attenuated polio virus vaccine which may mutate during its replication in the intestine [67]. In extreme cases, the mutations can result in the reversion of the avirulent vaccine virus to a virulent phenotype. When serotype 1 polio virus excreted from recently vaccinated individuals was examined there was a characteristic mutation of VP3, compared to the vaccine virus, that affected the protein's net charge (Fig. 4). The VP3 mutation correlated with an altered recognition by a monoclonal antibody (MAb 423), specific for a neutralisation site located on VP3 (Table 2) [66]. A series of polio virus mutants (provided by Dr. P.D. Minor, National Institute for Biological Standards and Control, London, UK), resistant to neutralisation by MAb 423 [68], were also characterised by 2DE. In these mutants the same correlation between VP3 mobility and MAb 423 recognition was

Table 2
Characterisation of polio virus isolates by 2DE and neutralisation by monoclonal antibodies to the VP3 proteins

Virus[a]	VP3 Mobility	Antibody response[b], MAb 423
Mahoney	Acidic	−
OPV-1	Basic	+
1883	Acidic	−
1869	Acidic	−
1318	Acidic	−
1256	Basic	+
1400	Basic	+
2687	Basic	+
3522	Basic	+
3859	Basic	+
4434	Basic	+

[a] Mahoney and OPV-1 are non-vaccine serotype 1 and serotype 1 poliovaccine virus isolates, respectively. The numbers represent clinical isolates isolated from faeces. All clinical isolates are from non-paralytic cases and are likely to represent excretion of vaccine virus.

[b] MAb 423 was raised against a neutralisation site on VP3 of the serotype 1 Sabin strain of polio virus. + = Neutralised by MAb 423; − = not neutralised by MAb 423. Modified from Ref. [66].

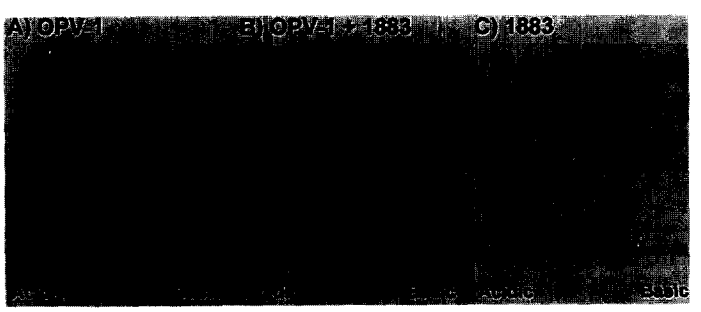

Fig. 4. Analysis the intracellular proteins of HEp-2 cells infected with serotype 1 polio virus isolates. Only a limited region of the complete protein profile is shown in this figure. The intracellular proteins were prepared and analysed as described previously [66]. The virus proteins VP0, VP2 and VP3 are indicated in (A). VP2 and VP3 are indicated by arrows in (B) and (C). Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles. Modified from Ref. [66].

Table 3
Analysis of serotype 1 polio virus antibody-escape mutants by 2DE

Virus	Mutation	Antibody response MAb 423	VP3 Mobility
Sabin	None	+	Basic
535	Ala(59) to Gln	–	Acidic
1096	Lys(60) to Gln	–	Acidic
505	Arg(71) to Gln	–	Acidic

[a] + = Neutralised by MAb 423; – = not neutralised by MAb 423.

observed irrespective of the amino acid substitution occurring in VP3 (Table 3).

The association of protein mutations, detected by 2DE, with complex phenotypic characteristics of viruses (e.g. virulence) has been examined. These multi-factorial phenotypes may be determined by mutations within more than one virus protein and the capacity of 2DE to characterise, non-specifically, many of the virus proteins simultaneously makes it a particularly attractive approach to this field of study. When combined with sensitive nucleic acid techniques (e.g. PCR) for fine gene mapping, it becomes a powerful approach to examine such complex phenotypes. 2DE has been used to look for virulence markers in a number of picornaviruses including encephalomyocarditis virus (EMCV) [54], TMEV [44,69] and polio virus [70]. EMCV variants can be differentiated on the basis of serological tests and pathogenesis in mice. Cerutis and Giron [54] used 2DE to compare the proteins of a virulent EMCV variant (EMCV-K) derived from an avirulent parental virus (EMCV-B). The two virus variants differed on the basis of a higher p*I* for the virion protein VP1 in EMCV-K compared to EMCV-B. This agreed with other reports demonstrating VP1 variation among EMCV pathogenic variants as determined by nucleotide sequencing of the genome RNA [71]. In addition, two new non-structural proteins (M_r 17 000 and 12 000) were detected for EMCV-K but not EMCV-B [54]. In a study of pathogenic variants of TMEV, no protein differences were observed between virulent and avirulent TMEV variants when compared by 2DE [44]. This was despite differences, between virulent and avirulent TMEV isolates, being demonstrated in the virus RNA genomes by RNase T1 oligonucleotide fingerprinting [72]. An analysis of a larger collection of TMEV isolates showed that 2DE did distinguish between pathogenic variants [69]. In the analysis of virulent variants selected from an avirulent polio virus serotype 1 strain, 2DE was used in combination with nucleotide sequencing to identify possible mutations conferring the virulence phenotype [70]. Although multiple nucleotide substitutions were identified in the virulent polio virus, compared to the avirulent parental polio virus, no protein mutations were demonstrated by 2DE. Virulent and avirulent variants of Marek's disease (MD) virus, an oncogenic virus of chickens, can be isolated. Protein analyses by 2DE have allowed the division of MD virus isolates into three groups on the basis of the 2D protein profiles prepared from virus infected cell lysates and purified virus nucleocapsids [73,74]. However, it was not possible to distinguish virulent from avirulent virus isolates by this approach. Recently, it has been shown that virulent and avirulent MD virus isolates can be distinguished on the basis of sequence differences detected using PCR [75]. These data indicate that protein analysis by 2DE alone will not always reveal differences between virulent and avirulent virus variants but can in some situations be used to screen virus isolates to identify virus proteins appropriate for detailed study by complementary methods. The studies described above predominantly looked for qualitative differences in the virus protein profiles, i.e. alterations in protein electrophoretic mobilities. Quantitative investigations of protein expression using 2DE have not received much attention and may be a fruitful area of investigation particularly for those virus groups with relatively complex control mechanisms for gene expression e.g. herpes viruses and pox viruses.

3.1.2. Characterisation of mutations in bacterial proteins

With increasing genome complexity and organisation, the analysis of proteins by 2DE becomes progressively more useful for detecting

and characterising mutations. Extensive work has been carried out on the analysis of bacterial proteins by 2DE to study either bacterial epidemiology and taxonomy or bacterial gene expression. The proteins of a number of bacterial genera have been analysed by 2DE. Comparative studies of the total protein content of bacteria have been reported for *Neisseria* spp. [76,77], *Treponema pallidum* [78], *Campylobacter* spp. [79], *Haemophilus* spp. [24] and *Mycoplasma* spp. [26,80]. For these comparative studies it is assumed that co-migrating protein spots are functionally equivalent proteins with amino acid homology. However, functionally equivalent proteins with as little as one alteration of a charged amino acid may appear as unique proteins when analysed by 2DE and bacterial strains may show a greater degree of variability by 2DE than by a "low-resolution" nucleic acid method (e.g. DNA–DNA hybridisation) [80]. In the case of the complex profiles obtained from the analysis of bacterial whole cell protein preparations, inter-laboratory comparisons of the data become difficult due to inevitable minor differences in analytical technique. When one laboratory has compared bacteria from different genera by the same analytical 2D gel system, characteristic protein profiles are found for each genus [24,76]. Examples of 2D protein profiles for *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli* and *Aeromonas* sp. derived from work in our laboratory are shown in Fig. 5. Jackson et al. [76] compared the proteins of multiple isolates of *Neisseria gonorrhoea*, *N. meningitidis* and *Branhamella catarrhalis* by 2DE. Quantitative and qualitative comparisons

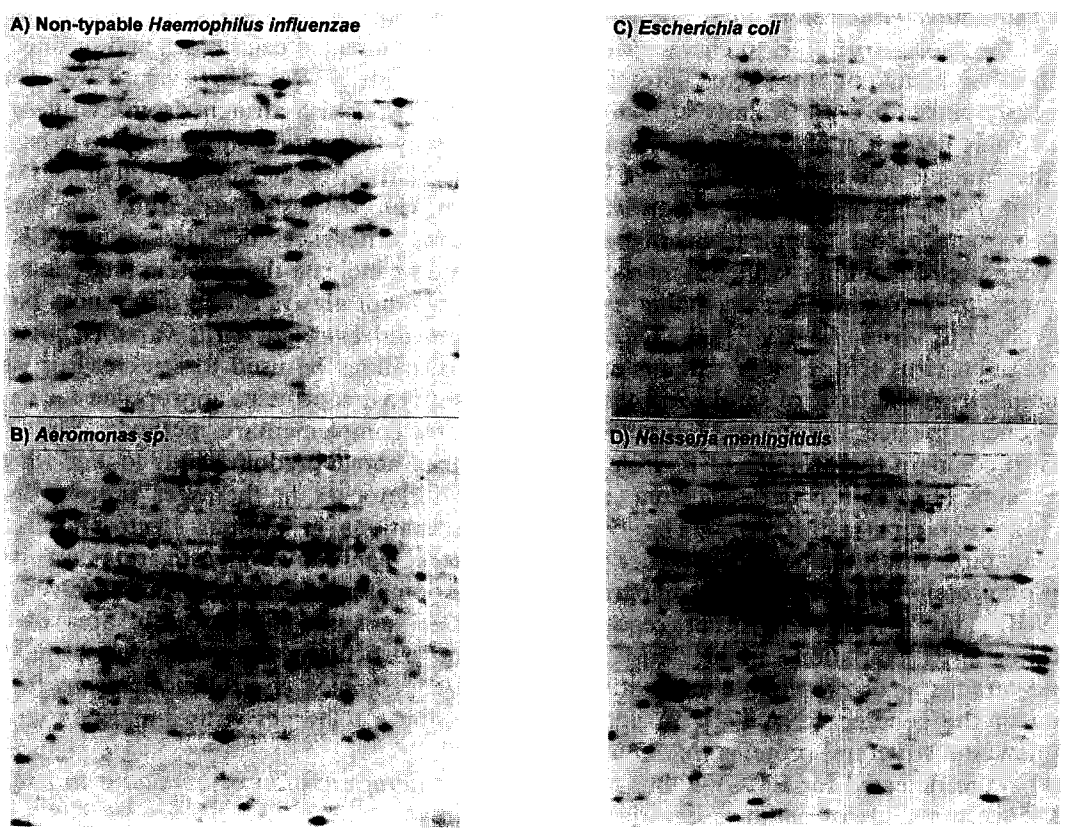

Fig. 5. 2D protein profiles of Gram-negative bacteria. Representative strains for four different genera of Gram-negative bacteria were grown on chocolate agar and the cellular proteins prepared and analysed by 2DE as described previously [24]. Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles.

of the approximately 200 proteins resolved by 2DE showed that the differences in the 2D protein profiles observed for these bacteria followed their generally recognised taxonomic classification [76]. On a more subtle level, qualitative differences in the 2D protein profiles of non-typeable *H. influenzae* isolates correlated with the clonal population structure demonstrated for these bacteria by other biochemical procedures, including DNA fingerprinting [81]. In the analysis of multiple clinical isolates of a bacterium some proteins are found to be genus specific and common to all isolates whereas other proteins show variation between isolates; for example among *Campylobacter pylori* isolates collected from widely different geographic locations [79] and among Mycoplasmas classified in different species [82–84]. Similar data were observed among four members of the *Haemophilus* genus (Fig. 6). Proteins with similar electrophoretic mobilities were identified in each of the 2D protein profiles. The non-typable and type b *H. influenzae* strains (Fig. 6A and B), which differ primarily on the basis of their capsular antigens, showed the closest similarity. The extent of the variation observed between clinical isolates of non-typable *H. influenzae* analysed by 2DE is shown in Fig. 7. For these data, the 2D protein profiles from three non-typable *H. influenzae* isolates ("Slaves") were matched to a single "Master" non-typable *H. influenzae* 2D protein profile using 2DE analytical software (Phoretix; Phoretix International) run on a desktop personal computer. The Master protein

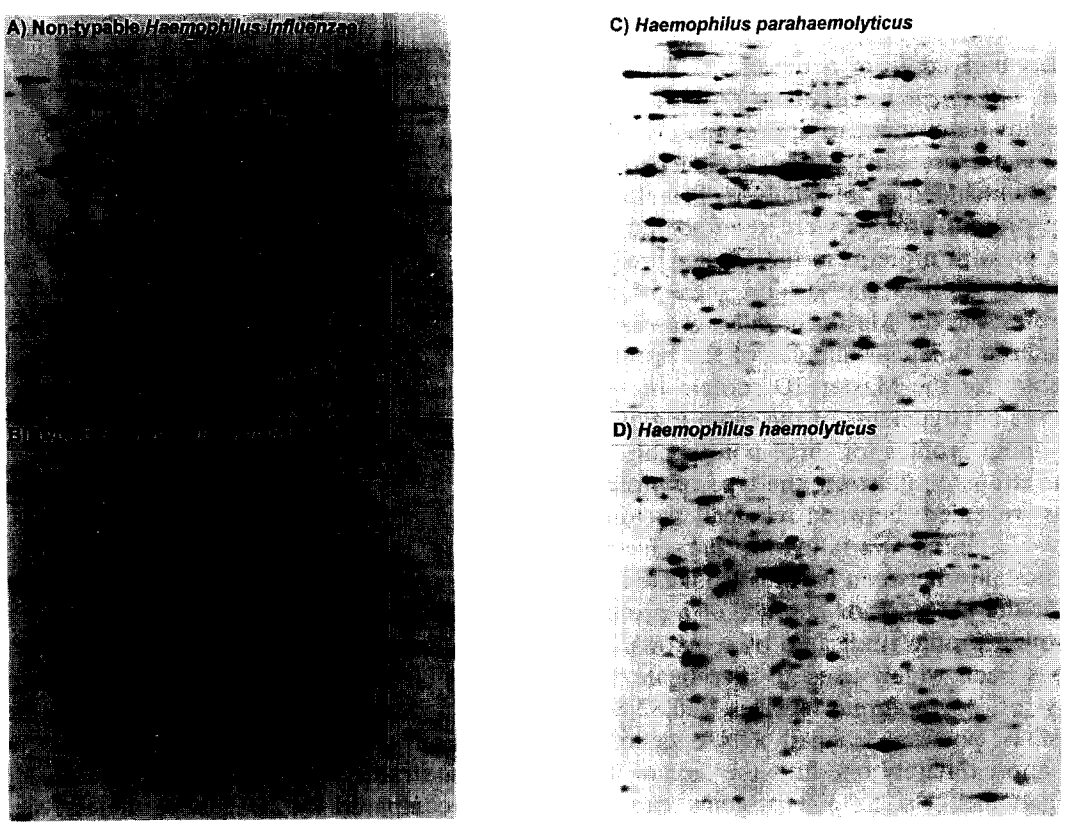

Fig. 6. 2D protein profiles of *Haemophilus* spp. Representative strains of four different species of *Haemophilus* were grown on chocolate agar and the cellular proteins prepared and analysed by 2DE as described previously [24]. Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles.

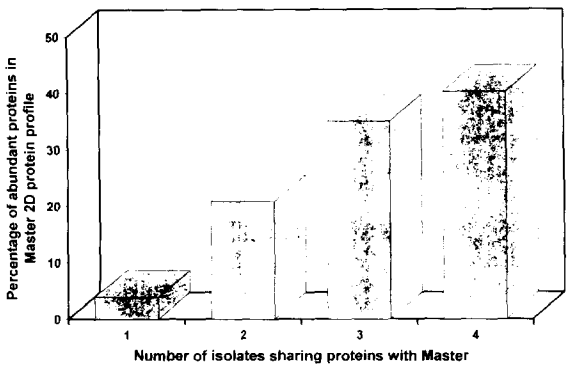

Fig. 7. Frequency of shared spots among non-typable *Haemophilus influenzae* strains. The 2D protein profiles of three strains of non-typable *H. influenzae* were matched to the 2D protein profile of a Master strain of non-typable *H. influenzae*. The 77 most abundant proteins in the Master protein profile were then scored as to their presence in the other three bacterial strains. The graph shows the frequencies of the proteins detected in 1 (i.e. Master 2D protein profile alone), 2, 3 or 4 strains.

profile was derived from a representative non-typable *H. influenzae* isolate. The 77 most abundant proteins (equivalent to approximately 34% of the detected proteins) of the Master protein profile were included in this analysis. Proteins in the Slave protein profiles were then scored for co-migration with proteins in the Master profile. In this series of bacterial strains, 40% of the scored proteins in the Master profile were shared by all bacterial isolates while 4% of the proteins were specific for the Master 2D protein profile. Fig. 8 shows enlargements of the same region of the 2D protein profiles for three non-typable *H. influenzae* strains to illustrate the type of protein mutations revealed between clinical bacterial isolates —i.e. either variation in the electrophoretic mobility between isolates (indicated by an star) or the presence or absence of a protein in the protein profile (indicated by a arrow).

2DE has been widely used to investigate the taxonomy of *Mycoplasma* species [26,82,84]. Mycoplasmas possess a small DNA genome which is approximately one-fifth the size of the *E. coli* genome. Up to 300 proteins can be identified when in vivo radiolabelled Mycoplasma cell lysates are analysed by 2DE [80,82]. Comparisons of isolates of the same *Mycoplasma*

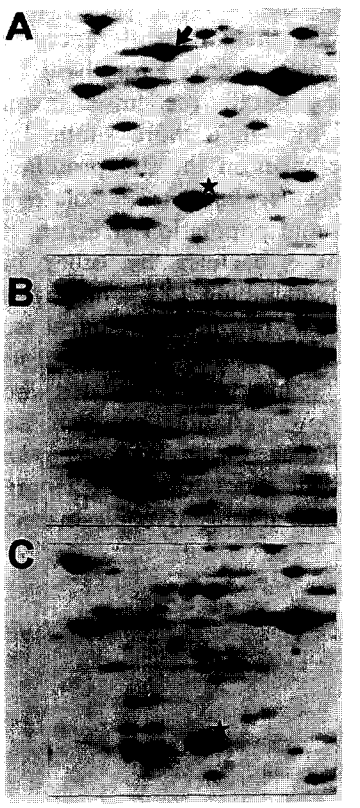

Fig. 8. 2D protein profiles of non-typable *Haemophilus influenzae*. Three clinical strains of non-typable *H. influenzae* were grown on chocolate agar and the cellular proteins prepared and analysed by 2DE as described previously [24]. The panels in this figure show the same enlarged region of the 2D protein profile for each strain. The arrows in (A) and (B) indicate a bacterial protein present in only these two *H. influenzae* isolates. The star indicates a common protein for all three isolates which differs in its mobility. Acidic proteins are to the left and high-molecular-mass proteins at the top of the profiles.

species reveals similar 2D protein profiles with a limited number of variable proteins; for example, among approximately 340 proteins detected for six strains of *M. arthritidis*, 25 proteins were identified as strain-variable [85]. More extensive variation was demonstrated when six *Mycoplasma* strains, representing four different species, were compared by 2DE; nevertheless, six common genus-specific proteins were identified [82]. The degree of similarity, demonstrated by 2DE, between these *Mycoplasma* strains was equiva-

lent to that obtained when they were compared by DNA–DNA hybridisation [82]. Further discrimination can be achieved by combining 2DE and immunoblotting to compare the major *Mycoplasma* antigens. This approach revealed the occurrence of common co-migrating genus-specific antigens as well as strain- and species-specific antigens [83,84]. 2DE and immunoblotting have been used to examine the V-1 surface antigen of *M. pulmonis* [86,87]. The V-1 antigen complex shows an unusual pattern of migration with a regular series of bands ranging from a molecular mass of 150 000 and p*I* of 4.5 down to a molecular mass of 20 000 and a p*I* of 5.5–6.0; the regularity of the migration may be due to some form of post-translational modification [86]. *M. pulmonis* strains, as well as cloned organisms from a single *M. pulmonis* strain, vary in the charge of the V-1 antigen as demonstrated using 2DE. Alterations in the V-1 antigen complex has been shown to correlate with altered surface properties of the organism; for example, *M. pulmonis* mutants lacking V-1 (as shown by 2DE) do not allow the adsorption of the *Mycoplasma* virus P1 [88].

The studies described above analysed the total cellular proteins for the comparison of bacterial isolates. In general, there is no information on either the identity or function of the proteins included in the analyses. Some investigations of bacterial taxonomy have used 2DE to characterise specific subsets of bacterial proteins, including ribosomal proteins and outer membrane proteins (OMPs). Ribosomes are conserved structures and the ribosomal components have been used in taxonomic investigations of different bacterial groups. Analysis of ribosomal proteins by 2DE has been used to look for relationships within and between bacterial genera [89,90]. Coefficients of similarity determined for ribosomal proteins of *Corynebacterium* and *Arthrobacterium* strains, analysed by 2DE, showed the same relationships between bacterial strains as observed by DNA analyses [90]. Mutations of ribosomal proteins detected by 2DE have been reported for *E. coli* [91]. Bacterial OMPs are important as the major antigens against which a protective immune response of the host is directed. Thus, an understanding of the mutations occurring in these proteins could be of practical importance in vaccine design as well as providing information on bacterial pathogenesis. Multiple differences in OMPs were observed between selected strains of *Campylobacter jejuni*, *C. coli*, *C. pylori* and *C. fetus* using 2DE and immunoblotting [79,92]. *C. pylori* did not possess the major OMP of M_r 44 000 and in *Campylobacter* strains in which the M_r 44 000 OMP was present, either one or two charge variants were identified [92]. Outer membrane proteins have also been compared in Lymphogranuloma venereum and trachomatis biovars of *Chlamydia trachomatis* [93]. Multiple differences in the OMPs were demonstrated for the two biovars in which the charge of an M_r 60 000 OMP differed whilst the low-molecular-mass OMPs (12 000–12 500) differed in both charge and molecular mass. Analysis of the nucleotide sequence of the M_r 60 000 OMP using the PCR has revealed polymorphism of the protein among different species and isolates of *C. trachomatis* [94].

There are many applications of 2DE in the study of bacterial gene expression and characterisation of specific protein mutations. The following discussion will specifically consider examples in the use of 2DE to identify and characterise protein mutations associated with virulence. The virulence of a microorganism is likely influenced by various factors ranging from the regulation of bacterial growth to the ability of the organism to survive in the host and evade the host's immune response. The identification of virulence-associated proteins requires the analysis of the complex interplay of many components of the bacterial cell. Classical methods of determining the effect of individual genes one at a time are unlikely to provide the necessary insights into this complex phenotype in the same way that the analysis of global gene expression by 2DE can achieve [7]. Thus the analysis of laboratory-induced mutants and naturally occurring variants by 2DE will continue to play a valuable role in identifying the determinants of microbial virulence. The common experimental strategy to identify mutations altering virulence

is to compare virulent and avirulent bacterial strains usually after growth of the organisms in vitro. However, different growth media [24] or growth in vitro [78] can lead to altered protein expression demonstrated by 2DE; this must be considered during data analysis. Analysis of *Mycoplasma arthritidis* strains by 2DE and immunoblotting demonstrated that two virulent strains were more closely related to each other than to avirulent strains. Differences were found for only six proteins among these four *M. arthritidis* strains, none of which were considered to be surface antigens [95]. Using 2DE, a detailed study has been made of protein expression associated with virulence in *Brucella abortis*, an important economic disease of cattle [96]. The proteins of an avirulent vaccine strain (S19) of *B. abortis* were compared with a closely related virulent field isolate (strain 2308). Qualitative and quantitative ($\geq$10-fold) differences in protein synthesis were scored and revealed approximately 90 protein differences between the two *B. abortis* strains [96]. Sowa et al. [96] proposed that many of these protein alterations were the result of regulatory differences in genes required for the maintenance of homeostasis rather than genetic differences between the virulent and avirulent bacteria. It was suggested that only between 14 and 25 proteins were likely to be involved in determining virulence. The attenuation of the S19 strain of *B. abortis* may be due to either the loss of a virulence protein or the expression of a new antigen leading to improved immune response by the host [96]. In common with many of these investigations using 2DE, the identities of the altered proteins remains unknown. In the case of *B. abortis*, 996 proteins were identified from the composite analysis of the S19 and 2308 strains equivalent to approximately 46% of the genome coding capacity. Thus, there may be other bacterial proteins playing a role in this complex phenotype. *Borrelia burgdorferi*, the causative organism of Lyme disease, produces an invasive disease and a variety of factors may be involved in determining its virulence. Extended passage of *B. burgdorferi* in vitro results in a loss of infectivity and virulence for the mammalian host [97]. Comparisons of low- and high-passage *B. burgdorferi* B31 by 2DE revealed two proteins of M_r 24 000 and 20 000 with reduced expression levels and another two proteins of M_r 35 000 and 28 000 absent in high-passage *B. burgdorferi*; these could be potential virulence proteins [98]. The abundant M_r 28 000 protein present in low-passage *B. burgdorferi* was identified as OspD a surface protein encoded by a 38 000-base pair linear plasmid which is lost in high passage *B. burgdorferi*. Thus, reduced virulence of *B. burgdorferi* is associated with the absence of the OspD gene due to the loss of the plasmid encoding the gene. The loss of the M_r 35 000 protein may also be due to plasmid loss [98]. The explanation for the reduced expression of the M_r 24 000 and 20 000 proteins in the high-passage organisms remains unclear.

A number of bacteria grow intracellularly during in vivo infection of the host. Interaction between some bacteria and eukaryotic cells in vitro leads to the altered synthesis of a number of bacterial proteins detectable by 2DE [99,100]. *Salmonella typhimurium* infection of macrophage cells in vitro leads to increased levels of up to 40 bacterial proteins, and approximately 140 other bacterial proteins show reduced levels as determined by 2DE [7,101]. Some of the proteins with increased biosynthetic levels are stress proteins (e.g. GroEL) which have high levels of synthesis under a variety of stress conditions. *S. typhimurium* mutants, unable to replicate in macrophages, are deficient in the expression of varying subsets of the induced proteins [101]. *S. typhimurium* virulence and its capacity to grow in macrophages is controlled by the *phoP* genetic locus which is made up of two regulatory genes *phoP* and *phoQ* controlling the expression of many other *S. typhimurium* genes in response to external stimuli. *S. typhimurium* mutants, constitutively expressing PhoP (PhoP^c), show significantly increased levels of expression of *phoP*-activated genes compared to wild type (PhoP$^+$) bacteria. When analysed by 2DE, PhoP^c mutants show abnormal levels of synthesis of 40 proteins compared to PhoP$^+$. Proteins were either activated in the PhoP^c mutant (consistent with the known activating role of the *phoP* locus) or

repressed, an unexpected observation [102]. The identities of the proteins resolved by 2DE in these mutants are unknown but it was proposed that repression in the synthesis of one of these proteins lead to the attenuated *S. typhimurium* phenotype. One gene of *S. typhimurium* whose transcription is activated by phoP is the *pagC* locus; mutants defective at *pagC* survive poorly in cultured macrophages and are less virulent in mice. Analysis by 2DE of one mutant defective in *pagC* showed the loss of a protein of M_r 18 000 and p*I* 8.0 which had a strong nucleotide sequence homology to a virulence gene (*ail* locus) of *Yersinia enterocolitica* [103]. The analysis of the *phoP* locus of *S. typhimurium* represents one example in which 2DE can be used to determine the global effect of mutations in regulatory proteins of bacteria.

3.2. Detection and characterisation of protein mutations in eukaryotes

The following section considers the analysis of naturally occurring and induced protein mutations in eukaryotes. 2DE is an important analytical tool for the detection and characterisation of protein mutations covering the detection of naturally occurring protein polymorphisms, the detection of mutagen induced protein mutations as well as the identification of disease associated protein mutations. 2DE has also been used to analyse proteins in insects, particularly *Drosophila*, to look at natural protein variation [104] as well as specific mutational lesions [105,106]. The following discussion will focus on the characterisation of protein mutations in animals and plants.

3.2.1. Detection and characterisation of protein mutations in animals

The application of 2DE for detecting protein polymorphisms in humans takes advantage of the high resolving power of 2DE to analyse rapidly many independent loci. Protein polymorphisms have been characterised by 2DE in a variety of cell types and body fluids, including, for example, fibroblasts [33,34], lymphocytes [35,107], erythrocytes [108], platelets [109] and plasma [4,110]. By collecting samples from a child and his or her parents the genetic nature of the polymorphism can be confirmed [107–109]. The early studies, using limited selection criteria for the proteins in the data, reported protein heterozygosity indices of between 0.6 and 1% [33,34]. Although later studies, employing more rigorous selection criteria for the proteins analysed, yielded higher values for the index of heterozygosity (2.4–3.1%), these were still lower than values obtained by 1DE in which proteins were generally located on the basis enzymatic reactions [35,107–109]. Heterozygosity indices (6.2%) determined from the analysis of plasma proteins are closer to those obtained by 1DE [110,111]. In a review of these data, and similar studies from other groups, Neel [111] concluded that technical aspects did not wholly explain the differences in the determination of protein heterozygosity observed between 1DE and 2DE. It was suggested that proteins detected by 2DE have naturally low mutation rates for a yet unknown reason [111]. Investigations of protein polymorphisms among inbred strains of mice demonstrated the existence of proteins possessing specific electrophoretic mobilities for the strains of mice investigated [112–115]. These investigations also revealed that the frequency of qualitative and quantitative protein variants detected differed according to the tissue analysed (that is, analysis of liver proteins revealed more genetic variants than similar comparisons of brain proteins). In addition, the proteins present in membrane bound structures are more highly conserved than cytosol proteins when compared by 2DE [113,114,116].

Various experimental strategies can be used to detect mutations following exposure of animals or cultured cells to mutagenic treatment. 2DE has been widely used as a means of detecting mutations affecting the coding sequences of the cellular genome. Laboratory studies commonly detect protein mutations expressed in the livers of the progeny of mice exposed to the mutagen under study. As discussed by Giometti [117], the analysis of the liver proteins has a number of technical advantages: (1) the 2D protein profiles are composed of well-separated proteins with

widely differing isoelectric points and molecular masses with little evidence of extensive post-translational modifications, (2) liver is a soft tissue from which proteins can be readily extracted for analysis, and (3) liver biopsies can be collected by partial hepatectomy allowing the mice to breed, thus permitting the demonstration of the genetic nature of the mutation. Essential to many of the studies of induced protein mutations has been the use of computer analysis to screen for qualitative and quantitative changes in the 2D protein profiles from mutagenised animals. Mice, heterozygous for a mutated structural gene, would be expected to show a 50% decrease, compared to homozygous non-mutant mice, in the protein spot on the gel. In the case of a point mutation, affecting the protein's electrophoretic mobility, a new spot may be present in the vicinity of the original protein. The inactivation of a regulatory gene is expected to have little effect on the 2D protein profile in a heterozygote but can result in the altered expression of the protein(s) under its control in homozygous animals [118]. To accurately determine the quantitative changes only those proteins showing minimal variation between analyses are generally included in the data. Proteins showing coefficients of variation of 15–20% for the amounts detected in replicate gels are taken as sufficiently reproducible [12,119,120]. The mouse liver protein database used by Giometti and her colleagues contains approximately 100 proteins fulfilling the necessary criteria of reproducibility [121]. Increased numbers of proteins can be screened for quantitative changes by using multivariate statistical analyses (for example, principal component analysis) to analyse the gel data [122].

A variety of chemical mutagens have been screened to determine their effects on protein synthesis. A comparative study of four chemicals, known to cause peroxisome proliferation and hepatocellular carcinoma, was carried out in mice. Multiple protein alterations were observed in mice treated with the chemicals, and each chemical altered specific subsets of the liver proteins [123]. Exposure to ethylnitrosourea (ENU) has been examined in mice [12,124] and cultured human lymphoblastoid cells [9]. Two protein mutations were detected among 67 F1 offspring of male mice exposed to ENU at 150 mg/kg giving an induced mutation rate of $0.88 \cdot 10^{-4}$ [124]. At least one of the induced mutations caused a charge shift in a protein that was consistent with the occurrence of a point mutation at the locus. A similar approach was used by Giometti et al. [12] who used densitometry to identify quantitative alterations in the 2D protein profiles of the livers of mice exposed to ENU. Four new protein spots were detected in ENU-treated mice but not in untreated control animals [12]. Each of the four mutations was accompanied by the reduction of approximately 50%, compared to untreated animals, of a nearby protein. These data are consistent with the occurrence of a point mutation affecting the mobility of one of the alleles at that locus. The four ENU-induced mutations affected proteins that were predominantly associated with either the mitochondria or microsomal cellular fractions and showed no tissue specificity [125]. One of the mutated proteins has been identified as ornithine aminotransferase [71]. 2DE combined with densitometry was also used to screen for quantitative mutations among 267 proteins (equivalent to 263 unselected loci) in cultured human lymphoblastoid cells exposed to ENU (50 μg ENU per ml for 40 min). From these analyses, 74 variants were identified at 54 loci; 65 variants exhibited an altered protein charge and/or molecular mass whereas 9 variants showed a loss of the normal gene product. Under these conditions, the induced mutation rate was calculated to be $1.1 \cdot 10^{-4}$ per allele [9].

The induction of protein mutations in mice by either γ-radiation or fission spectrum neutrons has been examined. No significant alterations in liver protein synthesis were detected among 369 offspring of male mice exposed to γ-radiation [12]. In contrast, two quantitative mutations were detected for three out of 530 F1 offspring of neutron irradiated male mice; no electrophoretic mobility mutants were detected. One of the two mutations observed was likely to have been a spontaneous mutation derived from the non-irradiated dam of the offspring [119]. It was

likely that the mutation caused by neutron radiation was due to the deletion or inactivation of the structural gene itself rather than the mutation of a regulatory gene [119]. Mutation rates calculated for mice exposed to either ENU or neutron radiation, from 2DE analyses, are in general lower than the rates estimated by other procedures [9,119,124]. Giometti et al. [119] proposed that survivable mutants occurred at a lower frequency in the genes assayed by 2DE than by other mutation screening assays. 2DE has been used to characterise protein expression in mice carrying known genetic deletions. Mice which are heterozygous carriers for one or other of eight recessive lethal mutations show no alterations in their liver protein synthesis which could be correlated with the presence of the mutation [112]. Similarly, mice heterozygous for deletions at the albino locus on chromosome 7 show similar 2D protein profiles in the liver compared to normal mice [118]. However, homozygous mice for these deletions show multiple protein abnormalities [118,126] which are believed to be due to a mutation (e.g. a deletion) within a regulatory gene [118]. Analyses of male mice carrying the X-linked scurfy (*sf*) mutation, show multiple differences in protein expression in the thymus and spleen compared to normal mice [127]. Heterozygous female carriers of *sf* are phenotypically unaffected, but no protein analyses of these female mice were presented [127].

The application of 2DE to locate disease-specific protein mutations in inherited diseases has had varying degrees of success. Yang et al. [43] compared 2D protein profiles of fibroblast cell lines from normal individuals and individuals with known inherited disease. No protein alterations were found to correlate with any of the inherited diseases investigated. Among the other genetic disease examined has been systemic lupus erythrematosus (SLE) for which three quantitative protein variants in lymphocytes and sera were observed for the majority of patients with SLE. These variations in protein expression were also observed for a smaller percentage of the normal control samples [128]. Lesch–Nyhan syndrome is due to a deficiency of hypoxanthine phosphoribosyl transferase (HPRT) activity. Characterisation of protein expression in lymphocytes from sufferers of this syndrome showed that, although they were HPRT deficient, a detectable HPRT protein was present in the 2D protein profile of lymphocytes [129,130]. Presumably, the mutation leading to HPRT deficiency did not affect the electrophoretic mobility of the enzyme. Quantitative alterations in eleven other lymphocyte proteins were detected but their specificity for the syndrome was not confirmed [130]. A benign variant of serum prealbumin, first detected by 2DE, was reported by Harrison et al. [131]. The variant, which was isoelectric with the normal protein but had a lower molecular mass, differed from the normal protein by a single amino acid substitution. Genetic analysis of the inheritance of the variant prealbumin showed it was an autosomal dominant [131].

3.2.2. Detection and characterisation of protein mutations in plants

2DE has been widely used to identify and characterise naturally occurring protein polymorphisms as a means to determine the genetic relationships between plant strains. As with the studies described above for other systems, the advantage of 2DE in this area of investigation is the capacity to compare many distinct characteristics (i.e. protein spots) simultaneously. This approach has been applied to investigations of the genetic relatedness of, for example, variants of maize (*Zea mays* L.) [132,133], barley [134,135], Douglas Fir (*Pseudotsuga menziesii*) [136] and pepper (*Capsicum annum* L.) [137,138]. In the detection of protein polymorphisms among inbred strains of pepper two analytical procedures were used for the first dimension of 2DE, specifically separation in either the presence or the absence of urea and detergent [138]. In these comparisons the proportion of variable spots detected in the absence of urea and detergent solubilisation was greater than detected in the presence of urea and detergent. One possible explanation for this difference between the two methods is that analysis of proteins under denaturing conditions detects

only the amino acid substitutions causing charge alterations. In contrast, under non-denaturing conditions, substitutions of amino acids causing minor charge differences in the net protein charge were also detected [138].

Fontes et al. [139] applied 2DE to the analysis of maize *floury-2* (*fl2*) mutants. Mutations within the *fl2* locus leads to a reduction in zein synthesis, the major storage proteins in the endosperm of maize kernels, and alters the protein body morphology. Analysis of the mutants by 2DE showed the overproduction of an M_r 75 000 protein (designated b-70) existing as two apparent isomers with p*I* values of 5.3 and 5.4. Although the component of b-70 with a p*I* of 5.3 was phosphorylated, it was not a modified product of the p*I* 5.4 component. The relationship of these two components of the M_r 75 000 protein remains to be established. A similar pattern of protein synthesis, in which synthesis of the M_r 75 000 protein was induced, was observed for two other zein regulatory mutants of maize [140]. Although b-70 is unlinked to the *fl2* locus, b-70 expression is controlled by the *fl2* product through the induction of its mRNA [139]. A further illustration of the application of 2DE to the analysis of plant protein mutations comes from the analysis of etiolated and de-etiolated *Arabidopsis thaliana* mutants [141]. A large number of overlapping protein alterations were detected in comparisons of the mutant and normal plants. Thus, the *response* of cellular protein synthesis to the mutation was detected in these analyses rather than the mutation at the locus itself. These authors commented that the simple comparison of normal and mutant plants by 2DE alone is inadequate to identify the mutant gene [141].

4. Conclusions and future developments

The applications described above clearly indicate the usefulness of 2DE to identify and characterise protein mutations in a wide range of biological systems. These mutations can be demonstrated either qualitatively, according to an altered protein electrophoretic mobility, or quantitatively. Quantitative changes may represent mutation(s) within regulatory gene or control sequences affecting protein biosynthesis, or in diploid organisms indicate the mutation of a single allele for a structural gene (i.e. a 50% drop in protein intensity). Despite these successes it must be borne in mind that 2DE cannot identify all possible mutations and that the absence of changes in the 2D protein profiles does not necessarily mean that no mutations are present.

Apart from some studies of "simple" organisms (e.g. viruses) and selected studies with higher organisms, many of the mutations revealed by 2DE were in proteins of unknown function —this does not imply that the protein in question has not been characterised by some other means, rather that its identity has not yet been linked with a protein spot resolved by 2DE. In studies monitoring natural and induced mutation rates this is not a significant problem as the proteins included in the data analysis are selected on the basis of their reproducibility in detection and clarity in resolution. In many instances, however, there is a need to know the identity of the protein(s) in which the mutation(s) occur, for example in the determination of virulence factors in microorganisms or in following metabolic defects in eukaryotes. Over the past few years, significant progress has been made in determining the characteristics and identities of the proteins resolved by 2DE. For selected organisms and tissues, these data have been combined into comprehensive protein databases that contain information on many of the characteristics of the separated proteins from basic information on molecular mass and p*I* to more detailed information on tissue (or cellular) location and identity (see for example [142–145]). These relational databases will provide an important route to link the detected protein mutation to a protein of known identity and function [143]. A number of procedures have been used to determine the identity of proteins resolved by 2DE, including immunoblotting with monospecific antisera, co-electrophoresis with proteins of known identity and protein micro-sequencing. Protein micro-se-

quencing is the most generally applicable of these methods for abundant proteins. Once an amino acid sequence is determined it can be used to search a nucleotide sequence database to determine the identity of the protein [146,147]. If the protein of interest cannot be identified from a pre-existing sequence, the partial amino acid sequence can be used as the basis of developing a gene cloning strategy to identify the gene sequence and carry out fine structural analysis [148]. The combination of 2DE with recombinant DNA techniques to characterise protein mutations has been documented for a mutant β-actin gene identified in in vitro transformed human fibroblast cells ([149] and references therein).

The development of micro-sequencing techniques for proteins resolved by 2DE provides a link between 2DE and nucleic acid-based technologies for mutation analysis. Thus, gene coding sequences of either highly variable proteins (e.g. identified from the comparison of bacterial isolates) or mutated proteins, correlating with specific phenotypes, can be determined. Once the nucleotide sequence is known the sensitivity of PCR can be brought in to play to develop rapid assays to characterise and screen for further mutations within the gene coding sequence. 2DE may then be used to further characterise the protein on the basis of cell and tissue location as well as quantitative changes induced through external stimuli. In this manner one can move away from simply examining the alterations of protein "spots" in a 2D profile to an in-depth understanding of structure and function of the genes in question.

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