Psychrobacter immobilis isolated from foods: characteristics and identification

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ABSTRACT: A total of 15 strains of *Psychrobacter immobilis* isolated from animal sources, e.g. cheese, fish and poultry, were tested. A commercial diagnostic kit NEFERMtest 24, conventional tests and determination of fatty acids were used for identification. By using the results of NEFERMtest 24 and numerical identification system TNW version 6.0 the identification was successful on the species level (46.7%) while the correct species identification by using conventional tests increased up to 86.7%. All 9 saccharolytic strains including 7 Czech isolates were identified in most cases on an excellent or very good level by both methods based on biochemical reactions. On the other hand, the identification of 6 asaccharolytic strains was unsuccessful especially by NEFERMtest 24. While 4 asaccharolytic strains were identified correctly on the basis of conventional tests on species or genus level, incorrect identification on species level, for example *Ralstonia paucula*, *Comamonas terrigena*, *Oligella ureolytica*, *Moraxella lincolnii* and *Psychrobacter phenylpyruvicus*, was found by using NEFERMtest 24. Determination of fatty acid composition by MIDI System confirmed the species identification of 9 out of the 10 tested strains of *P. immobilis* and 1 tested strain of *Psychrobacter* sp.

Keywords: *Psychrobacter immobilis*; characteristics; identification; foods

INTRODUCTION

The sanitary and nutrition quality of foods from animal sources is significantly influenced by the presence of psychrotrophic bacteria. The most important proteolytic species (e.g. *Pseudomonas fluorescens*, *Shewanella putrefaciens*) and lipolytic bacteria (e.g. *Acinetobacter* spp., *Psychrobacter immobilis*, *P. fluorescens*, *S. putrefaciens*) contribute to nutritive and sensory changes of foods (Pin and Baranyi, 1998; Gennari *et al*., 1999). These spoilage organisms can usually be found in refrigerated meat and food during aerobic and vacuum-packaged storage (Shaw and Latty, 1988). Some psychrobacters with optimal growth temperature of 35 to 37°C have been isolated from pathological specimens derived from humans (Gini, 1990; Lloyd-Puryear *et al*., 1991) and animals (Prieto *et al*., 1992; Gennari *et al*., 1999). The sources of isolation (CSF, blood, urethra, eyes) suggest that psychrobacters might be a cause of incidental infections (Lloyd-Puryear *et al*., 1991).

The name *Psychrobacter* was proposed for a group of psychrotrophic, aerobic, gramnegative, nonmotile, oxidase positive coccobacilli commonly found associated with fish and processed meat and poultry products (Juni and Heym, 1986; Gonzalez *et al*., 2000). Psychrobacters with their typically cocci or coccobacilli shape resemble strains of *Acinetobacter* or *Moraxella* microscopically. Because of a variety of findings regarding their relatively close relationship, a new family *Moraxellaceae* was proposed to accommodate the genera *Acinetobacter*, *Moraxella* and *Psychrobacter* (Rossau *et al*., 1991). It was indicated on the basis of phenotypic tests and phylogenetic analysis that *Moraxella phenylpyruvica* is more closely related to *P. immobilis* than to other *Moraxella* species and was transferred into the genus *Psychrobacter* as *P. phenylpyruvicus* comb. nov. (Bowman *et al*., 1996). In the last years the four new species of *Psychrobacter* were described – *P. urativorans* and *P. frigidicola* (Bowman *et al*., 1996), *P. glacincola* (Bowman *et al*., 1997) and *P. pacificensis* (Maruyama *et al*., 2000). It is evident that the members of the genus *Psychrobacter* are psychrotolerant or psychrophilic and halotolerant, which reflects the ubiquitous distribution of the genus in foods, marine and terrestrial environments, predominant in the Antarctic (e.g. ornithogenic soils, Antarctic Sea ice and Japan Trench deep sea water).

In this study the characteristics and identification of Czech isolates of *P. immobilis* are compared with the strains from different geographical areas on the basis of

biochemical, physiological properties and fatty acid composition.

MATERIAL AND METHODS

Bacterial strains

A total of 15 strains were studied (Table 1). The authors' seven strains were isolated from cheese on Nutrient and Pseudomonas Agar (Oxoid) after incubation at room temperature (22–24°C) for three days during a testing of psychrotrophic bacteria from milk and dairy products (Páčová and Urbanová, 1995, 1998). The other tested strains were isolated from different animal food sources (fish, poultry, sausage).

Phenotypic studies

The tested strains were cultivated on Columbia Agar at 25°C. Phenotypic properties were parallelly tested by a new commercial diagnostic kit NEFERMtest 24 (Pliva Lachema a.s., Brno, Czech Republic) including OXItest, and by conventional methods routinely used for identification of nonfermentative bacteria. The NEFERMtest 24 kit and reagents were all used according to the manufacturer's instructions. The kit contains the following biochemical reactions: indole production, arginine dihydrolase, lysine decarboxylase, urease, phosphatase, β-galactosidase, β-glucosidase, N-acetyl-β-D-glucosaminidase, γ-glutamyl transferase, reduction of nitrate and nitrite, Simmons' citrate, esculin hydrolysis, oxidative utilisation of glucose, fructose, inositol, sucrose, mannitol, xylose, cellobiose, galactose, lactose, maltose and trehalose. The following conventional tests were used for correct determination: catalase, β-galactosidase (ONPG), growth at 5, 15, 25, 30, 37, 42°C and in 6.5% and 10% NaCl, Simmons' citrate utilisation, production of fluorescein and indole, reduction of nitrate and nitrite (Barrow and Feltham, 1993), phenylalanine deaminase (Bøvre and Henriksen, 1976), phosphatase (Páčová and Kocur, 1978), hydrolysis of gelatine and Tween 80 (Páčová and Kocur, 1984), carbohydrate acidification on special King OF medium (Weyant *et al*., 1996). When conventionally tested, the strains were cultivated at 25°C for 3 to 7 days, except for the microtests (phenylalanine deaminase and phosphatase). The strains were identified by TNW numerical identification programme version 6.0 (Czech Collection of Microorganisms, Brno) on the basis of commercial kit NEFERMtest 24 and routine conventional tests simultaneously.

Fatty acid methyl ester analysis

The psychrobacters for whole-cell fatty acid analysis were harvested from 24 h cultures grown at 28°C on plates containing Trypticase Soy Broth (BBL) with agar, the strain P4 was cultivated on the same medium with 1% NaCl because of unsufficient growth. Preparation, separation, numerical comparison of the fatty acid methyl esters and identification of the cultures (TSBA library) were performed using the Microbial Identification

Table 1. *Psychrobacter immobilis* strains tested

Serial No.	Other Nos.	Original sources and other names
P1	CCM 4772; strain 300	Edam cheese; authors' isolates, Czech Republic
P2	strain 374	
P3	CCM 4773; strain 375	
P4	CCM 4774; strain 377	
P5	CCM 4775; strain 379	
P6	CCM 4440; strain 371	
P7	CCM 4771; strain 372	
P8	CCM 4776^{T}; CCUG 9708^{T}	poultry, carcass, U.K.
P9	CCM 4777, CCUG 9710	poultry, Sweden
P10	CCM 4778; CCUG 21728	cod, skin, U.K., Scotland
P11	CCM 4779; CCUG 21730	fish; U.K., Scotland
P12	CCM 4780; CCUG 21778	irradiated Vienna sausage, Japan
P13	CCM 2453; K. Hayashi H11-4	salted fish, Japan, "*Halococcus nondenitrificans*"
P14	CCM 2454; K. Hayashi H8-1	salted fish, Japan, "*Halococcus agglodenitrificans*"
P15	CCM 2455; K. Hayashi H7-1	salted fish, Japan, "*Halococcus acetoinfaciens*", *Psychrobacter* sp.

^{T} = type strain; CCM = Czech Collection of Microorganisms, Brno, Czech Republic; CCUG = Culture Collection of the University of Göteborg, Göteborg, Sweden

System according to the manufacturer's instructions (MIDI Inc., 1999).

RESULTS AND DISCUSSION

Phenotypic characteristics

The tested strains and their sources are listed in Table 1. Although the first Czech isolates of psychrobacters were determined from cheese, the other tested psychrobacters were detected mainly in fish and poultry. The Japanese strains (P13–P15) were deposited in CCM as not validly published species of the genus *Halococcus*. The results of commercial diagnostic kit NEFERMtest 24 and conventional tests are listed in Tables 2 and 3. In both cases, the test results correspond with literature (Juni and Heym, 1986; Bowman *et al*., 1996). All tested strains, including seven Czech isolates, were nonfermentative, oxidase and phenylalanine positive, predominantly saccharolytic rods. Nine saccharolytic strains (P1–P9) produced acid from xylose, glucose, lactose, cellobiose and galactose. Prolonged cultivation of NEFERMtest up to 72 h at room temperature can increase the percentage of acidification of carbohydrates in psychrotrophic bacteria (Páčová and Urbanová, 1995). Six strains (P10–P15) acidified carbohydrates neither in NEFERMtest 24 nor in conventional tests (Tables 2 and 3). Even though the occurrence of asaccharolytic strains of *Psychrobacter* was mentioned (Weyant *et al*., 1996), the used medium could not be suitable for strains isolated from the marine environment (P10, P11, P13, P14 and P15). Variable phenotypic reactions (Tables 2 and 3) are in accordance with literature (Weyant *et al*., 1996; Bowman *et al*. 1996).

Identification

Identification of *P. immobilis* strains by numerical programme TNW is listed in Table 4. Seven strains (46.7%) were correctly classified as excellent (EI), very good (VGI) or good (GI) identification on the species level by NEFERMtest 24. Asaccharolytic strains (P10–P15) were incorrectly identified as *Ralstonia paucula*, *Comamonas terrigena*, *Oligella ureolytica*, *Moraxella lincolnii* or *P. phenylpyruvicus* on the level of intermediate strains.

Table 2. Biochemical reactions of *Psychrobacter immobilis* in NEFERMtest 24

Strain Nos.	IND	ARG	URE	LYS	GLU	FRU	INO	SUC	PHS	bGA	bGL	NAG	MAN	XYL	CEL	GAL	NO3	NO2	ESL	GGT	LAC	MLT	TRE	SCI
P 1	–	–	–	–	w	–	–	–	+	–	–	–	–	w	w	w	+	–	–	+	–	–	–	–
P 2	–	–	–	–	w	–	–	–	–	–	–	–	–	w	+	+	+	–	–	+	–	–	–	–
P 3	–	–	+	–	w	–	–	–	–	–	–	–	–	+	+	+	+	–	–	+	+	–	–	–
P 4	–	–	–	–	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	+	–	–	–
P 5	–	–	+	–	w	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–
P 6	–	–	+	–	w	–	–	–	–	–	–	–	–	w	w	w	+	–	–	+	+	–	–	–
P 7	–	–	+	–	+	–	–	–	–	–	–	–	–	w	+	+	+	–	–	+	+	–	–	–
P 8	–	–	–	–	w	–	–	–	–	–	–	–	–	–	+	+	+	–	–	+	+	–	–	–
P 9	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
P 10	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
P 11	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
P 12	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P 13	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–
P 14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P 15	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–

+ = positive; – = negative; w = weakly positive
IND = indole; ARG = arginine dihydrolase; URE = urease; LYS = lysine; GLU = glucose; FRU = fructose; INO = inositol; SUC = sucrose; PHS = phosphatase; bGA = β-galactosidase; bGL = β–glucosidase; NAG = N-acetyl-β-D-glucosaminidase; MAN = mannitol; XYL = xylose; CEL = cellobiose; GAL = galactose; NO3 = nitrate reduction; NO2 = nitrite reduction; ESL = hydrolysis of esculin; GGT = γ-glutamyl transferase; LAC = lactose; MLT = maltose; TRE = trehalose; SCI = Simmons' citrate

Table 3. Phenotypic characteristics of *Psychrobacter immobilis* by conventional tests

All strains were positive for oxidase, catalase, phenylalanine deaminase, hydrolysis of Tween 80, growth in 6.5% NaCl, growth at 5, 15, 25, 30°C. All strains were negative for hydrolysis of gelatine, indole production, β-galactosidase, arginine dihydrolase, lysine and ornithine decarboxylase, nitrite reduction, haemolysis, fluorescein production, acidification of mannitol, maltose, fructose, sucrose and myo-inositol.

Strain Nos.	GLU	XYL	CEL	GAL	LAC	37C	NAC10	TYR	LEC	URE	PHS	NO3	SCI
P1	+	+	+	+	+	–	–	+	–	–	–	+	+
P2	+	+	+	+	+	–	–	+	w	–	–	+	–
P3	+	+	+	+	+	–	+	+	w	+	–	+	–
P4	+	+	+	+	+	–	+	+	w	–	–	–	–
P5	+	+	w	+	+	–	w	+	+	+	–	+	–
P6	+	+	w	+	+	–	–	+	+	+	–	+	–
P7	+	+	+	+	+	–	–	+	+	+	–	+	–
P8	+	w	+	+	+	–	–	–	w	–	–	+	–
P9	–	w	w	–	w	–	–	+	+	–	+	+	–
P10	–	–	–	–	–	–	–	–	+	+	+	–	–
P11	–	–	–	–	–	–	–	–	+	+	+	–	–
P12	–	–	–	–	–	–	–	+	–	+	–	+	–
P13	–	–	–	–	–	+	w	+	+	+	w	+	–
P14	–	–	–	–	–	+	+	+	–	–	–	–	–
P15	–	–	–	–	–	+	w	+	–	+	w	+	–

+ = positive; – = negative; w = weakly positive

GLU = glucose; XYL = xylose; CEL = cellobiose; GAL = galactose; LAC = lactose; 37C = growth at 37°C; NAC10 = growth in 10% NaCl; TYR = hydrolysis of tyrosine; LEC = lecithinase; URE = urease; PHS = phosphatase; NO3 = nitrate reduction; SCI = Simmons' citrate

Table 4. Identification of 15 strains of *Psychrobacter immobilis* by numerical TNW system, version 6.0

Identification		NEFERMtest 24		Conventional tests	
		No. of strains	%	No. of strains	%
Species correctly:	EI	5 (Nos. P2, 3, 4, 6,7)	33.3	9 (Nos. P1, 2, 3, 4, 5, 6, 7, 8, 9)	60.0
	VGI	1 (No. P8)	6.7		
	GI	1 (No. P1)	6.7		
	AI			3 (Nos. P10, 11, 13)	20.0
Genus correctly				1 (No. P12)	6.7
Species incorrectly:	IS	8 (Nos. P5, 9, 10, 11, 12, 13, 14, 15)	53.3		
Unidentified				2 (Nos. P14, 15)	13.3

EI = excellent identification; VGI = very good identification; GI = good identification; AI = acceptable identification; IS = intermediate strain

The number of correctly identified strains increased to 13 (86.7%) by means of 33 conventional tests, routinely used for identification of gramnegative, nonfermentative bacteria in CCM (Czech Collection of Microorganisms, Brno, Czech Republic). These tests contain basic differentiating tests for discrimination of *P. immobilis* from the other nonfermentative, oxidase positive and indole negative rods, first of all phenylalanine deaminase, hydrolysis of Tween 80 and gelatine. That is why also 4 asaccharolytic strains (P10 – P13) were correctly identified on the species or genus level by conventional tests (Table 4). Two asaccharolytic strains growing at 37°C (P14 and P15) were unidentified by TNW programme but determined as *P. immobilis* (P14) by MIDI System and *Psychrobacter* sp. (P15) by differential tables (Juni and Heym, 1986; Bowman *et al*., 1996). All seven Czech isolates were identified as *P. immobilis* on the level of excellent identification by both the commercial kit and conventional tests.

Rapid and accurate identification of bacterial pathogens isolated from food samples is important both for food quality assurance and for tracing of outbreaks of

Table 5. Identification from the results of fatty acid methyl esters analysis

Strain No.	Identification	Similarity index**
P1	*Psychrobacter immobilis*	0.54
P3	*Psychrobacter immobilis*	0.61
P4	*Psychrobacter immobilis*	0.36
P5	*Psychrobacter immobilis*	0.37
P6	*Psychrobacter immobilis*	0.39
P7	*Psychrobacter immobilis*	0.78
P12	*Psychrobacter immobilis*	0.48
P13	*Psychrobacter immobilis*	0.57
P14	*Psychrobacter immobilis*	0.54
P15	NM*– most related to *Psychrobacter*	

*NM – no match in TSBA library
**Similarity index – a numerical value which expresses how closely the fatty acid composition of an unknown strain compares with the mean fatty acid composition of the strains used to create the library entry listed as its match (0.50 or higher – good library comparison, between 0.30 and 0.50 probably a good match but an atypical strain).

Table 6. Differential characteristics between *P. immobilis* and *P. phenylpyruvicus* included in the matrix of TNW programme, version 6.0

Characteristics	*P. immobilis* (% positive)	*P. phenylpyruvicus* (% positive)
Growth at		
5°C	93	16
37°C	21	99
42°C	1	20
Acid from		
glucose	74	1
xylose	92	1
cellobiose	30	1
lactose	85	1
γ-Glutamyl transferase	75	1
Hydrolysis of tyrosine	74	1

bacterial pathogens within the food supply. Determination of fatty acid composition by gas chromatography has proved to be a simple method of identification, epidemiologic typing and subgrouping of closely related bacteria (Abel *et al*., 1963; Mukwaya and Welch, 1989; Birnbaum *et al*., 1994). MIDI System is one of the automated microbial identification systems containing databases for identification of both *P. immobilis* and *P. phenylpyruvicus* isolates, which can be detected in food samples. The results obtained by MIDI corresponded well with those obtained by identification on the basis of phenotypic characteristics. Nine out of the 10 tested isolates were identified as *P. immobilis* (Table 5). The strain P15 was not identified on the species level and therefore testing by other molecular-biological methods is necessary. As the basic characteristic of this strain corresponds to the genus *Psychrobacter*, the strain was determined as *Psychrobacter* sp.

Even though the genus *Psychrobacter* includes 6 species at present, two species – *P. immobilis* and *P. phenylpyruvicus*, isolated from similar animal and human sources, are important in view of microbial testing of food quality. Since many of the psychrobacters appear to be radiation resistant in contrast to the more common food-spoilage bacteria, they have been found surviving in foods following irradiation for preservation purposes (Firstenberg-Eden *et al*., 1980).). First of all from fish, fresh or ice-stored and spoiled, *P. immobilis* and *P. phenylpyruvicus* species are isolated with fluorescent pseudomonads as the highest increase in microflora frequency (Gennari *et al*., 1999; Gonzalez *et al*., 2000; Walton and Smith, 1999). Differentiation of the two abovementioned species on the basis of phenotypic characteristics is rather difficult, first of all in the asaccharolytic species *P. immobilis*. The species *P. immobilis* and *P. phenylpyruvicus* grow at 25°C, are catalase, oxidase, phenylalanine deaminase positive, non-proteolytic but lipolytic positive. *P. phenylpyruvicus*, in contrast with *P. immobilis*, is strictly asaccharolytic, nutritionally fastidious and grows well when blood is added to the medium (Bowman *et al*., 1996). Differential characteristics included in TNW programme version 6.0, e.g. growth at different temperatures, hydrolysis of tyrosine and production of γ-glutamyl transferase, for these species are mentioned in Table 6. With respect to their potential to contribute to spoilage of the products of animal origin it is necessary to pay attention to identification of both these species by microbial testing of quality.

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