J. Microbiol. Biotechnol. (2002), **12**(4), 674–678

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Analysis of Outer Membrane Proteins of *Yersinia enterocolitica* Isolated from Mountainspring Water and Pig

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Received: November 15, 2001
Accepted: March 31, 2002

Abstract *Yersinia enterocolitica* causes various diseases in humans, including enteritis. The onset of such diseases is closely related with the expression of important virulence factors, particularly outer membrane proteins (OMPs). The expression of OMPs depends on several factors, including temperature, and origin, biotype and serotype of the bacteria. Recently, concerns over food safety have increased along with the demand for the development of sensitive, rapid, and pathogen-specific detection methods. To develop a suitable detection method for *Y. enterocolitica* isolated from Korean moutainspring water and pig feces, the OMP expression patterns were analyzed phenotypically and immunologically using 12 representative strains from 51 *Y. enterocolitica* Korean isolates. A 38-kDa OMP was commonly observed in all strains. However, additional OMPs were also observed in different biotypes and serotypes as well as bacterial origins, by incubating *Y. enterocolitica* at a low temperature. The specificity of the 38-kDa OMP was confirmed by a Western blot analysis with antisera against *Y. enterocolitica* and *Brucella abortus*. The results, therefore, indicate that the 38-kDa OMP could be used as a marker for detecting *Y. enterocolitica* in the environment or for seromonitoring.

Key words: Analysis, *Yersinia enterocolitica*, OMP

Yersinia enterocolitica, existing as residues and normal flora in the lower intestinal tract of many domestic and wild animals and in nature [3, 20], are also enteroinvasive bacteria that cause various diseases such as gastroenterocolitis, mesenteric lymphadenitis, and septicemia [18, 25, 26, 28]. Infection usually occurs through a fecal-oral route by consuming food or water contaminated with the feces of infected animals, and pigs have been recognized as primary carriers for the infection of humans through contaminated pork [10, 14, 27]. However, mountainspring water has recently received attention as one of the most important sources of infection. Recently, cases of yersiniosis have been increasingly reported throughout the world, particularly in the developed countries, North America and Europe [19]. Therefore, a pathogen-specific, sensitive, and rapid method to detect *Y. enterocolitica* is required.

Generally, the bacterium has been differentiated based on the serotype and biotype, through its somatic antigenic properties using antisera and its biochemical characteristics, respectively [5, 24]. However, various problems have been encountered in differentiation because of increasingly frequent occurrence of variants. Therefore, new molecular biological techniques, such as an analysis of the genomic DNA and ribotyping, have recently been developed based on current field knowledge [9, 13, 16, 17]. The occurrence of the disease is closely related with the expression of important virulence factors, including outer membrane proteins (OMPs). However, the expression of such factors is influenced by environmental conditions, particularly temperature [1, 2, 4, 9, 23].

The OMP patterns of *Y. enterocolitica* isolates may differ depending on the origin of the bacterium, even though they exhibit the same serotypes and biotypes. Accordingly, the current study was undertaken to investigate the expression patterns of the OMPs of *Y. enterocolitica* Korean isolates phenotypically and immunologically.

Ninety-seven strains of *Yersinia* spp. were isolated from the feces of pigs and mountainspring water collected between 1995 and 1998 in Korea, using cold enrichment and filtration methods, respectively [20, 22]. Thirty-four *Y. enterocolitica* isolates from the feces of pigs and 17 from mountainspring waters, were identified, and their biotypes were determined by their basic biochemical properties [7, 18].

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The serotypes were determined through a microagglutination test using antisera produced from whole cell antigens of *Y. enterocolitica* in rabbit [6] or purchased from Denka Seikan Co. (Tokyo, Japan). Based on their origin, and serotypes and biotypes, 12 representative strains of *Y. enterocolitica* isolates were selected for analysis of OMPs of the bacterium.

The OMPs were prepared by the method previously described [8] with minor modifications. The selected bacterial strains were chosen to represent each serotype, biotype, and origin, and their OMPs were extracted. Briefly, the bacteria were initially cultured in 60 ml of tryptic soy broth (TSB, Difco Co. Detroit, MI, U.S.A.) at 26 or 37°C for 36 h without shaking, then harvested through centrifugation for 30 min at 5,000 rpm, washed twice with phosphate buffered saline (PBS, pH 7.4), and resuspended in 10 mM HEPES buffer (pH 7.4). After sonication with an ultrasonic homogenizer (1 min, 5 cycles, 60 power), the cell debris and unbroken cells were removed through centrifugation at 8,000 rpm for 1 h. The insoluble materials were sedimented by ultracentrifugation (Beckman Co.) at 20,000 rpm for 60 min. The sediment was then resuspended in 10 ml of 2% sodium lauryl sulfate in 10 mM HEPES buffer and the suspension was incubated with shaking for 2 h at room temperature. After incubation, the insoluble OMPs were harvested by centrifugation at 20,000 rpm for 60 min and resuspended in distilled H_2O. The protein concentration of the suspension was determined using a Bio-Rad DC Protein assay kit (Bio-Rad Co., Hercules, CA, U.S.A.).

SDS-PAGE was carried out as described by Lammeli [15]. The OMPs were separated using 12 and 5% acrylamide as the resolving and stacking gels, respectively, in a discontinuous system. Ten-microgram samples were incubated with an equal volume of the sample buffer [0.1 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue] at 100°C for 10 min or 37°C for 30 min. After loading the samples, the electrophoresis was carried out at 25 mA for 1.5 h. The gel was then stained with a 0.05% Coomassie Brilliant Blue R-250 solution or subjected to an immunological analysis. After destaining, the gel was air-dried and a picture was taken.

For an immunological analysis of the OMPs of the *Y. enterocolitica* isolates, the proteins separated as described above through SDS-PAGE were electrophoretically transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad) using a Bio-Rad Trans-blot Semi-Dry Transfer cell system (Bio-Rad). The NC membranes were incubated in 10% skim milk in Tris-buffered saline (TBS, pH 7.5) for 1 h at room temperature. After washing three times with TBS, the membranes were incubated with 1/200 diluted antibodies [6, 20] specific against *Y. enterocolitica* O:3, O:13, or O:16 for 3 h at 37°C. After the immunoreaction, the membranes were washed again as described above, and then reacted with 1/15,000 diluted alkaline phosphate-conjugated goat anti-rabbit IgG antibody (Sigma Co., St. Louis, MO, U.S.A.). After removing the unreacted antibodies by washing with TBS, the immunoreactive bands were visualized using an enhanced AP conjugate substrate kit (Bio-Rad) in the dark. For the specificity test of the OMPs of *Y. enterocolitica*, antisera against *Brucella abortus* (kindly provided by National Veterinary Research and Quarantine Service) was used instead of antisera against *Y. enterocolitica* O:3, O:13, or O:16.

Fifty-one strains of *Y. enterocolitica* were identified from 97 strains of *Yersinia* spp. isolated from the feces of pigs and from mountainspring water. The biotypes of the isolates were 3B, 3A, and 4 in an increasing order. The predominant serotypes of the isolates were O:3, O:13, and O:16. Although the relationship between the biotypes or serotypes and the origin of the isolates was not evident, the predominant biotypes and serotypes of the isolates from the feces of pigs and from mountainspring water were 3B and 3A, and O:3 and O:13, respectively [20].

Twelve representative strains of the 51 *Y. enterocolitica* isolates were selected based on the serotype, biotype, and origin of the isolates (Table 1), [22]. These strains were then used to compare the OMP profiles and for the immunological analysis.

Three major bands (38, 40, and 41.3 kDa) were observed in the OMP profiles of *Y. enterocolitica*, cultured at 26°C for 24 h and then treated for 10 min in boiling water. Four different OMP patterns were observed among the tested strains depending on the biotype, serotype, and origin. A 38-kDa OMP was observed in the all serotypes and biotypes, while 40-kDa OMP was detected only in biotype 3A, and a 41.3-kDa OMP in biotype 3A and serotype O:16 (Table 2). The isolates from the mountainspring water exhibited one more OMP (40 or 41.3 kDa) in addition to the 38-kDa OMP (Table 2). The OMP profiles of the representative strains were differentiated depending on the origin (Tables 1 and 2).

The OMP profiles of the *Y. enterocolitica* isolates cultured at 37°C for 24 h showed only a 38-kDa band, as revealed through the SDS-PAGE analysis, regardless of the biotype, serotype, and origins (Fig. 1), while two major

Table 1. *Yersinia enterocolitica* strains used in this study.

Origins	Serotypes	Biotypes	*Yersinia enterocolitica* isolates*
Feces of pigs	O:3	3B	Y4, Y12
		3A	Y24, Y30
		4	Y21, Y18
Moutainspring water	O:13	3A	Y36, Y37
	O:16	3A	Y42, Y45, Y1
		3B	Y40

*Representative strains of *Y. enterocolitica* isolates from Korea based on the origins, serotypes, and biotypes.

Table 2. Expression of outer membrane proteins of *Yersinia enterocolitica* isolates cultured at 26°C for 24 h.

Serotypes	Biotypes	Analysis by SDS-PAGE			Analysis by Western blot		
		38 kDa	40 kDa	41.3 kDa	38kDa	40kDa	41.3kDa
O:3	3B	+	-	-	+	-	-
	3A	+	+	-	+	-	-
	4	+	-	-	+	-	-
O:13	3A	+	+	-	+	-	-
O:16	3A	+	+	+	+	+	-
	3B	+	-	-	+	-	-

+; Expressed, -; Not expressed.

bands (29 and 38 kDa) were observed in the strains isolated from the pig feces (lanes 1–6) and a single 29-kDa band was observed in the strains isolated from the mountainspring water (lanes 7–12) (Fig. 2). However, no differences in the profiles were observed in the biotypes or serotypes.

In the analysis of the OMPs using polyclonal antibodies against *Y. enterocolitica* O:3, O:13, and O:16, which were the most predominant serotypes in Korea, the antibodies specifically reacted with a 38-kDa protein in all strains tested (Table 2). An additionally reacted band of 40 kDa was observed in two isolates, serotype O:16 and biotype 3A, from the mountainspring water (Table 2). In the Western blot analysis with an antibody against *B. abortus*, the 38-kDa major band of *Y. enterocolitica* did not react with the antibody.

Y. enterocolitica is regarded as a major source of food infection [3, 14, 27], resulting in various diseases such as gastroenterocolitis and septicemia, even though the bacterium forms a part of the normal flora found in the lower intestinal tract of many domestic and wild animals [3, 20, 25, 26, 27].

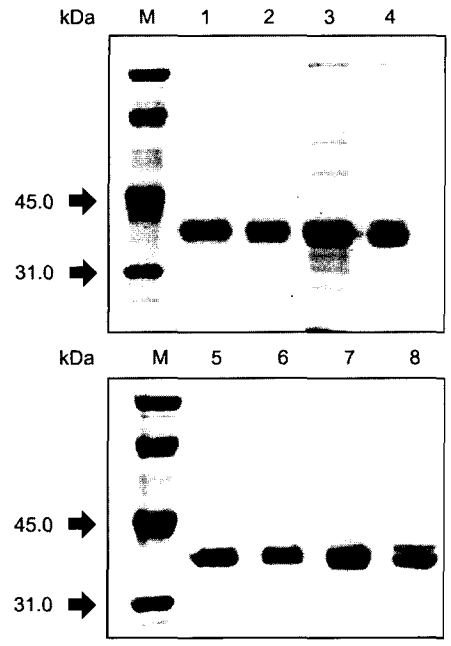

Fig. 1. Electrophoretic analysis of the outer membrane proteins (OMPs) of *Yersinia enterocolitica* isolated from mountainspring water and the feces of pigs in Korea.

The bacteria were cultured at 37°C (lanes 1–4) or 26°C (lanes 5–8) for 24 h, and OMPs were extracted as described in Materials and Methods. Lane M, Molecular weight marker; lanes 1 and 5, *Y. enterocolitica* 4; lanes 2 and 6, *Y. enterocolitica* 21; lanes 3 and 7, *Y. enterocolitica* 37; and lanes 4 and 8, *Y. enterocolitica* 42.

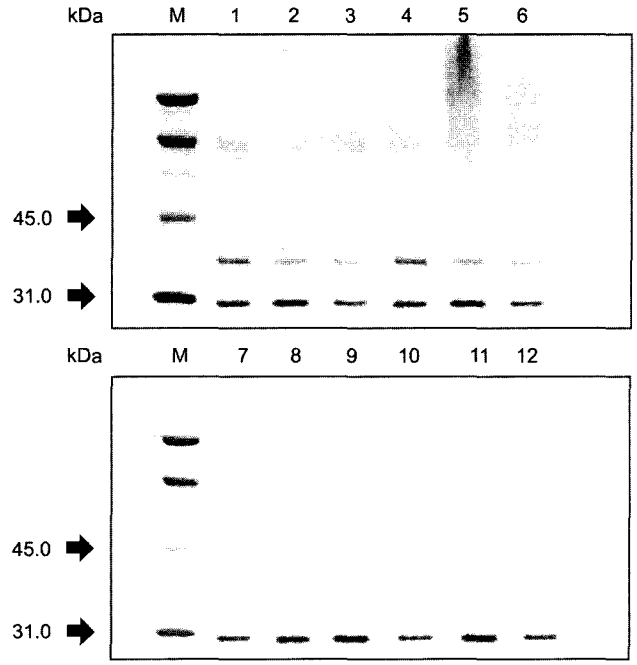

Fig. 2. Electrophoretic analysis of the outer membrane proteins (OMPs) of *Y. enterocolitica* isolated from mountainspring water and the feces of pigs in Korea.

OMPs were extracted from the bacteria cultured at 26°C and treated with a sample buffer at 37°C for 30 min. Lane M, Molecular weight marker; lane 1, *Y. enterocolitica* 4; lane 2, *Y. enterocolitica* 12; lane 3, *Y. enterocolitica* 18; lane 4, *Y. enterocolitica* 21; lane 5, *Y. enterocolitica* 24; lane 6, *Y. enterocolitica* 30; lane 7, *Y. enterocolitica* 36; lane 8, *Y. enterocolitica* 37; lane 9; *Y. enterocolitica* 40; lane 10, *Y. enterocolitica* 42; lane 11, *Y. enterocolitica* 45; and lane 12, *Y. enterocolitica* 1.

The virulence of this bacterium is closely related with the expression of the yersinia outer membrane protein (Yop), which is strictly regulated by several environmental factors, including Ca^{+2} concentration, temperature, nutrient limitations, and origin [15, 16-19, 26]. Generally, the bacterium is classified on the basis of phenotypical phenomena, such as its serotype and biotype [5, 24]. However, this method is not appropriate when analyzing virulence, since the incubation temperature usually regulates the expression of the OMPs. Therefore, in the current study, we examined phenotypically and immunologically the expression patterns of Yop of *Y. enterocolitica* isolated from pig feces and mountainspring water. In the analysis, a 38-kDa OMP was commonly observed in all tested strains, regardless of the origin, biotype, serotype, and incubation temperature. Moreover, the band with same size was also detected through Western blot analysis using antisera against *Y. enterocolitica* 0:3 and 0:16.

The results of the analysis suggested that the 38-kDa Yop could be the most antigenic OMP of the *Y. enterocolitica* isolates. However, when the purified OMP was incubated for 1 h at 37°C with an SDS-PAGE buffer instead of boiling at 100°C, a 29-kDa OMP was observed in all the tested strains. This might have been due to proteolysis of the 38-kDa Yop, as reported in other studies [1, 2, 4].

In the isolates from pig, additional Yops, namely a 40-kDa Yop in biotype 3A and 41.3-kDa Yop in serotype 0:16 and biotype 3A, were identified, and these characteristics could be used as markers to differentiate the serotypes or biotypes of the isolates. The current results are in agreement with a previous report [14], which suggested that the 38-kDa Yop can be an essential and significant antigenic OMP, even though several Yops are required to express the virulence of *Y. enterocolitica*. In conclusion, the current results suggested that Yop could be used as the target protein for seromonitoring or detection of *Y. enterocolitica* in the environment, particularly in foods. In addition, the 38-kDa Yop could also be used in the serological discrimination of false positive reactions between *B. abortus* and *Y. enterocolitica* O:9 infections.

Acknowledgments

This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare (HMP-98-M-1-0007), and Brain Korea 21 Project, Korea.

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