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# Electrophoretic Analysis of Total and Membrane Proteins of *Xanthomonas campestris* Pathovars, the Causal Agents of the Leaf Streak of Cereals and Grasses in Iran

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With 2 figures

**Abstract**

Forty-five Iranian isolates of *Xanthomonas campestris* obtained from wheat, barley and grasses were compared with reference strains using polyacrylamide gel electrophoresis (PAGE) of the whole-cell and membrane proteins.

The PAGE profiles of the whole-cell and membrane proteins of the Iranian isolates obtained from barley, with the exception of IBLS 11 and IBLS 12, were identical and clearly distinguishable from those of the other isolates. The barley group isolates, which were pathogenic only to barley, were similar to UPB 458 (NCPPB 2389), the reference strain of pathovar *hordei*. The isolates obtained from wheat and grasses, as well as IBLS 11 and IBLS 12, which can infect wheat, barley and some wild grasses, had similar banding patterns; only IBLS 40 isolated from *Hordeum* sp. displayed the same profile as the barley group. Reference strains UPB 443 (NCPPB 2821) and UPB 513, which correspond to pathovars *undulosa* and *translucens*, respectively, were related to the wheat group. IBLS45, isolated from *Bromus* sp., had a banding pattern that differed from those observed for strains of the barley and wheat groups.

The results suggest that this method can be useful for discriminating different pathovars of *X. campestris* attacking cereals and grasses, and sodium dodecyl sulphate (SDS)–PAGE of membrane proteins was not more sensitive than SDS–PAGE of total proteins for differentiating the isolates.

**Zusammenfassung**

**Elektropboretische Analyse der Gesamtproteine und der Membranproteine von *Xanthomonas campestris*-Pathovaren, den Erregern der Blattstreifigkeit von Getreide und Gräsern im Iran**

Fünfundvierzig von Weizen, Gerste und Gräsern stammende iranische *Xanthomonas campestris*-Isolate wurden mit Hilfe der Polyacrylamid-Gelelektrophorese (PAGE) der gesamten Zellproteine und der Membranproteine mit Referenzstämmen verglichen.

Mit Ausnahme von IBLS 11 und IBLS 12 waren die PAGE-Profile der gesamten Zellproteine und der Membranproteine der iranischen Isolate von Gerste identisch und zeigten deutliche Unterschiede zu den Profilen der anderen Isolate. Die nur für Gerste pathogenen Isolate der Gerstengruppe glichen UPB 458 (NCPPB 2389), dem Referenzstamm der Pathovar *hordei*. Die von Weizen und Gräsern gewonnenen Isolate und auch IBLS 11 und IBLS 12, die Weizen, Gerste und einige Wildgräser befallen können, zeigten ähnliche Bandenmuster: nur der von *Hordeum sp.* isolierte IBLS 40 wies das gleiche Profil wie die Isolate der Gerstengruppe auf. Die Referenzstämme UPB 443 (NCPPB 2821) und UPB 513, die den Pathovaren *undulosa* bzw. *translucens* entsprechen, waren mit der Weizengruppe verwandt. Der von *Bromus sp.* isolierte IBLS 45 zeigte ein Bandenmuster, das sich von denen der Stämme der Gersten- und der Weizengruppe unterschied.

Die Ergebnisse legen nahe, daß dieses Verfahren zur Unterscheidung verschiedener, Getreide unf Gräser befallender *X. campestris*-Pathovare von Nutzen sein kann. Bei der Unterscheidung der Isolate war die SDS–PAGE der Membranproteine nicht empfindlicher als die SDS–PAGE der Gesamtproteine.

## Introduction

Among the bacterial diseases of cereals, the 'leaf streak' or 'black chaff' caused by various pathovars of *Xanthomonas campestris* is the most important seed-borne disease of wheat and barley in many countries (Akhtar and Aslam, 1985; Alizadeh and Rahimian, 1989; Noval, 1989; Duveiller, 1989; Demir and Üstun, 1992). Under favourable conditions the yield losses caused by this disease can reach 40% in susceptible triticale and bread wheat cultivars (Schaad and Forster, 1985; Cunfer, 1988; Mehta, 1990).

The characteristic symptoms of the disease, which can be observed on the aerial parts of the plants, particularly on the leaves, appear as translucent water-soaked stripes. Bacterial exudates are usually present on the diseased leaves as milky drops or dry yellow granules or translucent layers. On the glumes, the symptoms appear as black lesions, hence the term 'black chaff'.

Five pathovars (*hordei*, *cerealis*, *undulosa*, *secalis*, *phleipratensis*) of *X. campestris* were reported as the causal agents of the disease on cereals, and were distinguished by their host range. In Iran, the causal agents of the bacterial leaf streak of barley and wheat were first reported in south-eastern Iran as *X. c.* pvs *hordei* and *cerealis*, respectively (Alizadeh and Rahimian, 1989).

In order to study this further, 45 bacterial isolates of *X. campestris* were obtained from cereals and grasses growing in

different regions of Iran. From their phenotypic characters and their host range, they were separated into two groups (Alizadeh et al., 1995): the major discriminative characteristic of the strains was their pathogenicity toward wheat, barley and some grasses. Group I strains can infect only barley whereas group II strains are pathogenic to wheat, barley and some grasses. The host range of each group was not thoroughly defined and many grasses, which might be potential hosts for these groups, have not yet been tested. Instead of such an ambiguous and time-consuming approach, it would be more efficient to develop a simple technique that would allow differentiation between the bacterial isolates, and assessment of their host specificity.

The main objective of the investigations reported below was to evaluate the potential of the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) technique for distinguishing between 45 isolates of *X. campestris* obtained from cereals and grasses grown in different regions of Iran, through comparison with reference strains.

## Materials and methods

### Bacterial strains

Four reference strains of *X. campestris* pathovars, pathogenic to wheat and barley, were obtained from H. Maraite and C. Bragard (Unité de phytopathologie, UCL, Louvain-la-Neuve, Belgium) and 45 Iranian isolates of the pathogen, previously identified as *X. c.* pvs *hordei* and *cerealis*, were used (Table 1). All strains were routinely grown on nutrient agar (NA, Difco™, Detroit, MI, USA) at 25°C and maintained at 3–4°C, or lyophilized for long-term storage.

### Polyacrylamide gel ectrophoresis of whole-cell and membrane proteins

All isolates were studied by PAGE of the whole-cell proteins. In order to extract the whole cell proteins, bacterial cells grown on NA medium at 25°C for 24 h were harvested from the plates in 15 ml sterile distilled water. The optical density (*OD*) of the suspension at 600 nm was adjusted identically. A 10 ml sample of the suspension was centrifuged for 10 min at 15 000 *g* and the pellets were resuspended in 1 ml of sample buffer (Laemmli, 1970). SDS, 2-mercaptoethanol, and glycerol were added to the samples at final concentrations of 2, 5, and 10%, respectively (Laemmli, 1970). The protein samples were boiled for 5 min and clarified by low-speed centrifugation. The supernatants were used for electrophoresis.

The membrane proteins of some representative strains (IBLS1, 2, 3, 11, 20, 31, 40, 42 and 45, UPB 513 and 545, NCPPB 2389 and 2821) were isolated according to the method of Qhobela et al. (1991); after growing the bacteria in 0.8% nutrient broth (Difco) and 5% NaCl at 27°C for 24 h, the bacterial suspensions were centrifuged at 4 000 *g* for 15 min. The bacterial pellets were resuspended in 10 ml of 10 mM Tris-HCl buffer, pH 7.4, and sonicated with a cell-disrupter (Sonifier Model B.15, Branson Ultrasons, OSI, Paris, France) for 3 min in 30-s intervals with 30 s cooling between intervals. After centrifugation of the crude sonic extract for 20 min at 6000 *g*, the supernatants were centrifuged at 110 000 *g* for 35 min. The pellet containing the membrane fractions was suspended in 0.5 ml of 10 mM Tris-HCl buffer, pH 7.4 Laemmli sample buffer, 1 ml, was added and the samples were boiled for 2 min and stored at −20 C until use.

Discontinuous, one-dimensional SDS–PAGE was performed in a Protean II vertical electrophoresis unit (Bio-Rad, Richmond, CA, USA). Preparation, casting, assembling and running of the gel were done as prescribed by the manufacturer; acrylamide stacking concentration was 5% and concentrations of the resolving gels were 10, 12

Table 1
Origin and hosts of bacterial strains

| Strain | Pathovar group | Host | Isolation Year | Country | Source, origin or reference |
|---|---|---|---|---|---|
| Reference strains: | | | | | |
| UPB 458 (NCPPB2389) | *hordei* | *Hordeum vulgare* | 1970 | India | G. S. Shekhavat (India) |
| UPB 443 (NCPPB2821) | *undulosa* | *Triticum turgidum* | 1966 | Canada | J. Wilkie (New Zealand) |
| UPB513 | *translucens* | *Triticale-secale* | 1987 | Mexico | E. Duveiller (CIMMYT, Mexico) |
| UPB545 | *translucens* | *Hordeum vulgare* | 1987 | Mexico | E. Duveiller (CIMMYT, Mexico) |
| Iranian strains | | | | | |
| IBLS1, 2, 3, 4, 5, 6, 8, 9, 10, 13, 14, 15 and 16 | Group I | *Hordeum vulgare* | 1988–1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS11, 12, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 and 31 | Group II | *Triticum aestivum* | 1988–90 | Iran | A. Alizadeh et al. (1995) |
| IBLS32 | Group II | *Aegilops* sp. | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS33 | Group II | *Aegilops ventricosa* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS34 | Group II | *Aegilops cylindrica* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS35 | Group II | *Aegilops cylindrica* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS36 | Group II | *Bromus* sp. | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS37 | Group II | *Bromus tectorum* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS38 | Group II | *Hordeum maritimum* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS39 | Group II | *Hordeum maritimum* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS40 | Group I | *Hordeum* sp. | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS41 | Group II | *Lolium strictum* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS42 | Group II | *Lolium strictum* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS43 | Group II | *Schlerochloa dura* | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS44 | Group II | *Heteranthelium* sp. | 1990 | Iran | A. Alizadeh et al. (1995) |
| IBLS45 | Group III | *Bromus* sp. | 1990 | Iran | A. Alizadeh et al. (unpublished data) |

NCPPB, National Collection of Phytopathogenic Bacteria, Harpenden, UK;UPB, Unit of Phytopathogenic Bacteria, UCL, Louvain-la-Neuve, Belgium; IBLS, Iranian isolates of bacterial leaf streak of cereals; CIMMYT, Centro Internacional de Mejor amiento de Maiz y Trigo.

and 15%. A Tris–glycine electrode buffer (28.8 g glycine, 6 g Tris, 0.1 g SDS, per litre $H_2O$, pH 8.8) was used for the runs.

The gels were stained overnight in 0.1% Coomassie brilliant blue R in (5 : 1 : 5, v/v/v) methanol, acetic acid and water (Scalla et al., 1978), and destained in the same solvent, and kept in 7% acetic acid.

The isolates obtained from barley, wheat and grasses were first observed separately, then, representative isolates of each group were compared with the standard strains.

### Electron microscopy

Total membrane of some isolates (IBLS1, 2, 11 and NCPPB 2389) were suspended in 10 mM Tris-HCl and centrifuged again for 35 min at 110000 *g*. The pellets were fixed in 4% glutaraldehyde and 1% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, for 12 h and washed three times in the same buffer. Fixed pellets were then treated for 6 h with 1% osmium tetroxide dissolved in PBS, pH 7.3. Samples were dehydrated with a graded series of ethanol and then embedded in a low-viscosity resin (Spurr, 1969). Ultrathin sections were stained with 4% uranyl acetate followed by 1% lead citrate and examined under a Hitachi H-300 (Elexience, 91370, Verriere-le-Buissen, France) transmission electron microscope operating at 75 kV.

## Results

### Electron microscopy

The purity of the samples was verified through electron microscope observations of total-membrane preparations which displayed a large number of circular and C-shaped membrane elements (Fig. 1) known as characteristic morphology of membranes in other Gram-negative bacteria (Dianese and Schaad, 1982; Dos-Santos and Dianese, 1985).

### Polyacrylamide gel electrophoresis of whole-cell proteins

Visual analysis of the banding patterns of total and membrane proteins on all gels at various concentrations of polyacrylamide clearly showed the existence of two different groups among the isolates studied (Fig. 2 and Table 2).

The many bands distributed over the entire length of the gel can be used as discriminative bands between the two groups. The two intense bands between 66 200 and 97 000 daltons in the banding patterns of IBLS1, 2, 5, and 9, were observed in all the other isolates of group I. In addition, some bands between 14 400 and 45 000 may also allow group I isolates to be differentiated from group II isolates. Interestingly, membrane protein electrophoresis was not more discriminating than total protein electrophoresis: thus, discriminative markers for the two groups

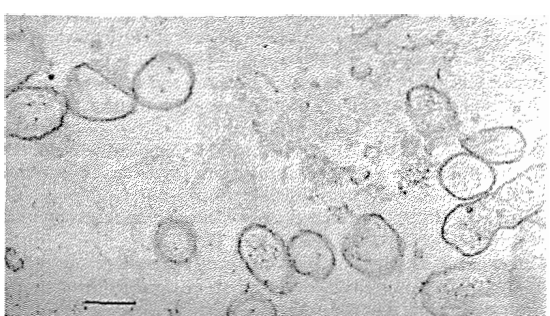

Fig. 1 Electron micrograph of membrane fragments of *Xanthomonas campestris* isolate IBLS2. The fragments were obtained after centrifugation of sonicated bacterial suspension first at 6000 *g* for 20 min to eliminate the cell debris, then at 110000 *g* for 35 min. Scale bar = 0.25 μm

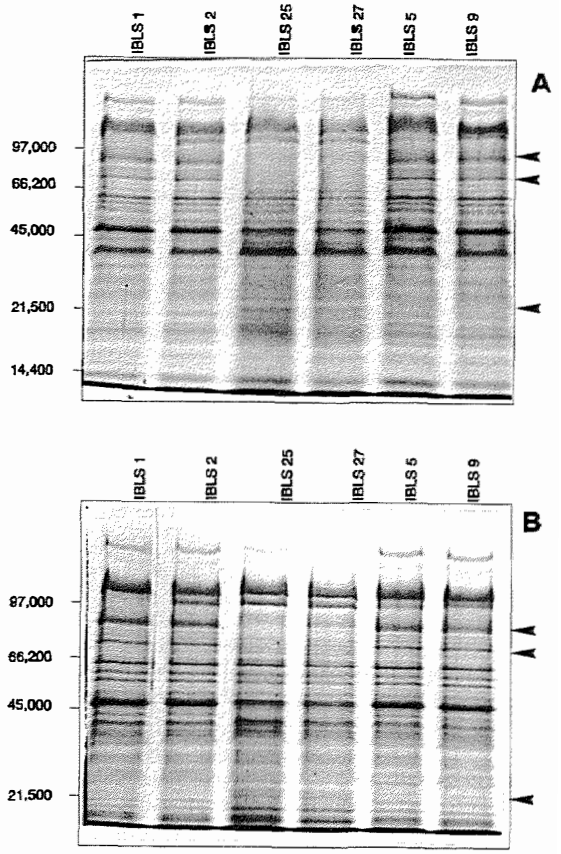


Fig. 2 Electrophoretic profiles of total proteins of Iranians isolates of *Xanthomonas campestris* pathovars, the causal agents of bacterial leaf streak of cereals, in 15% (A) and 10% (B) polyacrylamide gel. Values to the left are molecular weights (daltons). Arrows to the right show some of the discriminating bands

of isolates can be found in the cytoplasm and in the bacterial cell envelope.

Among the 16 barley isolates studied, 14 had similar banding patterns; only IBLS12 and IBLS11 were different from the other barley isolates. All wheat isolates were similar but exhibited a banding pattern that differed from that observed for the barley group. The majority of the grass isolates, as well as IBLS12 and IBLS11, had the same banding pattern as the wheat group, but IBLS40 displayed a barley group profile and IBLS45 was different from the first two groups mentioned.

A comparison with reference strains showed that banding patterns of UPB 458 (NCPPB 2389) and UPB 545 were similar to those of the barley group whereas UPB 443 (NCPPB 2821) and UPB 513 had the same banding pattern as the wheat group isolates.

## Discussion

### Polyacrylamide gel electrophoresis of membrane proteins

The SDS–PAGE technique, which was used for analysing the whole-cell and membrane proteins of the isolates, is relatively simple and inexpensive for the classification and identification of bacteria (Starr, 1981; Vera Cruz et al., 1984; van den Mooter et al., 1987). The results obtained correlate with those obtained

Table 2
Grouping of Iranian strains of *Xanthomonas campestris* pathovars, causing bacterial leaf streak of cereals and grasses, using polyacrylamide gel electrophoresis of whole-cell proteins

| Electrophoretic Group | Discriminative bands | Iranian strains | Pathogenic[a] to Barley | Pathogenic[a] to Wheat | Related reference strains |
|---|---|---|---|---|---|
| I | Two bands between 66.2 and 97 kDa and some bands between 14.4 and 45 kDa. | IBLS1, 2, 3, 4, | + | – | UPB 458 (NCPPB2389) |
| | | 5, 6, 7, 8, 9, | + | – | |
| | | 10, 13, 14, 15, | + | – | UPB 545 |
| | | 16 and 40 | + | – | |
| II | Absence of the two bands at 66.2 and 97 kDa and presence of some bands between 14.4 and 45 kDa | IBLS11, 12, | + | + | UPB 443 (NCPPB2821) |
| | | 17, 18, 19, 20, | + | + | |
| | | 21, 22, 23, 24, | + | + | UPB 513 |
| | | 25, 26, 27, 28 | + | + | |
| | | 29, 30, 31, 32, | + | + | |
| | | 33, 34, 35, 36, | + | + | |
| | | 37, 38, 39, 41, | + | + | |
| | | 42, 43 and 44 | + | + | |
| III | Discriminative bands distributed over the entire length of the profile | IBLS45 | + | + | — |

NCPPB, National Collection of Phytopathogenic Bacteria, Harpenden, UK; UPB, Unit of Phytopathogenic Bacteria, UCL, Louvain-la-Neuve, Belgium; IBLS, Iranian isolates of bacterial leaf streak of cereals.
[a]Alizadeh et al. (1995).

by other molecular identification and classification techniques, such as DNA–DNA hybridization (Kersters and De Ley, 1980; Jackman, 1982; Owen and Jackman, 1982; Vauterin et al., 1990), RFLP analysis (Qhobela et al., 1991; Qhobela and Claflin, 1992), fatty acid methyl ester analysis (Vauterin et al., 1991b, 1992) and phenotypic features (Kersters and De Ley, 1975; Vera Cruz et al., 1984).

Using the SDS–PAGE technique, Vera Cruz et al. (1984) were able to differentiate *X. campestris* pv. *oryzae* from *X. c.* pv. *oryzicola*. Thaveechai and Schaad (1986) discriminated *X. campestris* pv. *campestris* from other pathovars. Similar conclusions were reached for the pathovars *manihotis* and *cassavae* (van den Mooter et al., 1987), *gramins*, *phleipratensis*, *poae* and *arrhenatheri* (van den Mooter et al., 1987), and also some isolates of pathovar *campestris* (Vauterin et al., 1991a), *citri* (Vauterin et al., 1991b), and *holcicola* (Qhobela et al., 1991).

SDS–PAGE has been used in other investigations of *X. campestris* pathovars isolated from grasses and cereals. Kersters et al. (1989) studied more than 120 *X. campestris* strains belonging to 15 different pathovars: pathovars *cerealis*, *hordei*, *phlei*, *phleipratensis*, *poa*, *secalis*, *translucens* and *undulosa* displayed only a single large-protein electrophoretic cluster, although the strains of *X. campestris* pv. *phlei* were split into a small subcluster. Vauterin et al. (1992) compared various pathovars of *X. campestris* that are pathogenic to cereals and grasses but could not discriminate between the eight pathovars using SDS–PAGE technique.

In contrast, the results presented in this paper show that, under the experimental conditions used, the SDS–PAGE method can be useful for differentiating the pathovars of *X. campestris* causing the bacterial leaf streak of cereals. The grouping thus obtained correlated with that obtained from the phenotypic features and host range (Alizadeh et al., 1994). Using SDS–PAGE, there was no clear advantage to using membrane proteins rather than total proteins, although membrane protein extraction requires additional equipment that is not available in every diagnostic laboratory.

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