ELSEVIER

Journal of Chromatography B, 771 (2002) 251–260

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Separation and surveys of proteins of *Helicobacter pylori*

I. Nilsson*, M. Utt

Division of Bacteriology, Department of Medical Microbiology, Dermatology and Infection (MMDI), University of Lund, Solvegatan 23, S-223 62 Lund, Sweden

Abstract

The analysis of *Helicobacter pylori* proteins is a demanding task for the elucidation of virulence factors, antigens and vaccines, all important for diagnosis, therapy and protection. In the ''pre-genomic era'' the purification of proteins was mostly performed by using various techniques such as detergent treatment of the bacterial cells, ultra-centrifugation, various chromatographic methods, antibody detection, N-terminal sequence determination and finally cloning and identification of the corresponding gene. In this review, the most representative methods used for purification, separation and identification of *H. pylori* proteins will be presented as well as some important developments in the ''post-genomic era'' that have improved the performance of these characterisation techniques.

Keywords: Reviews; *Helicobacter pylori*; Proteomics; Proteins

Contents

*Corresponding author. Tel.: +46-46-173-237; fax.: +46-46-189-117.
E-mail address: ingrid.nilsson@mmb.lu.se (I. Nilsson).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00113-7

1. Historical perspective

1.1. A new era in gastric microbiology

For centuries the human stomach had been considered an inhospitable environment for bacterial growth because of its acidic pH. However, in 1983 B.J. Marshall and J.R. Warren [1] at the Royal Perth Hospital successfully cultured a spiral microaerophilic bacterium from the human gastric mucosa and demonstrated an association between the presence of this organism and gastric inflammation. The microbe was first named *Campylobacter pyloridis* (later corrected to *C. pylori)* but was later taxonomically placed in the genus *Helicobacter*, which now consists of more than 20 species including non-gastric *Helicobacter* species. A new era in gastric microbiology started and observations from laboratories in Europe, USA, Canada and Asia rapidly appeared in scientific journals, although there was still considerable scepticism about the pathogenic capacity of this microbe. Langenberg et al. [2] reported on the ability of *Helicobacter pylori* to produce high quantities of urease in bacterial cultures, providing an explanation for the urease activity observed in mammalian stomachs some 60 years earlier [3]. The ability to hydrolyse urea is an important characteristic of *H. pylori*, as well as of other *Helicobacter* species that colonise the gastric mucosa of different mammals.

1.2. A world-wide infection

The discovery that *H. pylori* causes acute and chronic type-B gastritis initiated interest in research and epidemiological studies were undertaken to study the prevalence and incidence of *H. pylori* infection in developed and developing countries as well as its relationship to age, socio-economic class, and ethnic background. *H. pylori* causes gastric infection in more than half of the human population worldwide and in >80% of populations from developing countries. The prevalence of *H. pylori* in gastric ulcer disease is >90% and cure of the infection results in cure of gastric ulcer disease. Food additives (chemicals), nitrates, environmental and host genetic factors also contribute to the gastritis and peptic ulcer disease [4].

2. Methods used for isolation and identification of major virulence factors and immunogenic proteins common to all *H. pylori* strains

2.1. Urease

H. pylori urease is a well-defined virulence protein, which has been purified to homogeneity. It is an enzyme consisting of two sub-units, UreA (M_r 26 500) and UreB (M_r 61 600).

Dunn et al. [5] purified surface associated urease starting with water extraction of bacterial cells followed by size exclusion chromatography, and anion-exchange chromatography. The kinetics, estimation of M_r for the native enzyme and subunits and p*I* were determined. Partial amino-terminal sequence of the two subunits showed similarities to urease found in plants and other bacteria [6]. Hawtin et al. [7] investigated the structure and localisation by using monoclonal antibodies. By cryo-immuno-electron microscopy Phadnis et al. [8] showed that urease was almost exclusively found within the cytoplasm in fresh log-phase cultures, but a significant fraction of the enzyme was localised on the outer membrane in sub-cultured preparations by comparing the enzyme sensitivity to flurofamide, a potent urease inhibitor. It was suggested that *H. pylori* cells could undergo spontaneous autolysis during culture and the urease would then become surface-associated due to this autolysis.

For characterisation of the intracellular urease, Hu and Mobley [9] used whole cells that were ruptured by French pressure cell lysis, and soluble proteins were chromatographed on DEAE-Sepharose, phenyl-Sepharose, MonoQ and Superose 6 resins. The native polymer consists of six of each sub-unit with an M_r of 550 000 containing two Ni^{2+} ions per native molecule as an essential cofactor for enzymatic activity. The p*I* of the native enzyme was estimated between 6.0 and 6.3 and the K_m value was found to be 0.2 m*M*. They also demonstrated that urease subunits of other bacterial species were recognised by antiserum, raised against the 66 000-M_r subunit of *H. pylori* urease, indicating that some antigenic determinants are conserved among ureases from different species. Furthermore, the urease gene cluster has been cloned and the *ureA* and *ureB* genes sequenced [10], and recombinant proteins have been produced and purified [11].

2.2. Flagella

Flagellar motility has been shown to be essential for the ability of *H. pylori* to spread in the gastric mucus layer and colonise the gastric epithelium [12]. This motility is caused by four to six sheathed flagella with filaments consisting of two flagellin types, encoded by genes *flaA* and *flaB* and both are essential for full motility [13]. Luke and co-workers [14] purified the flagella by suspending *H. pylori* cells in PBS followed by homogenisation and centrifugation. The supernatant was treated with a detergent buffer (Triton X-100, EDTA, Tris). The detergent supernatant mixture was centrifuged at high speed and the purified protein used to prepare a mono-specific antiserum. By SDS–PAGE a 54 000-M_r protein was observed and also shown by transmission electron microscopy with immunogold labelled antibodies. Two flagellin species in *H. pylori* were characterised [15] by using various techniques for purification, including CsCl density gradient ultra-centrifugation, and differential ultra-centrifugation followed by molecular sieving with a Sephacryl S-500 column or MonoQ anion-exchange column, isoelectric focusing (IEF) and SDS–PAGE. Two flagellin species of p*I* 5.2 and with apparent subunits of 57 000 and 56 000 M_r were obtained. The minor 57 000-M_r species was located proximal to the hook, and the major 56 000-M_r flagellin composed the remainder of the filament. By immunoelectron microscopy with poly- and monoclonal antibodies two different flagellin species were shown to be located in different regions of the flagellar filament.

H. pylori also possess flagellar hooks, composed of flgE subunits. Mutants, defective in hook production, are non-motile and aflagellate despite the production of both flagellin subunits as reported by O'Toole et al. [16].

Geis et al. [17] reported on a flagellar sheath covering the flagella, possibly protecting the bacterium from the acid pH in the gastric milieu. Sheaths were isolated by sucrose density-gradient centrifugation and examined by electron microscopy and SDS–PAGE. Major fatty acids and lipopolysaccharide-specific fatty acids were analysed by gas–liquid chromatography. Using these methods they were able to demonstrate that the flagellar sheaths of *H. pylori* are membranes containing lipopolysaccharides and proteins. Luke and Penn [18] identified a flagellar sheath protein by one- and two-dimensional electrophoretic analysis (1-DE, 2-DE) and immunoblotting. A murine monoclonal antibody reacted with a 29 000-M_r protein used in immunogold-electron microscopy. This antigen was detected in 11 strains of diverse geographic origin and found to be distinct from the abundant urease subunit UreA, of similar molecular mass.

2.3. Cytotoxins

Mucosal injury depends on the release of several bacterial products and virulence factors, e.g. cytotoxins. *H. pylori* comprise two different types of isolates, cytotoxic (type I) and non-cytotoxic (type II). Type I strains produce a vacuolating cytotoxin (VacA) [19], whereas type II strains do not. Almost all type I strains possess a gene coding for cytotoxin-associated gene A (CagA), a highly immunogenic, high M_r surface protein not present in Type II strains [20].

2.4. Vacuolating cytotoxinA (VacA)

All *H. pylori* isolates contain the VacA toxin gene, but it is not always expressed. The toxin induces cytoplasmic vacuolation in mammalian cell lines in vitro [21–23]. VacA is an oligomeric toxin composed of 95 000-M_r monomers, proteolytically cleaved into 37 000- and 58 000-M_r fragments (P37 and P58). The ability to induce vacuoles is localised mostly but not entirely in P37, whereas P58 is mostly involved in cell targeting [24]. The toxin is activated by its exposure to pH values below 5.5. At low pH, the monomerisation of the toxin occurs with exposure of hydrophobic segments most likely responsible for VacA penetration into the lipid bilayer and forming ion selective channels [25,26]. Immunogenic proteins are produced by toxigenic strains as observed by immunoblotting using sera from *H. pylori* infected patients with various clinical diagnoses [21]. It has been suggested that cytotoxin-positive strains are associated with increased pathological abnormalities, including the presence of polymorphonuclear leucocytes, as a sign of inflammation and tissue damage in contrast to less severe tissue damage in patients harbouring toxin-negative strains. Cover and co-workers [27] isolated the VacA toxin from Brucella broth cultures of two control strains and 28

clinical isolates. Cultures were centrifuged to get cell-free supernatants and after a concentration step the retentates were sterilised by passage through a 0.22-μm pore size filter and incubated with HeLa cells. Culture supernatants from 15 of the 28 (53.6%) clinical isolates tested produced vacuolisation monitored by crystal violet staining.

Native VacA was purified from culture supernatants after ammonium sulphate precipitation and chromatography on Matrex Cellufine Sulphate at pH 6.5 followed by gel filtration on Superose 6 [28]. Yahiro et al. [29] used chromatography on hydroxyapatite, Superose 6HR 10/30 and ResourceQ to purify the toxin from culture supernatant precipitated with ammonium sulphate. Reyrat et al. [30] utilised a one-step purification of the VacA toxin from the culture supernatant using monoclonal affinity chromatography. In most studies the vacuolisation of mammalian cell lines was scored visually under a phase-contrast microscope or by Giemsa-staining. The activity of purified toxin was investigated by neutral red uptake and vacuole formation or by serial dilutions of the toxin applied to the cell line.

2.5. Cytotoxin-associated protein (CagA)

The cytotoxin-associated protein is an immunodominant antigen and a specific immunogenic marker of diseases caused by *H. pylori*. It resides within the *cag* pathogenicity island (PAI). Several genes in the *cag*-PAI code for proteins involved in the induction of a pro-inflammatory cytokine, IL-8, from the gastric epithelial cells [31]. Israel et al. [32] described, in an animal model, by a whole genome micro array, that the ability to regulate an inflammatory response of epithelial cells was dependent on an intact *cag*-PAI. Furthermore, alterations in activity of reactive oxygen species-scavenging enzymes by CagA-positive *H. pylori* strains may further increase the already elevated risk of gastric cancer in infected persons [33].

In a study by Gerstenecker et al. [34] the CagA protein was purified by subjecting CHAPS-solubilised cells to a size exclusion chromatography in the presence of low concentrations of sodium dodecylsulphate (SDS). Fractions, already defined by SDS–PAGE and immunoblot, containing the 120 000-M_r protein were once again subjected to chromatography using identical conditions. The purified antigen was used for *H. pylori* antibody detection in an enzyme immunoassay (EIA) and results compared with results of a whole cell EIA. The specificity was found to be much higher for the purified 120 000-M_r antigen (92%) compared to the sonicate EIA (60.7%). Both EIAs showed a high sensitivity. They concluded that the specificity could be increased by further purification of the 120 000-M_r polypeptide using alternative solubilisation methods and multidimensional chromatography.

A recombinant fragment (a fusion protein) of the CagA protein was constructed by Xiang et al. [35] and used in an EIA and immunoblot for screening of sera from patients with gastro duodenal disease. Data from this study indicated that the recombinant protein was a reliable antigen for detection of *H. pylori* infections (specificity 96.6%, sensitivity 96.2%). A DNA fragment of the *cagA* gene, extracted from a supernatant of 6 *M* guanidine–HCl treated cells containing an enriched fraction of the 128 000-M_r protein, was digested and sub cloned. The recombinant protein was purified from inclusion bodies by suspending in a buffer (Tris–HCl, urea, EDTA, and DTT), loaded on a Q-Sepharose column and then passed over to a column of carboxymethyl-Sepharose using buffers with low pH (3.5–4.8).

In a study by Cover et al. [36] the recombinant CagA was purified by sequential column chromatography (MonoQ 5/5 anion-exchange column and Superose 6 10/50 column) and the CagA fraction was used for immunisation. A rabbit anti-CagA antibody detected by immunoblot the expression of CagA by *H. pylori* strains. Sera from patients infected with *cagA*$^+$ strains reacted with recombinant CagA antigen in an EIA to a significantly greater extent than either sera from patients infected with strains lacking *cagA* or sera from uninfected persons. Data demonstrated an association between infections with *cagA*$^+$ *H. pylori* and the presence of duodenal ulceration and that serologic testing with CagA was a sensitive method for detecting infection with *cagA*$^+$ strains.

2.6. Heat shock proteins

Heat shock proteins (Hsps) are highly conserved immunogenic molecules [37]. Increased synthesis of

these proteins occurs in response to many environmental stresses. *H. pylori* produce at least two Hsps, the HspA (13 000-M_r) sharing homology at the amino acid level with the GroES family and HspB (54 000-M_r) identified to be a homologue of the class belonging to the GroEL proteins. The HspA was found to possess a nickel-binding domain and most probably to play a role in the integration of nickel into the urease molecule [38]. It has been suggested that Hsps might be involved in the pathogenesis of *H. pylori*-related diseases through the induction of an autoimmune response [39,40].

Purification and identification of a major surface-exposed protein (54 000-M_r) in *H. pylori* was performed by Dunn et al. [41]. Proteins were recovered from water extracts of whole cells followed by co-purification with urease by size exclusion chromatography and then separated from urease by anion-exchange chromatography. The Hsp54 was identified as a chaperonin 60 homologue on the basis of its N-terminal amino acid sequence and its cross-reactivity with monospecific antibodies against a variety of known chaperonin 60 homologues. Evans et al. [42] purified a 62 000-M_r protein that was recognised by a monoclonal antibody against Hsp65 common antigen of *Mycobacterium leprae* and Austin et al. [43] purified a protein termed Hp60K. However, based on N-terminal amino acid sequences, all appeared to represent the same protein. It was also reported that this major antigen of all *H. pylori* strains induces a strong antibody response in humans infected with *H. pylori* [41,44].

Ferrero and co-workers [45] constructed recombinant HspA, HspB and UreB proteins of *H. pylori* and *Helicobacter felis* to investigate if these proteins were able to induce protective immunity in mice. Fusion proteins were purified from culture lysates by affinity and anion-exchange chromatography and the purity of the recombinant protein preparations was analysed by SDS–PAGE and immunoblotting. The authors reported that a protein (HspA) belonging to the GroES class of Hsps induced immunity and could be a potential component of a future vaccine.

2.7. Neutrophil activating protein (HP-NAP)

H. pylori infection elicits an inflammatory cell response, and the severity of mucosal injury appears to be directly correlated with the extent of neutrophil infiltration [46]. Evans et al. [47] released the HP-NAP from the bacterial cells by water washing and following two-step gel-filtration chromatography using agarose and Superdex-200 columns. The most active fractions were pooled and applied to a MonoQ ion-exchange column and one major protein (15 000-M_r) was obtained. By immunoblot, using an antiserum from an immunised rabbit, it was shown that this protein was immunogenic and its pro-adhesive capacity was demonstrated using a neutrophil adhesion assay. An important role of HP-NAP in immunity was suggested by Satin et al. [48] who found that vaccination of mice with this protein induced protection against *H. pylori* challenge (80%).

3. Post-genomic era and proteomics

Proteomics is a development from two-dimensional gel electrophoresis (2-DE) of proteins. The ‘‘process’’ of proteomics involves the characterisation of proteins from either whole cells or sequential extraction to fractionate proteins in terms of cellular location. 2-DE is usually performed on these preparations using immobilised pH gradients in the first dimension to separate proteins on the basis of their p*I* followed by SDS–PAGE (second dimension) that subsequently separates proteins on the basis of their size (Fig. 1).

Obtained protein profiles are analysed and documented using image analysis software, and may be further characterised using techniques such as peptide-mass fingerprinting mass spectroscopic or N-terminal sequencing. Proteomics can easily provide data on expression levels, subcellular location, post-translational modifications, protein structure and domains [49].

3.1. New strategies for protein identification

Since the first complete bacterial genome (*Haemophilus influenzae* Rd.) was reported in 1995 [50] protein identification strategies have changed remarkably. From November 2001, 53 complete bacterial genomes can be found at Entrez Genomes (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/eub_g.html) and 74 more are in progress.

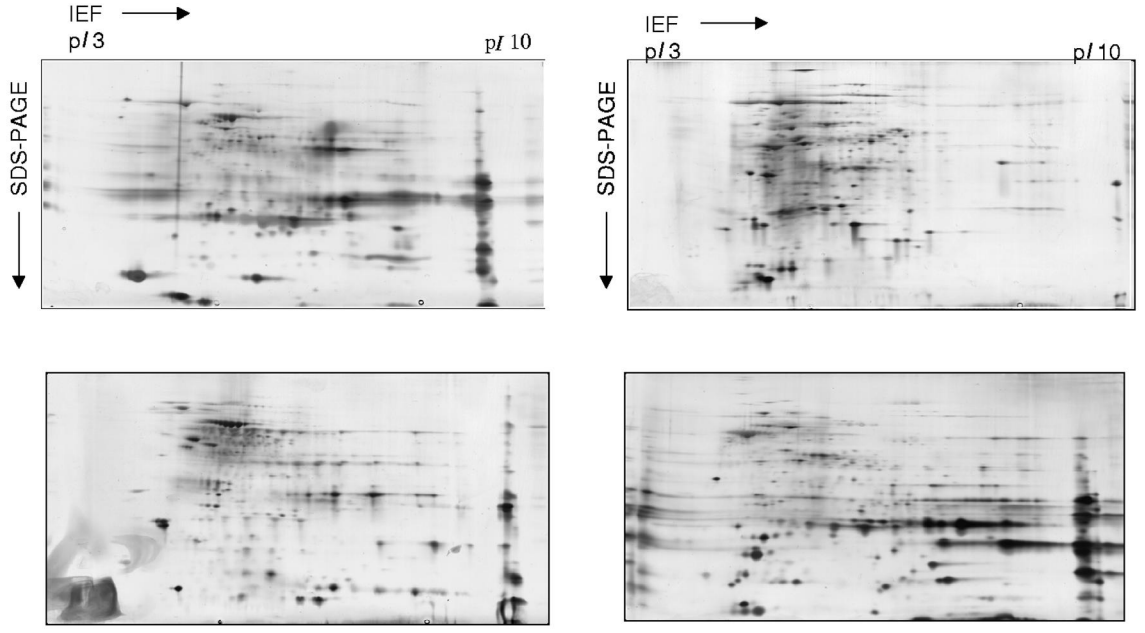

Fig. 1. 2-DE profiles illustrating *Helicobacter* proteins extracted by various methods resolved by IEF in the pH range 3–10 followed by SDS–PAGE gradient gel (8–16%) and stained by silver.

Bioinformatics, which compares new sequence data with those already available in databases, may now be performed for an initial identification. Based on different analysis and comparisons, similarities can be found and a function proposed for a new sequence.

A second feature is the separation of bacterial whole cell proteins or a fraction of it by 2-DE and final identification with mass spectroscopic analysis using peptide fingerprints and MS–MS sequence data, which may then be used to search in databases for identification and function prediction. This has become possible due to the development of immobilised pH gradients [51] (broad and narrow range) and achievements in mass spectroscopy, particularly in MALDI–TOF-MS and ESI-MS technology. Automation of spot cutting and workstations for automatic tryptic digestion for MS–MS analysis are already on the market, meaning there will be less or no ''hands on'' after the 2-DE gel is developed. Furthermore, accurate models and bioinformatic tools for better prediction have been developed.

3.2. Chromatographic fractionation and enrichment

Many proteins in the cell have a low copy number but might carry important function or strong immunogenic determinants. These proteins will probably not be detected in stained gels of a whole cell lysate. Chromatographic fractionation and enrichment helps overcome the above problem. Chromatography was used in combination with 2-DE for enrichment of less abundant proteins of *H. influenzae*. Heparin affinity chromatography was used and 110 novel proteins were identified [52]. Another chromatographic matrix (hydroxyapatite) was used for enrichment of less abundant proteins of *Escherichia coli* [53]. Butt et al. [54] used MonoQ anion-exchange chromatography to enrich proteins of *E. coli* prior to 2-DE and this purification step increased the presence of some proteins 13-fold. In addition, peptide fingerprint MS spectra were improved. Utt et al. [55] used heparin affinity chromatography fractionation and narrow pH gradients in

the basic region 6–11, for enrichment of less abundant proteins of *H. pylori*. For the first time a hypothetical protein (HP0231) was shown to bind to heparin and its immunogenic properties were also demonstrated.

3.3. Genome of H. pylori

The complete genome of *H. pylori* strain 26695 was published in Nature in August 1997 [56]. It has 1 667 867 base pairs and 1590 predicted coding sequences. A second complete genome of strain J99 was released in 1999 and both are available at www.tigr.org. Comparison of the two genomes revealed differences in sequence and in the number of coding regions [57].

More than 70% of predicted proteins in *H. pylori* have a calculated p*I* higher than pH 7 compared to ~40% in *H. influenzae* and *E. coli* [56]. Today, 549 annotated entries can be found in the SWISSPROT and SWISSNEW databases (Table 1), including 78 hypothetical and 19 putative proteins. Many of these proteins were first identified by comparison of nucleotide and amino acid sequences in databases with known protein sequences, which allowed a putative function to be proposed for the new protein. These types of approaches will be more extensively used and can be applied to sequenced genomes of recently discovered and potentially pathogenic microbes, e.g. *Helicobacter hepaticus* [58].

3.4. 2-DE and 2-DE immunoblotting of H. pylori proteins

2-DE has been applied to study *H. pylori* proteins from several aspects, including the evaluation of different stress responses. Slonczewski and co-workers [59] used 2-DE in combination with N-terminal sequence analysis to study pH stress of *H. pylori* and protein expression. Urease expression was found to increase in growing cells at pH 5.7 compared to pH 7.5. Interestingly, expression of the stress chaperons GroES (HP0011) and GroEL (HP0010) was shown to increase at pH 7.5, compared to growth at pH 5.7. The TsaA protein (HP1563), responsible for detoxification of radicals and perhaps, also other toxic molecules in cell cultures with pH 7, was up-regulated, whereas the homologue AhpC from *E. coli* is up-regulated in acidic conditions. In a study by Nilsson et al. [60] cell surface protein profiles of the spiral and coccoid forms of *H. pylori* were compared. The surface proteins of the two forms were released by acid glycine (pH 2.2) treatment. Differences in the expression of resolved polypeptides were demonstrated in the 2-DE silver-stained gels mainly on the acidic side. However, the antibody reactivity to antigens of the two morphological forms, analysed by immunoblot, was found to be similar.

Defining surface proteins of the human gastric pathogen *H. pylori* is of great interest as several of these proteins are candidates for optimising immunodiagnostic tests, such as immunoblot assays (Fig. 2), and for vaccine design. 2-DE in combination with antibody detection and N-terminal amino acid sequence analysis of reacting proteins, was used for the identification of *H. pylori* antigens [55,61,62]. Jungblut et al. [63] studied *H. pylori* whole cell proteins extensively by 2-DE and 152 proteins were identified by MS. This 2-DE map is available at http://www.mpiib-berlin.mpg.de/2D-PAGE/. For immunoblotting a single patient serum was used and antibody reactivity to a relatively small number of immunogenic proteins was recorded. These results suggest that several antigens might be minor components in whole cell lysates and therefore difficult to detect without enrichment methods.

Attempts have been made to identify disease

Table 1
Information of *H. pylori* protein sequences (strain 26695 and strain J99) can be found on URL databases

http://www.tigr.org./tigr-scripts/CMR2/GenomePage3.spl?database=ghp
http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=nthp01
http://www.mpiib-berlin.mpg.de/2D-PAGE/EBP-PAGE/index.html
http://srs.embl-heidelberg.de8000/srs5/

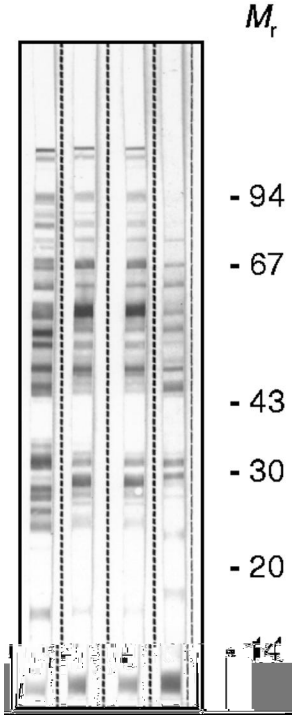

Fig. 2. Immunoblot strips probed with sera from *H. pylori* infected patients.

specific marker proteins [62,64,65]. 2-DE and immunoblot analysis of such proteins were performed by Enroth et al. [66] using *H. pylori* clinical isolates from patients with gastric cancer, duodenal ulcer and gastritis. Variability in the 2-DE protein profiles and antibody reactivity was observed between *H. pylori* strains isolated from the different patient groups. Eight variable proteins were N-terminal sequenced and the authors suggest the presence of disease specific proteins for each clinical group that could be of diagnostic interest and concluded that variation exists between *H. pylori* strains at the protein level.

Chakravarti et al. [67] used proteomic and genomic approaches for identification of potential vaccine candidates of *H. pylori*. An outer membrane fraction, purified from disrupted cells, was initially treated with Triton X-100, and following centrifugation treated with a detergent (Zwittergent) followed again by centrifugation. Proteins separated by 1-D SDS–PAGE were transferred to PVDF membrane and those reacting against monoclonal antibodies were N-terminal sequenced and identified by database search. A second approach was also used. Outer membrane proteins were separated by 2-DE and transferred to PVDF membrane. Spots were trypsin-digested and extracted peptides analysed by MALDI–TOF-MS. These approaches in combination with bioinformatics and genomic methods have been described as general routes for antigen discovery.

4. Summary

Sample fractionation and enrichment of proteins using a chromatographic step prior to IEF and 2-D electrophoresis improves the possibility of identifying proteins present at a low concentration. It may also improve the ratio of immunogenic versus non-immunogenic proteins in a complex antigen preparation. In some studies, broad pH gradients have been used to separate *H. pylori* proteins [60–63,66,68] and the basic proteins, which are prevalent in *H. pylori,* might have been resolved to a lower degree. Thus, isoelectric focusing using an appropriate pH-gradient permits a higher resolution of several proteins.

By 2-DE-immunoblotting it is possible to identify specific immunogenic proteins as well as evaluate cross-reacting proteins and/or antibodies, e.g. when complex antigens are used in diagnostic tests, a more precise identification of immunogenic proteins will be necessary, for example for production of recombinant proteins, either using classical N-terminal mi-

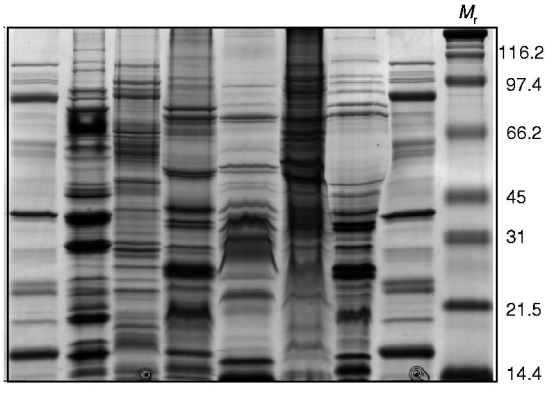

Fig. 3. 1-D SDS–PAGE showing profiles of *Helicobacter* proteins extracted by different methods and separated in an 8–16% gradient gel followed by CBB staining.

crosequencing or more advanced MS–MS sequencing.

Furthermore, the 2-DE technique has facilitated identification of proteins in a complex antigen to a higher degree than proteins separated by 1-DE (Fig. 3) and also overcomes the problem of co-migrating proteins which is common with 1-DE. Bioinformatics and functional proteomic approaches facilitate the investigation of *H. pylori* proteins that have pathological implications.

References

[1] B.J. Marshall, J.R. Warren, Lancet i (1984) 1311.
[2] M.-L. Langenberg, G.N.J. Tytgat, M.E.I. Schipper, P.J.G.M. Rietra, H.C. Zanen, Lancet ii (1984) 1348.
[3] J.M. Luck, T.N. Seth, Biochem. J. 18 (1924) 1227.
[4] H.L.T. Mobley, in: H.L.T. Mobley, G.L. Mendz, S.L. Hazell (Eds.), *Helicobacter pylori* Physiology and Genetics, ASM Press, Washington, 2001, p. 397, Chapter 34.
[5] B.E. Dunn, G.P. Campbell, G.I. Perez-Perez, M.J. Blaser, J. Biol. Chem. 265 (1990) 9464.
[6] H.L.T. Mobley, in: H.L.T. Mobley, G.L. Mendz, S.L. Hazell (Eds.), *Helicobacter pylori* Physiology and Genetics, ASM Press, Washington, 2001, p. 179, Chapter 16.
[7] P.R. Hawtin, A.R. Stacey, D.G. Newell, J. Gen. Microbiol. 136 (1990) 1995.
[8] S.H. Phadnis, M.H. Parlow, M. Levy, D. Ilver, C.M. Caulkins, J.B. Connors, B.E. Dunn, Infect. Immunol. 64 (1996) 905.
[9] L.T. Hu, H.L. Mobley, Infect. Immunol. 58 (1990) 992.
[10] A. Labigne, V. Cussac, P. Courcoux, J. Bacteriol. 173 (1991) 1920.
[11] L.T. Hu, P.A. Foxall, R. Russell, H.L. Mobley, Infect. Immunol. 60 (1992) 2657.
[12] A. Labigne, H. de Reuse, Infect. Agents Dis. 5 (1996) 191.
[13] C. Josenhans, A. Labigne, S. Suerbaum, J. Bacteriol. 177 (1995) 3010.
[14] C.J. Luke, E. Kubiak, A. Cockayne, T.S.J. Elliot, C.W. Penn, FEMS Microbiol. Lett. 71 (1990) 225.
[15] M. Kostzrynska, J.D. Betts, J.W. Austin, T. Trust, J. Bacteriol. 173 (1991) 937.
[16] P.W. O'Toole, M. Kostrzynska, T. Trust, Mol. Micobiol. 14 (1994) 691.
[17] G. Geis, S. Suerbaum, B. Forsthoff, H. Leying, W. Opferkuch, J. Med. Microbiol. 38 (1993) 371.
[18] C.J. Luke, C.W. Penn, Microbiology 141 (1995) 597.
[19] J.L. Telford, P. Ghiara, M. Dellórco, M. Commanducci, D. Burroni, M. Bugnoli, M.F. Tecce, S. Cencini, A. Covacci, Z. Xiang, J. Exp. Med. 179 (1994) 1653.
[20] A. Covacci, S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, Proc. Natl. Acad. Sci. USA 90 (1993) 5791.
[21] S.H. Phadnis, D. Ilver, L. Janzon, S. Normark, U.T. Westblom, Infect. Immunol. 62 (1994) 1557.
[22] S. Kamiya, M. Kai, A. Ozawa, H. Kobayashi, T. Shirai, S. Harasawa, T. Miwa, Eur. J. Gastroenterol. Hepatol. 6 (1994) 23.
[23] T.L. Cover, Mol. Microbiol. 20 (1996) 241.
[24] J.M. Reyrat, V. Pelicic, E. Papini, C. Montecucco, R. Rappuoli, J.L. Telford, Mol. Microbiol. 34 (1999) 197.
[25] M. Molinari, C. Galli, M. de Bernard, N. Norais, J.M. Ruysschaert, R. Rappuoli, C. Montecucco, Biochem. Biophys. Res. Commun. 248 (1998) 334.
[26] C. Montecucco, E. Papini, M. de Bernard, M. Zoratti, FEBS Lett. 452 (1999) 16.
[27] T.L. Cover, C.P. Dooley, M.J. Blaser, Infect. Immunol. 58 (1990) 58603.
[28] R. Manetti, P. Massari, D. Burroni, M. de Bernard, A. Marchini, R. Olivieri, E. Papini, C. Montecucco, R. Rappuoli, J.L. Telford, Infect. Immunol. 63 (1995) 4476.
[29] K. Yahiro, T. Niidome, T. Hatakeyama, H. Aoyagi, H. Kurazono, P.I. Padilla, A. Wada, T. Hirayama, Biochem. Biophys. Res. Commun. 238 (1997) 629.
[30] J.M. Reyrat, M. Charrel, C. Pagliaccia, D. Burroni, P. Lupetti, M. de Bernard, X. Ji, N. Norais, E. Papini, R. Dallai, R. Rappuoli, J.L. Telford, FEMS Microbiol. Lett. 165 (1998) 79.
[31] C. Audibert, B. Janvier, B. Grignon, L. Salaun, C. Burucoa, J.C. Lecron, J.L. Fauchere, Res. Microbiol. 151 (2000) 191.
[32] D.A. Israel, N. Salama, C.N. Arnold, S.F. Moss, T. Ando, H.P. Wirth, K.T. Tham, M. Camorlinga, M.J. Blaser, S. Falkow, R.M. Peek, J. Clin. Invest. 107 (2001) 611.
[33] D.T. Smoot, T.B. Elliot, H.W. Verspaget, D. Jones, C.R. Allen, K.G. Vernon, T. Bremner, L.C. Kidd, J.D. Groupman, H. Ashktorab, Carcinogenesis 21 (2000) 2091.
[34] B. Gerstenecker, B. Eschweiler, H. Vögele, H.K. Koch, U. Hellerich, M. Kist, Eur. J. Clin. Microbiol. Infect. Dis. 11 (1992) 595.
[35] Z. Xiang, M. Bugnoli, A. Ponzetti, A. Morgando, N. Figura, A. Covacci, R. Petracca, C. Pennatini, S. Cencini, D. Armellini, R. Rappuoli, Eur. J. Clin. Microbiol. Infect. Dis. 12 (1993) 739.
[36] T.L. Cover, Y. Glupczynski, A.P. Lage, A. Burette, M.K.R. Tummuru, G.L. Perez-Perez, M.J. Blaser, J. Clin. Microbiol. 33 (1995) 1496.
[37] I. Kansau, A. Labigne, Aliment. Pharmacol. Ther. 10 (1996) 51.
[38] S. Suerbaum, J.M. Thiberge, I. Kansau, R.L. Ferrero, A. Labigne, Mol. Microbiol. 1 (1994) 959.
[39] J. Winfield, W. Jarjour, Immunol. Rev. 121 (1991) 193.
[40] R. Negrini, L. Lisato, I. Zanella, L. Cavazzini, S. Gullini, V. Villanacci, C. Poiesi, A. Albertini, S. Ghielmi, Gastroenterology 101 (1991) 437.
[41] B.E. Dunn, R.M. Roop II, C.C. Sung, S.A. Sharma, G.I. Perez-Perez, M.J. Blaser, Infect. Immunol. 60 (1992) 1946.
[42] D.J. Evans, D.G. Evans, L. Engstrand, D.Y. Graham, Infect. Immunol. 60 (1992) 2125.
[43] J.W. Austin, J.W.P. Doig, M. Stewart, T.J. Trust, J. Bacteriol. 174 (1992) 7470.

[44] B.E. Dunn, G.I. Perez-Perez, M.J. Blaser, Infect. Immunol. 57 (1989) 1825.
[45] R.J. Ferrero, T. Jean-Michel, K. Imada, M. Wuscher, M. Huerre, A. Labigne, Proc. Natl. Acad. Sci. USA 92 (1995) 6499.
[46] P.M. Craig, M.C. Territo, W.E. Karnes, J.H. Walsh, Gut 33 (1992) 1020.
[47] D.J. Evans, D.G. Evans, T. Takemura, H. Nakano, H.C. Lampert, D.Y. Graham, D.N. Granger, P.R. Kvietys, Infect. Immunol. 63 (1995) 2213.
[48] B. Satin, G. DelGiudice, V.D. Bianca, S. Dusi, C. Laudanna, F. Tonello, D. Kelleher, R. Rappuoli, C. Montecucco, F. Rossi, J. Exp. Med. 191 (2000) 1467.
[49] T. Rabilloud, M. Chevallet, in: T. Rabilloud (Ed.), Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods, Springer, Berlin, 2000, p. 9, Chapter 2.
[50] R.D. Fleischmann, M.D. Adams, O. White et al., Science 269 (1995) 496.
[51] A. Görg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, Electrophoresis 21 (2000) 1037.
[52] M. Fountoulakis, H. Langen, S. Evers, C. Gray, B. Takacs, Electrophoresis 18 (1997) 1193.
[53] M. Fountoulakis, M.F. Takacs, P. Berndt, H. Langen, B. Takacs, Electrophoresis 20 (1999) 2181.
[54] A. Butt, M.D. Davison, G.J. Smith, J.A. Young, S.J. Gaskell, S.G. Oliver, R.J. Beynon, Proteomics 1 (2001) 42.
[55] M. Utt, I. Nilsson, Å. Ljungh, T. Wadström, J. Immunol. Methods 259 (2002) 1.
[56] J.F. Tomb, O. White, A.R. Kerlavage et al., Nature 388 (1997) 539.
[57] R.A. Alm, L.S. Ling, D.T. Moir, B.L. King, Nature 14 (97(67135)) (1999) 176.
[58] S. Suerbaum, C. Josenhans, M. Frosch et al., Int. J. Med. Microbiol. 291 (Suppl. 31) (2001) M-17.
[59] J.L. Slonczewski, D.J. McGee, J. Phillips, C. Kirkpatrick, H.L. Mobley, Helicobacter 5 (2000) 240.
[60] I. Nilsson, M. Utt, H.-O. Nilsson, Å. Ljungh, T. Wadström, Electrophoresis 21 (2000) 2670.
[61] C.P. McAtee, M.Y. Lim, K. Fung, M. Velligan, K. Fry, T. Chow, D.E. Berg, Clin. Diagn. Lab. Immunol. 5 (1998) 537.
[62] B. Kimmel, A. Bosserhoff, R. Frank, R. Gross, W. Goebel, D. Beier, Infect. Immunol. 68 (2000) 915.
[63] P.R. Jungblut, D. Bumann, G. Haas, U. Zimny-Arndt, P. Holland, S. Lamer, F. Siejak, A. Aebischer, T.F. Meyer, Mol. Microbiol. 36 (2000) 710.
[64] Y. Yamaoka, T. Kodama, D.Y. Graham, K. Kashima, Dig. Dis. Sci. 43 (1998) 1482.
[65] J.T. Wang, C.S. Chang, C.Z. Lee, J.C. Yang, J.T. Lin, T.H. Wang, Biochem. Biophys. Res. Commun. 244 (1998) 360.
[66] H. Enroth, T. Akerlund, A. Sillen, L. Engstrand, Clin. Diagn. Lab. Immunol. 7 (2000) 301.
[67] D.N. Chakravarti, M.J. Fiske, L.D. Fletcher, R.J. Zagursky, Vaccine 19 (2001) 601.
[68] C.P. McAtee, K. Fry, D.E. Berg, Helicobacter 3 (1998) 163.