Polish Journal of Microbiology
2012, Vol. 61, No 1, 33–40

ORIGINAL PAPER

Prevalence of *cag*A, *vac*A, *bab*A2 and *ice*A Genes in *H. pylori* Strains Isolated from Colombian Patients with Functional Dyspepsia

AZUCENA ARÉVALO-GALVIS[1], ALBA A. TRESPALACIOS-RANGEL[1]*, WILLIAM OTERO[2], MARCELA M. MERCADO-REYES[1] and RAÚL A. POUTOU-PIÑALES[3]

[1]Laboratorio de Microbiología Especial, Grupo de Enfermedades Infecciosas. Departamento de Microbiología Facultad de Ciencias. Pontificia Universidad Javeriana. Bogotá, D.C. Colombia
[2]Unidad de Gastroenterología, Universidad Nacional de Colombia, Bogotá, D.C. Colombia
[3]Laboratorio de Biotecnología Molecular. Grupo de Biotecnología Ambiental e Industrial (GBAI) Departamento de Microbiología, Facultad de Ciencias. Pontificia Universidad Javeriana. Bogotá, D.C. Colombia

Received 23 September 2011, revised 20 January 2012, accepted 29 January 2012

Abstract

The clinical outcome of *Helicobacter pylori* infection has been particularly associated with virulence genotypes. These genotypes are useful as molecular markers in the identification of patients that are infected and at high risk for developing more severe gastric pathologies. Our main objective was to determine the prevalence of virulence genotypes *cag*A, *vac*A, *ice*A and *bab*A2 of *H.pylori*, in patients with functional dyspepsia who are infected with the bacteria. *H. pylori* genotypes *bab*A2 and *cag*A as well as *vac*A and *ice*A allelic variants were identified by PCR in 122 isolates resulting from 79 patients with functional dyspepsia. A high prevalence of genes *cag*A+ (71%), *vac*As1am1 (34%), *bab*A2 (57%) and *ice*A1 (87%) was found. The most frequent combined genotype found were *cag*A+/*vac*As1am1/*bab*A2+/*ice*A1 and *cag*A–/*vac*As1am1/*bab*A2+/*ice*A1, regardless of any family history of gastric cancer or MALT lymphoma. The very virulent genotype *cag*A+/*vac*As1am1/*bab*A2+/*ice*A1 prevailed in the studied patients with functional dyspepsia. Our results provide information about the prevalence of four of the more important virulent factors and constitute new evidence on the prevalence of the most virulent *H. pylori* genotype in patients with functional dyspepsia.

Key words: *Helicobacter pylori*, virulence genotypes, *cag*A, *vac*A, *ice*A, *bab*A2

Introduction

H. pylori colonizes the gastric mucosa causing chronic gastritis, gastric ulcers, duodenal ulcers, gastric cancer and lymphoma of mucosa-associated lymphoid tissue (MALT) (Amieva and El-Omar, 2008). The clinical outcome related with this infection has been associated with host genetic factors, environmental factors and pathogen virulence factors (Yamaoka, 2008). In this sense, the presence of virulence factors, change depending upon the geographic area and plays an important role in the *H. pylori* pathogenicity. Some of these factors are encoded by *cag*A, *vac*A, *bab*A2 and *ice*A genes (Van Doorn *et al.*, 1998).

Once *H. pylori* reach the gastric mucosa, it makes use of several adhesins. One of the most studied adhesins is BabA, which is encoded by the gene *bab*A2 and binds to Lewis-b blood group antigens. Contact between *H. pylori* and epithelial cells activates the *ice*A gene transcription, whose function remains unknown; however, some authors have associated IceA as well as BabA with peptic ulcer and gastric adenocarcinoma development (Zambon *et al.,* 2003; Amieva and El-Omar, 2008). After the gastric epithelium adherence occurs, the bacterium secretes the virulence factor CagA that allows colonizing the gastric mucosa. The *cag*A gene is a key marker in the cag pathogenicity island (cagPAI). CagA is injected into the host cells through type IV secretion system, which triggers the activation of several signaling pathways and generates cellular changes and induction of inflammatory interleukins (Kusters *et al.*, 2006; Arévalo *et al.*, 2009). The presence of *cag*A gene in *H. pylori* strains has been associated with the development of peptic ulcer and gastric cancer in humans (Nogueira *et al.*, 2001).

Another important virulence factor of *H. pylori* is the vacuolating cytotoxin A (VacA), which is encoded by the gene *vac*A. This gene has four regions: a signal sequence consisting of the allele s1, which has three subtypes: s1a, s1b, s1c and s2; an intermediate sequence; a deletion sequence (d); and a middle sequence that may have the m1 or m2 alleles. The combinations of

* Corresponding author: A.A. Trespalacios-Rangel, Laboratorio de Microbiología Especial, Grupo de Enfermedades Infecciosas. Departamento de Microbiología, Facultad de Ciencias. Pontificia Universidad Javeriana. Bogotá, D.C. Colombia; **e-mail: alba.trespalacios@gmail.com**

s and m alleles determine the toxin vacuolating activity responsible for major damages of the epithelial cells (Van Doorn *et al.*, 1998; Ogiwara *et al.*, 2009). *H. pylori* isolates presenting the allelic combinations *vac*As1m1 are considered more virulent because they produce more quantity of toxin besides the fact that s1 allele has been identified in strains causing duodenal ulcer, the allele m1 has been related with peptic ulcer and gastric cancer (Atherton, 1998; Yamaoka *et al.,* 2000; Miehlke *et al.,* 2001). On the contrary, isolates with the gene combination *vac*As1m2, which produce a moderate amount of toxin, and *vac*As2m2 strains, which produce little or no toxin, have rarely been related with clinically significant diseases (Atherton, 1998; Van Doorn *et al.,* 1998). Additionally, the relationship of these genes with the development of gastric pathology might have a connection with the treatment success or failure, since aggressive genotypes are easier to eradicate and less virulent ones have been related with treatment failure (Correa *et al.*, 2000; van Doorn *et al.*, 2000; Yamaoka *et al.*, 2000; Sugimoto and Yamaoka, 2009).

In Colombia, there have been some studies reporting the frequency of *vac*A, *cag*A, and *ice*A, (Cittelly *et al.*, 2002) *bab*A2 and *ice*A, (Montealegre *et al.*, 2009) alleles of *vac*A (López *et al.*, 2009) or *bab*A2, *oip*A and *cag*E (Quiroga *et al.*, 2005). Considering that gastric cancer is a leading cause of death, our objective was to determine the association and prevalence of *cag*A, *vac*A, *ice*A and *bab*A2 genes in patients with functional dyspepsia, due to the significance of these virulence factors in *H. pylori* pathogenicity (Atherton, 1998; Van Doorn *et al.,* 1998).

Experimental

Materials and Methods

Study type and ethics. This is a cross-cutting study previously approved by the ethics committees of the participating institutions.

Exclusion criteria. Patients matching the following criteria were excluded from the study: people with severe concomitant diseases such as chronic renal failure, decompensated heart failure, HIV, respiratory failure, tumors or malignant diseases, previous gastric surgery, as well as anticoagulated people, people addicted to recreational drugs or alcohol, pregnant or lactating women, and women in reproductive age using no family planning method. We also excluded those who had received prior treatment to eradicate *H. pylori* or who were under treatment with antisecretory or inhibitors of the proton pump 10 days before entering the study. Patients were excluded if during the last month they were under antimicrobial therapy against *H. pylori* (tetracycline, amoxicillin, bismuth, clarithromycin, furazolidone, levofloxacin, metronidazole or tinidazole).

Patients. The study includes a total of 79 patients who attended the upper endoscopy service at the *Clínica Fundadores* in Bogotá, D.C., Colombia, from February 2009 to February 2010. They attended service arguing dyspepsia (50.6%), reflux (18%), dysphagia (7.6%), weight loss (2.5%), anemia (1.3%) and other symptoms (20%); all of them met the inclusion criteria, and signed the informed consent after receiving a detailed explanation of the study delivered by a physician.

Patient's distributions were as follow: females 54 (68%); ranging from 20 to 66 years old, males 25 (32%); ranging between 22 to 65 years old. Colombian distribution of patients were 72 (91%) from Andean region which include 100% of women, 3 (4%) from Pacific region, 2 (3%) from West region, and 2 (3%) from Caribbean region. 38% (n = 30) of patients revealed knowledge of a gastritis family history, 28% (n = 22) of patients revealed knowledge of a gastric cancer family history, 1.3% (n = 1) of patients revealed knowledge of a peptic ulcer family history and 1.3% (n = 1) of patients revealed knowledge of MALT-lymphoma family history.

Endoscopic study. Patients included in the study underwent upper gastrointestinal endoscopy under fasting conditions of minimum six hours, in left lateral position, and in clean and aseptic conditions (Trespalacios *et al.,* 2010). Patients were not sedated but were supplied with 20mg of lidocaine (roxicaina, topical solution, Ropshn Therapeutics) to anesthetize the pharynx. Endoscopy was performed by a gastroenterologist with a video endoscope Exera Olympus CV 145. During the endoscopy five biopsies were taken from the antrum and four biopsies from the body. One of the antrum biopsies was used for rapid urease test; two biopsies were used for histological stainings (hematoxylin-eosin and Giemsa); the other two biopsies were used for *H. pylori* culture.

Transport of samples. Biopsies from the antrum and the body intended for *H. pylori* culture were placed in 500µl of Brucella broth with 20% (v/v) glycerol and kept refrigerated until processing (Ansorg *et al.*, 1991).

Isolation of *H. pylori*. Isolation was performed from antrum and body biopsies of each patient and the remaining biopsies were kept at –70°C (Ansorg *et al.*, 1991). Biopsies were macerated in aseptic and sterile conditions with a wooden applicator previously treated in a 1% (w/v) of activated charcoal solution until total homogeneity was achieved (Correa *et al.,* 2000). Subsequently the homogenate was grown on Wilkins Chalgren agar modified for *H. pylori*, enriched with IsoVitaleX (BD) and Dent antibiotic supplement (Oxoid), and incubated at 37°C in microaerophilic conditions for 3 to 14 days.

Phenotypic characterization of *H. pylori*. After incubation, small transparent color colonies were selected and assayed by Gram stain (Gram-negative

curved rods), oxidase (positive), catalase (positive) and rapid urease tests (positive) (Ansorg *et al.*, 1991). Colonies who corresponded with this characteristic were isolated in modified Wilkins Chalgren agar and incubated at 37°C in microaerophilic conditions for 3 to 5 days to obtain pure cultures.

***H. pylori* DNA extraction.** Pure colonies were cultured in liquid media, (Duque-Jamaica *et al.*, 2010) and after 12 hours of incubation (~10^6–10^7 cells ml^{-1}). Liquid culture was verified for Gram, urease activity, catalase activity and oxidase activity. Later 2 ml of liquid culture were taken and then homogenized and centrifuged at 10,000 rpm during 4 minutes. The supernatant was discarded and gDNA extraction was performed from the pellet using the Kit DNAzol® (Invitrogen).

Genotyping *cag*A, *vac*A, *bab*A2, and *ice*A genes. Isolates gDNA were used to detect the presence of *cag*A, *vac*A, *bab*A2, and *ice*A by PCR. Primers used and conditions for different PCR reactions are displayed in Table I. Each PCR reaction was carried out in a final volume of 25 µl as follows:

cagA, vacA* (s1a, s1b, s1c, m1, m2), *babA2: 5.1 µl of molecular grade water (Difco), 1X PCR buffer (Promega), 200 µM dNTPs (Invitrogen), 0.6 µM primers, 1.5 mM $MgCl_2$ (Promega), 0.25 U of Taq DNA polymerase (Promega) and 10 µl of DNA.

***vacA*s1/s2:** 10 µl of molecular grade water (Difco), 1X PCR buffer (Promega), 200 µM dNTPs (Invitrogen), 0.3 µM primers, 1.5 mM $MgCl_2$ (Promega), 0.25 U of Taq DNA polymerase (Promega) and 1 µl of DNA.

***iceA*:** 5.6 µl of molecular grade water (Difco), 1X PCR buffer (Promega), 200 µM dNTPs (Invitrogen), 0.5 µM primers, 1.5 mM $MgCl_2$ (Promega), 0.25 U of Taq DNA polymerase (Promega) and 10 µl of DNA (Table I).

We used a C1000 Thermal Cycler thermocycler (BioRad) for gDNA amplification. PCR products were visualized in 2% (w/v) agarose gel electrophoresis in 1X TAE Buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0 ± 0.2), at 80 V for 1:30 h. Gels were stained with ethidium bromide solution (5 µg ml^{-1}).

Statistical analysis. PASW 18.0 software was used to generate a database where the data obtained were processed and statistically analyzed. Percentages and 95% confidence intervals were used for qualitative variables. Measures of central tendency and dispersion were calculated for quantitative variables. Significant differences in proportions were established at $\alpha = 0.05$.

Results

Patients and endoscopic study. The 90% of the patients included in the study, have presented functional dyspepsia and/or a chronic gastritis without atrophy or intestinal metaplasia. The *H. pylori* infection was confirmed by histology, culture and rapid urease tests. The concordance of the outcomes of the three tests were 81.6% (histology *vs.* rapid urease test, 73–90.2 of 95% CI), 82% (rapid urease test *vs.* culture, 73.5–90.4 of 95% CI) and 96% (Histology *vs.* Culture, 91.6–100 of 95% CI).

Isolation of *H. pylori* and phenotypic characterization. A total of 114 samples were taken, distributed as follows: 53/114 (46.5%) from the antrum and 61/114 (54%) from the body; from which 122 *H. pylori* isolates were obtained. The prevalence of *H. pylori* as function of isolation origin was 59/122 (48.4%) for antrum and 63/122 (51.6%) from the body. A total of 36/79 (46%) patients presented more than one isolates. By age the prevalence was 78 isolates in 52 patients ≤ 49 years (150%) and 44 in 27 patients ≥ 50 years (163%). By gender 80 isolates in 54 female patients and 42 isolates in 25 male patients were found. All isolates grew as small transparent color colonies and satisfy the following characteristics Gram-negative curved rods, oxidase positive, catalase positive and urease positive (Ansorg *et al.*, 1991).

Frequency of *cag*A, *vac*A, *bab*A2, and *ice*A genes. Molecular characterization showed that genes frequency were: *cag*A+ 71% (86/122), *bab*A2+ 57% (70/122), *ice*A1 87% (106/122), *vac*As1am1 34% (42/122), vacAs1am2, 32% (39/122), *vac*As1bm1 8% (10/122), *vac*As1bm2 3% (3/122), *vac*As1cm1 4% (5/122), *vac*As1cm2 7% (9/122), *vac*As2m1 4% (5/122) and *vac*As2m2 7% (9/122). No significant differences were found between antrum and body distribution of these genotypes with P values ranging from 0.073 to 0.87. Nine of 79 patients (11%) presented the same genotype in both antrum and body, whereas in 28/79 (35%) of patients was possible to isolate different genotypes. In one of these patients (1/28; 4%), 4 different genotypes of *H. pylori* were found. In the rest 42 (53%) of patients only was possible to isolate *H. pylory* strains from one each other gastric location.

When analyzing the presence of all the studied genes, 28 combinations were covering the total isolates. The most frequent combinations were *cag*A+/*vac*As1am1/*bab*A2+/*ice*A1 and *cag*A–/*vac*As1am1/*bab*A2+/*ice*A1; both appeared in 15% (17/114) of the samples, with a greater distribution in males (24%) than in females (15–19%). In the same way these genotypes were the most common regardless of the age (Table II).

In this study, 28% of patients reported a family history of gastric cancer. We obtained 36 isolates from those patients, corresponding to 30% (36/122) of the total isolates covered in our study; meaning a prevalence of 164% (36/22). The most frequent virulence genotypes in this population were: *cag*A+ (27/36; 75%), *bab*A2+ (18/36; 50%), *ice*A1 (29/36; 81%), *vac*As1am2 (29/36; 81%) and *vac*As1am1 (14/36; 39%). Further

Table I
Primers and PCR conditions

Genes	Primers	Product size (bp)	Initial denaturation temperature °C (min)	Denaturation temperature °C (min)	Annealing temperature °C (min)	Extension temperature °C (min)	Cycles	Final extension temperature °C (min)	Ref.
*cag*A	TTGACCAACAACCACAAACCGAAG	183	94 (9)	95 (0.5)	50 (0.75)	72 (0.75)	40	72 (5)	(Van Doorn *et al.*, 1998)
	CTTCCCTTAATTGCGAGATTCC								
*vac*As1/s2	ATGGAAATACAACAAACACAC	259 (s1) 286 (s2)	95 (2)	95 (0.5)	52 (0.5)	72 (0.5)	40	72 (5)	(Atherton *et al.*, 1995; Erzin *et al.*, 2006)
	CTGCTTGAATGCGCCAAAC								
*vac*As1a	GTCAGCATCACACCGCAAC	190		94 (1)	52 (1)	72 (1)	35	72 (5)	(Erzin *et al.*, 2006)
	CTGCTTGAATGCGCCAAAC								
*vac*As1b	AGCGCCATACCGCAAGAG	187		94 (1)	52 (1)	72 (1)	35	72 (5)	(Erzin *et al.*, 2006)
	CTGCTTGAATGCGCCAAAC								
*vac*As1c	TTAGTTTCTCTCGCTTTAGTRGGGYT	220		94 (1)	52 (1)	72 (1)	35	72 (5)	(Erzin *et al.*, 2006)
	CTGCTTGAATGCGCCAAAC								
*vac*Am1	GGTCAAAATGCGGTCATGG	290	95 (2)	94 (0.5)	52 (0.5)	72 (0.5)	40	72 (5)	(Atherton *et al.*, 1995)
	CCATTGGTACCTGTAGAAAC								
*vac*Am2	GGAGCCCCAGGAAACATTG	352	95 (2)	94 (0.5)	52 (0.5)	72 (0.5)	40	72 (5)	(Atherton *et al.*, 1995)
	CATAACTAGCGCCTTGCAC								
*ice*A1	GTGTTTTTAACCAAAGTATC	247	95 (2)	94 (0.5)	50 (0.5)	72 (0.5)	40	72 (5)	(Van Doorn *et al.*, 1998; Chomvarin *et al.*, 2008)
	CTATAGCCASTYTCTTTGCA								
*ice*A2	GTTGGGTATATCACAATTTAT	229	95 (2)	94 (0.5)	50 (0.5)	72 (0.5)	40	72 (5)	(Chomvarin *et al.*, 2008)
	TTTCCCTATTTTCTAGTAGGT								
*bab*A2	AATCCAAAAAGGAGAAAAAGTATGAAA TGTTAGTGATTTCGGTGTAGGACA	832	95 (5)	92 (1)	52 (1)	72 (1)	35	72 (5)	(Mizushima *et al.*, 2001; Erzin *et al.*, 2006)

Table II
Most prevalent genotypes according to: gastric location (antrum or body), gender (male or female) and age (≤ 49 or ≥ 50 years). Several patients had more than one isolate.

Genotypes	Gastric location Isolate/# samples from location (%)			Gender Isolate/# samples from gender (%)			Age (years) Isolate/# samples from age group (%)		
	Antrum	Body	P	Female	Male	P	≤ 49	≥ 50	P
cagA+/vacAs1am1/babA2+/iceA1	**9/53(17)**	**8/61(13)**	0.74	**8/54(15)**	**6/25(24)**	0.74	**7/52(14)**	**7/27(26)**	0.05
cagA–/vacAs1am1/babA2+/iceA1	**9/53(17)**	**8/61(13)**	0.15	**8/54(15)**	**7/25(24)**	0.018	**7/52(14)**	**7/27(26)**	0.98
cagA+/vacAs1am2/babA2–/iceA1	4/53(8)	**10/61(16)**	0.23	**10/54(19)**	2/25(8)	0.006	**6 /52(12)**	**6/27(22)**	0.31
cagA+/vacAs1am2/babA2+/iceA1	4/53(8)	**8/61(13)**	0.05	6/54(11)	4/25(16)	0.23	**7/52(14)**	3/27(11)	0.81
cagA–/vacAs1am2/babA2+/iceA1	4/53(8)	4/61(7)	0.59	6/54(11)	2/25(8)	0.1	5/52(10)	3/27(11)	0.59
cagA+/vacAs1am1/babA2–/iceA1	1/53(2)	7/61(12)	0.007	5/54(9)	3/25(12)	0.59	4/52(8)	4/27(15)	0.59
Other 22 genotyes	28/53(53)	18/61(30)	––	32/54(59)	12/25(38)	––	24/52(46)	16/27(59)	––

In bold the most prevalent genotypic combination. Genotypesthiner writing: have been previously associated with treatment failure

genotype analysis shows that the most frequent combined genotypes were *cag*A+/*vac*As1am2/*bab*A2–/*ice*A1, *cag*A+/*vac*As1am1/*bab*A2+*ice*A1 and *cag*A+/*vac*As1am1/*bab*A2–*ice*A1 (Table III). Only one patient reported a family history of MALT lymphoma; in this patient just one isolate was found possessing the genotype *cag*A+/*vac*As1am1/*bab*A2+/*ice*A1.

In 27 of 79 (34%) of patients was possible to isolates more than one *H. pylori* strain (multiple infection). The frequency of multiple infections in female was 32% (17/54), in male was 40% (10/25); by age was 29% (15/52) patients' ≤ 49 years and 44% (12/27) patient's ≥ 50 years.

Table III
Distribution of most frequent genotypes among isolates from patients with a family history of gastric cancer.

Genotypes	Frequency of genotypes # isolates with a specific genotype / # total isolates or patients wit family history of GC (%)	
	Isolates	Genotypes prevalence
*cagA+/vacAs1am2/**babA2–**/iceA1*	5/36 (14)	5/22(23)
cagA+/vacAs1am1/babA2+/iceA1	4/36 (11)*	4/22(18)
*cagA+/vacAs1am1/**babA2–**/iceA1*	4/36 (11)	4/22(18)
cagA+/vacAs1am2/babA2+/iceA1	3/36 (8)	3/22(14)
*cagA–/vacAs2m2/**babA2–**/iceA1*	2/36 (6)	2/22(9)
*cagA+/vacAs2m1/**babA2–**/iceA1*	2/36 (6)	2/22(9)
cagA–/vacAs1am1/babA2+/iceA1	2/36 (6)*	2/22(9)
cagA+/vacAs1cm2/babA2+/iceA1	2/36 (6)	2/22(9)
Other 12 genotypes	12/36 (33.3)	12/22(54.5)

GC: Gastric cancer. Genotypes in red: previously associated with treatment failure. Genotypes in bold: previously described as less virulent (Correa *et al.*, 2000; Sugimoto and Yamaoka, 2009).
* Most frequent genotypes in full study.

Discussion

The clinical development of *H. pylori* infection is determined by the interaction of several factors of the host and the bacteria. Among the factors of the microorganism, there are evidences of the involvement of certain *H. pylori* genotypes in more severe pathologies (Van Doorn *et al.,* 1998; Höcker and Hohenberger 2003; Caner *et al.,* 2007). In this study we determined the frequency of *cag*A, *vac*A, *bab*A2, and *ice*A in 122 *H. pylori* isolates from 79 strictly selected patients with functional dyspepsia.

Isolation of *H. pylori* and phenotypic characterization. Our 122 isolates were almost equally distributed in the body and the antrum, 49.6% and 50.4% respectively; suggesting that any of the two sites can be used as sample sources for the cultivation of *H. pylori*, long as patients had not been taking proton pump inhibitors.

Genotyping *cag*A, *vac*A, *bab*A2, and *ice*A genes. As in previous investigations, (Höcker and Hohenberger, 2003; Mégraud and Lehours, 2007) in this study the *cag*A gene was found in 71% of the isolates, a finding that is consistent with previous domestic studies conducted by Citelly *et al.* (2002) who found it in 63.7% of the isolates and Quiroga *et al.* (2005) who reported 72.9% (Cittelly *et al.*, 2002; Quiroga *et al.*, 2005). Studies in other Latin American countries have found similar prevalence of the *cag*A gene, *i.e.* in Brazil, Ribeiro *et al.*, (2003) and Oliveira *et al.* (2003) reported the presence of *cag*A in 67% and 79.8% respectively, and in Cuba the prevalence reported was of 70.7% (Gonçalves Oliveira *et al.,* 2003; Lima Ribeiro *et al.*, 2003; Martínez Echavarría *et al.*, 2008).

Analysis of *vac*A gene alleles showed the presence of eight combinations between s1 and s2 with m alleles. The most frequent combination was the allele *vac*As1m1. These results are consistent with other

studies (Cittelly *et al.*, 2002; Quiroga *et al.*, 2005; Torres *et al.*, 2009) and consistent with the findings of Yamaoka *et al*. in 1999, who found a prevalence of 45% for *vac*As1am1 in Colombian patients with chronic gastritis (Yamaoka *et al.*, 1999).

The fact that virulent strains were the most frequent in patients with functional dyspepsia, in opposition to the concept that in this pathology less aggressive strains are the ones generally present, (Rudi *et al.*, 1999; Zambon *et al.*, 2003) is of great interest. Bearing in mind that this study did not include patients with gastric cancer and/or duodenal ulcer, it is not possible to determine whether differences exist regarding the aggressiveness of the strains and the outcome of severe pathologies. However, considering that these virulent strains have also been associated with more severe histological changes such as atrophy or intestinal metaplasia (increased risk of gastric cancer), (Nogueira *et al.,* 2001; Soltermann *et al.*, 2007; Umit *et al.*, 2009) it is of great importance to investigate in a future, whether these *H. pylori* isolates increase the risk of gastric cancer.

Moreover, we determined the presence of *bab*A2 and found that 57% of the isolates were *bab*A2+, which coincides with other South American studies that report gene frequencies ranging from 46% to 82.3% (Lobo Gattia *et al.*, 2005; Quiroga *et al.*, 2005; Paniagua *et al.,* 2009; Torres *et al.*, 2009). In the case of the populations studied, *bab*A2 genes are more often found in populations where the prevalence of duodenal ulcer and gastric cancer has been high (Gonçalves Oliveira *et al.*, 2003). It is important to note that *H. pylori bab*A2+ strains have been associated with increased risk of peptic ulcer and gastric cancer, while *bab*A2- strains have been associated with less severe forms of gastritis (Höcker and Hohenberger 2003). Although in this study the frequency of *bab*A2+ isolates (57%) was close to that of *bab*A2– isolates (43%); it is advisable to monitor patients with characteristics similar to those of patients included in this study to prevent future gastric complications.

In relation to the presence of *ice*A gene, the most frequent allele was *ice*A1 (87%); this result differs completely from that reported in previous studies (Yamaoka *et al.*, 1999; Cittelly *et al.*, 2002) in which the *ice*A2 allele was the most frequent. The high frequency of allele *ice*A1 is striking for two reasons: first, its role in gastroduodenal diseases is unknown; and secondly, its association with diseases is not clearly documented, and whiles some authors have found *ice*A1 association with peptic ulcer (Peek *et al.*, 1998; Van Doorn *et al.*, 1998) other authors have interpreted this finding as not significant (Yamaoka *et al.*, 1999; Podzorski *et al.*, 2003). The results of this study and the controversy regarding the *ice*A1 allele suggest a high probability of peptic ulcer development, but taking into account that the patients studied showed non atrophic chronic gastritis and that other works suggest that this genetic variant should not be dominant, the study of such associations and the assessment of this gene as a potential pathology marker should acquire importance.

Furthermore, we studied the distribution of each gene in the antrum and the body (Table II), finding that isolates *cag*A+ and *vac*As1am1 were distributed evenly across both. The allele *bab*A2+ was found more predominantly in the antrum, a location that could be related to the development of duodenal ulcer. Our results are consistent with those reported by Lopez *et al*., (López-Vidal *et al.,* 2008) who evaluated the presence of *cag*A and *vac*A in terms of anatomical location in patients with and without cancer. They found that in patients without cancer, the *cag*A+ strain distribution was uniform in both antrum and body. Concerning *vac*A, the authors did not report statistical significance of the genotype distribution in the stomach (López-Vidal *et al.,* 2008).

We analyzed also the complete combined genotype in all patients and observed the presence of 28 different combinations. The most frequent combined genotype was *cag*A+/*vac*As1am1/*bab*A2+/*ice*A1 in 15% of the cases, more often found in males than in females. This genotype was more common in patients aged ≥ 50 years; however, its presence did not differ significantly compared with patients aged ≤ 49 (Table II).

Few studies have analyzed the combination of these four genes and this is the first study in Colombia with a simultaneous evaluation of this genotypes; therefore, it is important to bear in mind that if patients infected with more virulent genotypes are untreated or, alternatively, if treatment failure has occurred, they could develop more severe diseases such as ulcers and adenocarcinomas; especially considering the high prevalence of gastric cancer and the reported associations between pathology and virulence genotypes.

Considering that patients included in the study had functional dyspepsia and having found which of genotypes were the most virulent, we studied the complete genotype distribution taking into account the family history of gastric cancer and MALT lymphoma. We found that 28% of the isolates came from 22 patients who reported having in their family history gastric cancer. The most frequent genotype in this group was *cag*A+/*vac*As1am2/*bab*A2+/*ice*A1, (Table III); at the same time, the presence of less virulent genotypes was less frequent. Only one patient reported a family history of MALT lymphoma and had the genotype *cag*A+/vacAs1am1/*bab*A2+/*ice*A1.

It is known that family history of gastric cancer is associated with increased risk of atrophic gastritis, intestinal metaplasia and gastric cancer; on the other hand, the infection by genetically similar strains of *H. pylori*

in patients with a family history of gastric cancer is an important factor to be considered in the clinical development of infection, (Miehlke *et al.,* 2001; Chang *et al.,* 2002) especially if there is a maternal history for this type of cancer (Yamaoka *et al.,* 2000). By this reason we propose a rigorous monitoring of patients with a family history of gastric cancer and presenting infection by virulent genotypes of *H. pylori*.

In relation to full virulence genotypes, we found that 27 patients were infected by more than one genotype of *H. pylori*; a finding that is consistent with other reports in Latin America and it was reason of prevalence over 100% at present study (Cittelly *et al.*, 2002; Podzorski *et al.*, 2003; Lobo Gattia *et al.*, 2005; García C *et al.*, 2006). It has been suggested that the presence of various *H. pylori* genotypes in the same patient could be an advantage for the bacteria to easily survive in different ecological niches of the gastric mucosa, or that it could generate a competition among strains to survive and predominate in the mucosa environment (Blaser 1997; Figueiredo *et al.,* 2001). When multiple infections occur, it is not known with certainty which one of genotypes is causing the pathology or the treatment failure.

In conclusion, we found that the most frequent genotypes in *H. pylori* isolates were: *cag*A+, *vac*As1am1, *bab*A2 and *ice*A1. This is new evidence and an important finding, taking into account that these genotypes are the most virulent, that is often associated with the development of severe pathologies, and that 90% of the patients included in this study did not reveal any precursor lesions for gastric cancer. Our finding reinforces the importance of the interplay between the virulence of *H. pylori* and host susceptibility in the development of more severe illness (Amieva and El-Omar, 2008). In relation to patients with or without a family history of gastric cancer, there were no significant differences regarding the genotypes of isolates.

Acknowledgments

This work was supported by COLCIENCIAS (Colombia), grant number 120340820464, PUJ project ID 002242. Finally, we thank Sandra Constantino for copy-editing the English manuscript.

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Polish Journal of Microbiology
2012, Vol. 61, No 1, 41–49

ORIGINAL PAPER

Impact of Operational Parameters on Bacterial Community in a Full-Scale Municipal Wastewater Treatment Plant

AGNIESZKA CYDZIK-KWIATKOWSKA*, MAGDALENA ZIELIŃSKA and IRENA WOJNOWSKA-BARYŁA

University of Warmia and Mazury in Olsztyn, Department of Environmental Biotechnology

Received 16 June 2011, revised 12 December 2011, accepted 14 December 2011

Abstract

A bacterial community in activated sludge from a full-scale municipal wastewater treatment plant was monitored throughout the year with the use of FISH, RISA and DGGE techniques. In the investigated range of temperatures (11.9–21.6°C), a rise in temperature resulted in a lower total bacteria richness, while organic load rate changes from 0.09 to 0.21 g COD·g $TSS^{-1}\cdot d^{-1}$ were positively correlated with the number of bands in RISA patterns. The most diverse pattern (29 different bands) was characteristic for the activated sludge sample collected at the end of January at wastewater temperature of 11.9°C. The ammonia-oxidising bacteria community did not change during the study, and comprised of 4 different bacterial populations with one dominant species closely related to *Nitrosospira* sp. REGAU (GenBank accession number AY635572.1). The percentage of ammonia-oxidising bacteria in the activated sludge varied from 6.2 to 19.5% and depended on temperature ($R=0.61$, $p=0.02$) and organic load rate ($R=-0.55$, $p=0.04$).

Key words: ammonia-oxidising bacteria, DGGE, FISH, molecular analysis of activated sludge, RISA

Introduction

One of the significant aspects of microbial community investigations is the evaluation of the impact of environmental conditions on bacterial consortia. In engineered systems, *e.g.* wastewater treatment plants there is a possibility to manipulate the technological parameters of the process and as a result to influence the microbial communities involved in removal of pollutants. It has recently been suggested that the diversity of specific bacterial groups in activated sludge influences the functioning of the reactor (Daims *et al.*, 2001). The presence of many microorganisms able to conduct a specific process increases the probability that a change of environmental conditions does not worsen the effectiveness of wastewater treatment, since one of the species will adapt and maintain the specific metabolic pathway (LaPara *et al.*, 2002). Taking this into consideration, the operational parameters of the wastewater treatment plant should be selected to favour the development of a highly diversified bacterial community.

Ammonia-oxidising bacteria (AOB) are of universal importance in activated sludge community since ammonia oxidation is the limiting step for nitrogen removal from wastewater. The growth rate of AOB is slow and their population activity may be affected by operational/environmental factors *e.g.* solids retention time, ammonium and organic carbon concentration in the influent, pH or temperature (Nogueira *et al.*, 2002; Avrahami *et al.*, 2003; Kuo *et al.*, 2006). Research by Siripong and Rittmann (2007) showed that monitoring of changes in the nitrifiers community can assist plant operators in preventing nitrification failure and the washing out of these bacteria from the system. It is therefore important to evaluate an impact of the operational parameters of the wastewater treatment process on AOB community in activated sludge.

The use of molecular techniques allows an insight to be gained into changes in bacterial communities in activated sludge in relation to environmental conditions. Much recent research focuses on investigating bacteria involved in wastewater pollutants removal (Onuki *et al.*, 2000; Ariesyady *et al.*, 2007; Zhao *et al.*, 2008) but information about the relation between bacteria diversity and operating parameters is still limited. Juretschko *et al.* (2002) showed that consortia of microorganisms are influenced by wastewater composition and that diversity of bacteria is higher in systems treating municipal wastewater in comparison to industrial sewage treatment plants. Rowan *et al.* (2003) revealed that the bacterial diversity is linked to the type of bioreactor. The research indicated a higher diversity of

* Corresponding author: A. Cydzik-Kwiatkowska; Słoneczna 45G, 10-709 Olsztyn, Poland; phone: +48 895234185; fax: +48 985234131; **e-mail: agnieszka.cydzik@uwm.edu.pl**

AOB in the trickling filter than in the biological aerated filter (BAF), and that the performance of the trickling filter was better than the BAF. Cydzik-Kwiatkowska and Wojnowska-Baryła (2008) proved that a higher AOB diversity was observed when only carbonates were present in the wastewater in comparison to conditions in which sodium acetate was introduced to the reactors and that a lower ammonia nitrogen load favoured a more diverse AOB community.

In the present study we focused on determining the impact of technological parameters on ammonia-oxidising and total bacteria community in activated sludge from a full-scale municipal wastewater treatment plant operating in temperate climate. In our research we combined fluorescent *in situ* hybridisation (FISH), Denaturing Gradient Gel Electrophoresis (DGGE), Ribosomal Intergenic Spacer Analysis (RISA) and DNA sequencing in order to obtain comprehensive results.

Experimental

Materials and Methods

Description of wastewater treatment plant. A one-year study was conducted on a full-scale activated sludge wastewater treatment plant (WWTP) located in Poland (temperate climate). The WWTP treats on average 30,000 m^3 of municipal wastewater per day and discharges it to the Łyna River. The treatment unit of the plant involves grates, grit chambers, primary clarifiers, an anaerobic chamber, predenitrification chamber, aeration chambers for simultaneous nitrification and denitrification (16,380 m^3), and secondary clarifiers.

Activated sludge sampling. 14 activated sludge samples were collected at different dates from the WWTP. Three samples were collected in autumn: 1 – 14.10.2005, 2 – 8.11.2005, and 3 – 5.12.2005, three in winter: 4 – 03.01.2006, 5 – 31.01.2006, and 6 – 27.02.2006, four in spring: 7 – 27.03.2006, 8 – 24.04.2006, 9 – 10.05.2006, and 10 – 01.06.2006, and four in summer: 11 – 27.06.2006, 12 – 26.07.2006, 13 – 4.09.2006, 14 – 20.09.2006. Samples were collected directly from the aeration tank, transported on ice to the laboratory (< 30 min) and processed immediately.

Analytical methods. Complete operating results were delivered by the WWTP laboratory, which monitors the activated sludge process daily. Analyses were performed according to Polish Standards (www.pkn.pl). Computations of technological parameters such as sludge retention time (SRT) or nitrogen use for biomass synthesis were performed as described in Metcalf and Eddy (1991).

Molecular analyses of activated sludge samples. Genomic DNA was isolated in duplicate from 400 mg of centrifuged sludge sample using FastDNA® SPIN®Kit (Q-BIOgene), according to the manufacturer's protocol. Quality and quantity of isolated DNA was measured spectrophotometrically using Biophotometer (Eppendorf). The DNA extracted from a sample was mixed and frozen at –20°C for further analyses.

All PCR reactions were performed in Eppendorf Mastercycler Gradient (Eppendorf). The bacterial RIS (Ribosomal Intergenic Spacer) was amplified with primers *1* and *2* (Table I). Amplified fragments contained RIS plus approximately 380 bp corresponding to flanking regions of genes coding for 16S and 23S rRNA. The PCR mixture contained 50 ng of extracted total DNA, 0.5 µM of each primer, 100 µM of deoxynucleoside triphosphate mixture (Promega), 1.5 U of GoTaq® DNA Polymerase (Promega), 6 µl of reaction buffer supplied with polymerase, 1.5 mM $MgCl_2$ and sterile water to a final volume of 30 µl. The PCR amplification was carried out using the following program: 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 43°C for 45 s, extension at 72°C for 1 min, and a final elongation at 72°C for 5 min. The presence of PCR products was confirmed by analysing 5 µl of the product on a 0.8% agarose gel stained with ethidium bromide.

After successful DNA amplification, 5 µl of PCR products were applied to 6% polyacrylamide gel (29:1 acrylamide:bisacrylamide). Electrophoresis was carried out at 60 V in 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA; pH 8.0). In order to avoid overlapping of bands and fully exploit the microbial diversity, each sample was electrophoresed for 60, 120 and 180 min and all photos obtained were used for further analysis. After electrophoresis, the gel was

Table I
PCR primers used in the study

Primer	Sequence (5'-3')	Annealing temperature	Target sequence	Source
amoA-2R *amoA-1F*	ccc ctc tgc aaa gcc ttc ttc [a]ttt cta ctg gtg gt	60°C	*amoA*	Rotthauwe *et al.* (1997) (modified by Nicolaisen and Ramsing, 2002)
1 *2*	ttg tac aca ccg ccc gtc a gt act tag atg ttt cag ttc	43°C	*RIS*	Dolzani *et al.* (1995)

[a] – cgc cgc gcg gcg ggc ggg gcg ggg gcg ggg

Table II
Probes used for hybridisation

Probe	Sequence (5'-3')	Label	% formamide	Specificity
EUB338	GCTGCCTCCCGTAGGAGT	FITC	20	Eubacteria
Nso190	CGATCCCCTGCTTTTCTCC	Cy3	55	Majority of AOB: *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus, Nitrosovibrio*, *Nitrospira*

stained with SYBRgold (Molecular Probes) at 10,000x dilutions in 1x TBE buffer for 30 min, viewed with an ultraviolet transilluminator and recorded with CCD camera (Gel Logic 200, Eastman Kodak Company). Bands were detected automatically from digital images of the gel using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company). The size of PCR products was estimated using 100 bp O'GeneRuler ladder (Fermentas).

Amplification of *amoA* gene fragment for DGGE was performed as in RIS reactions, except for applied primers and annealing temperature (Table I). DGGE electrophoresis was performed with a D-CODE Universal Mutation System (Bio-Rad). The PCR samples were applied to 8% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) in a 0.5 × TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.5) with a denaturing gradient ranging from 30 to 70%. Denaturation of 100% corresponded to 7 M urea and 40% formamide. Electrophoresis was run at a constant voltage of 80 V at 60°C. After electrophoresis, the gel was processed as described above. The most intense band was cut from the gel, reamplified using specific primers (amoA-1F with no GC clamp) and sequenced by a specialist laboratory (www.oligo.ibb.waw.pl). The nucleotide sequence was compared with sequences in the GenBank using the BLASTn program (Altschul *et al.*, 1997) and deposited in the GenBank under accession no. GU073249).

The biomass used for FISH analysis was fixed according to Amann *et al.* (1990). The prepared sample was vortexed and kept at 4°C for 1 day. After centrifugation (3 min, 4,000 rpm), the supernatant was removed and the sample was resuspended in a mixture of 1x PBS and 96% ethanol (volume ratio of mixture components 1:1) and stored at a temperature of –20°C. For analysis, 10 µl of sample was spread on each well of the glass slides (Marienfeld Laboratory Glassware), dried for about 10 min at 46°C, and dehydrated by serial immersions of the slide in 50, 80, and 96% (v/v) ethanol (3 min each). The composition of the hybridisation buffer was the following: 180 µl of 5 M NaCl, 20 µl of 1 M TrisHCl (pH 8.0), 550 µl of formamide, 250 µl of ultra clean water and 2 µl of 10% (w/v) SDS. The washing buffer, containing 100 µl of 5 M NaCl, 1000 µl of 1 M TrisHCl (pH 8.0), 500 µl of 0,5 M EDTA (pH 8.0), 50 µl of 10% (w/v) SDS, and distilled water to complete the buffer to 50 ml, was preheated in a water bath to 48°C. Two molecular probes, as proposed by Mobarry *et al.* (1996) were used for hybridisation (Table II). In order to detect Eubacteria, only one molecular probe EUB338 was used (Chae *et al.*, 2008) that would make it simpler to utilize the FISH method in wastewater treatment plants.

A mixture of 140 µl of hybridisation buffer and molecular probes (10 pmol/µl) was prepared and placed in each well. The slide was transferred into the hybridisation chamber and incubated at 46°C for 3 h. After hybridisation the slide was incubated in the washing buffer for 10 min in a preheated water bath at 48°C. The slide was rinsed with cold distilled water and dried. Slides were mounted in VectaShield (Vector Laboratories) embedding medium. Epifluorescence microscopy by Nikon Eclipse (Nikon, 100x objective) was used for examination. For a reproducible and statistically correct result, 30 images for each probe taken from 30 different positions of the slide were analysed (Hall *et al.*, 2003).

The area covered by specific probe Nso190 and the area covered by the EUB338 probe in the same field of the slide glass, taken as images, were calculated by using ImageJ software (National Institutes of Health, USA). The occupation ratio of the area given by Nso190 to the area from EUB338 gives a direct measurement of the number of active ammonia-oxidising bacteria (Rittmann *et al.*, 1999).

Statistical analysis. The relationships between bacterial richness and chemical parameters in the effluent of the treatment facility were determined by correlation analyses using the STATISTICA 7.0 programme (StatSoft, USA). The strength of the correlation was evaluated according to Stanisz (2000). Analyses were carried out at a confidence interval of 95%. In the text, after ± the standard deviation is given. On the basis of RISA patterns, distance matrix analyses were performed according to the method of Nei Li (Nei and Li, 1979), using the DGGEstat 1.0 programme (van Hannen, the Netherlands Institute for Ecological Research, NIOO-KNAW, the Netherlands). Binary sequences were generated by determining the number and position of bands in individual lanes compared to all of the band positions detected in all of the lanes. The samples were clustered using the unweighted pair group method of arithmetic averages (UPGMA), bootstrapping was conducted with 1000 replicates.

Results and Discussion

In a temperate climate one of the major factors influencing biological wastewater treatment efficiency is temperature. In this study the temperature of wastewater fluctuated considerably throughout the year. The maximum temperature amplitude of wastewater during the study period was 9.7°C (Fig. 1a).

The operating data for the facility are presented in Table III. On the base of organic (B_C) and nitrogen (B_N) load rates it can be concluded that the plant represented a system with a low load rate. The average concentration of organic compounds expressed as COD in the influent was 416.1 ± 66.1 mg COD/l, while in the effluent during the year it averaged 37.0 ± 5.1 mg COD/l (Fig. 1b). COD removal maintained at the level of 90.8 ± 2.5%. The average concentration of total nitrogen (TN) in the influent was 62.1 ± 8.9 mg N/l. The highest TN concentration in the effluent of 20.8 and 16.1 mg N/l was observed at the turn of winter and spring (Fig. 1c). During the rest of the year this value did not exceed 13 mg N/l. A high percentage of total nitrogen removal (84.1 ± 6.4%) provided evidence of nitrification. It was calculated that 6.4 ± 2.9 mg N/l was used for biomass synthesis. Regarding phosphorus concentration, in the influent it averaged 10.6 ± 3.0 mg P/l, while in the effluent it was in the range of 0.3–1.7 mg P/l, except the sample for January, when the concentration of phosphorus increased to 4.1 mg P/l (Fig. 1d). The average efficiency of phosphorus removal in the system was 89.4 ± 8.9%.

An identification of microorganisms involved in pollutants removal gives us some information about properties of activated sludge, however, the complexity of factors influencing activated sludge in WWTP results in extremely variable microbial structure that is unlikely to occur in another WWTP. It is therefore more important to reveal general dependences between the microbial community as a whole and technological parameters of the process. In our research the bacterial

Table III
Summary wastewater treatment plant operating data during the study period.

Parameter	Mean value ± standard deviation
Sludge volume index (ml/g)	260.0 ± 77.0
Total suspended solids (mg TSS/l)	5229.0 ± 1117.2
Organic loading (g COD·g TSS^{-1}·d^{-1})	0.15 ± 0.03
Nitrogen loading (g N·g TSS^{-1}·d^{-1})	0.02 ± 0.005
COD/TKN (g COD/g N)	6.8 ± 1.3
Sludge yield (g TSS/g COD)	0.3 ± 0.1
HRT (h)	13.0
SRT (d)	21.6 ± 14.9

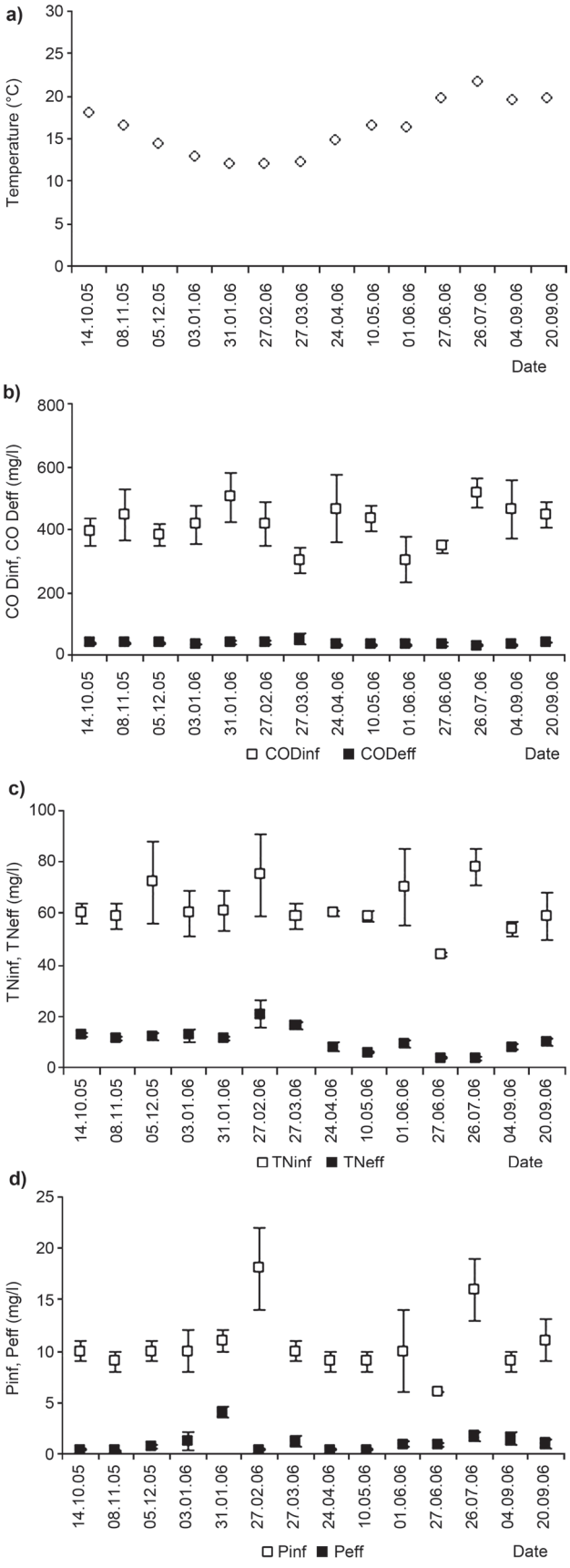

Fig. 1. Temperature (a) and influent ($_{inf}$) and effluent ($_{eff}$) quality of wastewater: COD (b), total nitrogen (c), phosphorus (d).
Each point is the mean of 4 measurements, vertical bars indicate standard deviation.

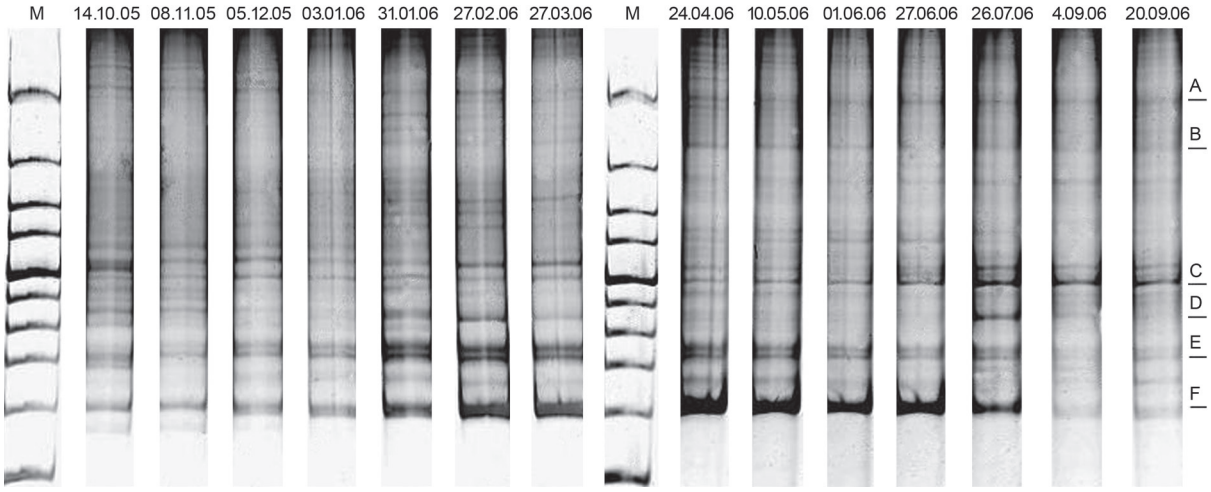

Fig. 2. RISA fingerprints of bacterial community from activated sludge.
Lanes are labelled with the dates of sampling.
Exemplary bands are marked with capital letters, M – molecular weight marker 100 kb O'GeneRuler (Fermentas).

community changed moderately throughout the year while the effluent quality remained relatively stable. The RISA fingerprints were complex, and the mean number of bands per lane was 22.1 ± 4.2 (Fig. 2). Several bands were present only temporarily (*e.g.* bands labelled B and D), while some bands were detectable throughout the whole year, though their intensity varied sometimes with time (*e.g.* bands A, C, E, F). The intensity of band F was significantly higher during the period from March to June, suggesting that this species was present in the highest number in spring. It is probable that the bands detected in all the samples are the crucial bacterial populations for activated sludge performance. The richness of bacterial consortia expressed by the number of different bands in the RISA pattern fluctuated during the year (Fig. 3). The most diverse patterns were characteristic for activated sludge samples collected at the end of the winter and at the beginning of the spring. The highest number of bands (29) was noted in the sample collected 31.01.2006.

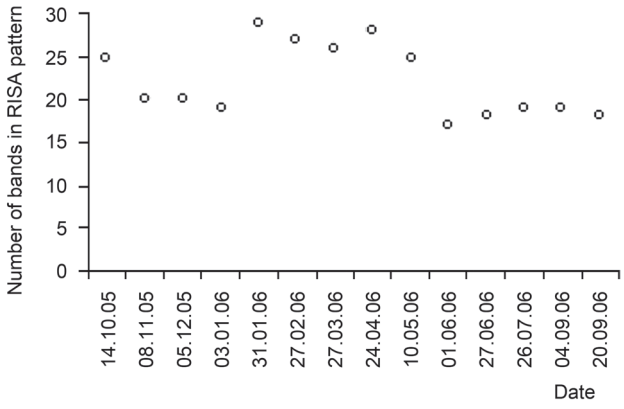

Fig. 3. The number of bands in RISA patterns obtained for activated sludge samples collected at given dates.
Grey vertical lines separate samples from different seasons of the year.

The higher temperature of wastewater leads to an increase in the population density and metabolic activity, and shortens the generation time of bacteria in activated sludge (Saleem *et al.*, 2003). Our research showed that a statistically significant correlation occurred between bacterial richness of sample and the temperature of wastewater (Fig. 4a). The value of Pearson's correlation rank (R = – 0.61) indicates that the negative correlation between the analysed parameters was strong. Moreover, richness of microorganisms was correlated with organic load rate (B_C) of the system (Fig. 4b). During the year of sampling B_C varied from 0.09 to 0.21 g COD · g $TSS^{-1} \cdot d^{-1}$ and bacteria richness increased with increasing B_C. Similar results were obtained by Xia *et al.* (2008). Authors investigated the microbial community structure response to different ratios of carbon to nitrogen (C/N 3:1, 5:1, 10:1) in wastewater in a suspended carrier biofilm reactor with simultaneous nitrification and denitrification. On the basis of DGGE and FISH analyses it was shown that total diversity of microbial community structure increased with increasing organic carbon concentration in wastewater. It is worth noting that the correlation obtained in our study is probably true only in a limited range of organic load rate. At a very high B_C, the diversity tends to decrease because a large quantity of organic carbon in wastewater stimulates dynamically growing bacteria with a very short generation time and these compete out other species and dominate the bacterial population.

Cluster analysis of RISA fingerprints revealed that the bacterial community changed gradually with the passage of time (Fig. 5). Three distinct branches were formed in the dendrogram. The first four samples formed the first branch, the following five the second branch which was connected to the third branch clasping the last five samples. The bootstrap values for the

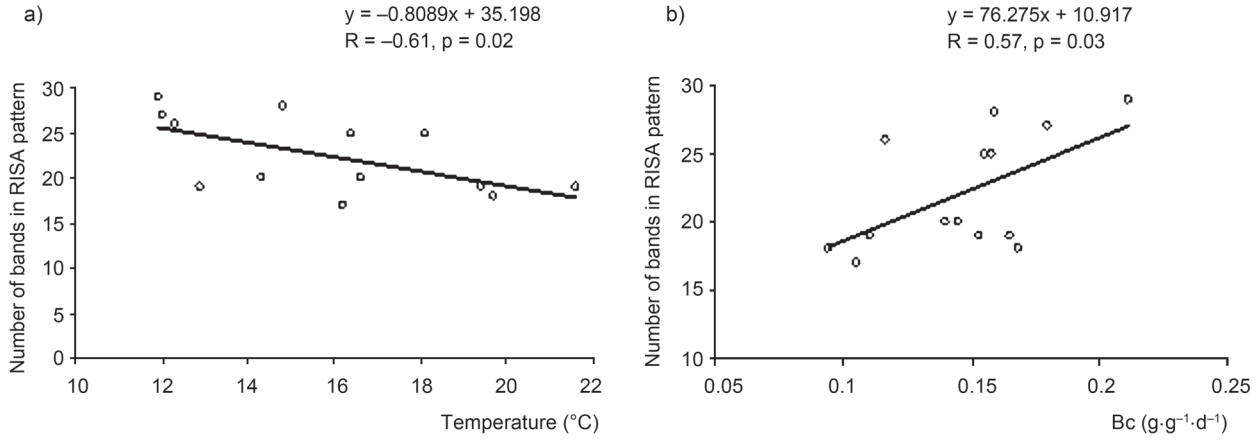

Fig. 4. Relationship between the number of bands in RISA pattern and a) wastewater temperature, b) organic load rate during the study

third branch was 90.7, suggesting that summer bacterial communities were the most similar to each other, and differed significantly from consortia developed in other seasons of the year. LaPara and Ghosh (2006) noted a similar situation in research carried out in a full-scale municipal wastewater treatment facility. DGGE fingerprints obtained on the basis of activated sludge samples from 25th June to 3rd September formed a distinct branch in the dendrogram.

DGGE of PCR-amplified *amoA* gene fragments revealed that there were 4 different AOB populations

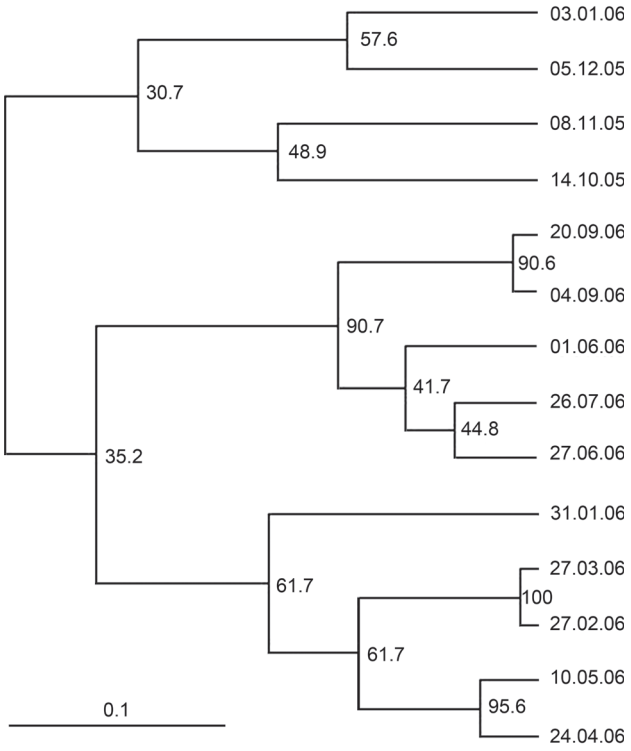

Fig. 5. Dendrogram revealing the relatedness of RISA fingerprints of the bacterial community from aeration tank of investigated wastewater treatment plant.

Branches are labelled with the dates of sampling; bootstrap values for branches are given at the nodes.

per sample, and that the ammonia-oxidising bacteria community did not change throughout the study (data not shown). The coexistence of various nitrifiers with different growth and survival characteristics may be valuable for maintaining the stability and performance of nitrifying bioreactors. Sequencing of the dominant band indicated that the partial *amoA* gene sequence was similar (89% similarity) to the sequence of ammonia monooxygenase from *Nitrosospira* sp. REGAU (GenBank accession number AY635572.1). LaPara and Ghosh (2006) investigated the AOB community in a full-scale municipal wastewater treatment facility using the nested PCR-DGGE technique. The fingerprint obtained was also dominated by one band. This band was, however, not phylogenetically related to any known AOB. Siripong and Rittman (2007), using the T-RFLP technique, examined the impact of temperature, sludge age, input of industrial wastewater and other technological parameters on the diversity of nitrifiers in seven water reclamation plants in two different seasons (winter and summer). Authors have shown that only the seasonal temperature variations changed the nitrifying community, in particular the balance between *Nitrosospira* and *Nitrosomonas*, however, both *Nitrosospira* and *Nitrosomonas* coexisted in winter and summer samples. Authors' conclusion is that a combination of low temperature and high SRT may favour *Nitrosospira*. Limpiyakorn *et al.* (2006) investigated AOB communities in 12 full-scale sewage activated sludge systems in three different seasons using PCR-DGGE-cloning-sequencing of 16S rRNA genes. However, in this case in all samples one of the identified sequence types of the *Nitrosomonas oligotropha* cluster was the dominant AOB in every system and every season studied.

FISH analyses revealed that the percentage of ammonia-oxidising bacteria in activated sludge decreased from 19.5 to 14.2% in autumn (Fig. 6), and to 6.2–9.7% in winter. In spring, the percentage of AOB increased to 14.9% and remained at a similar level until the end

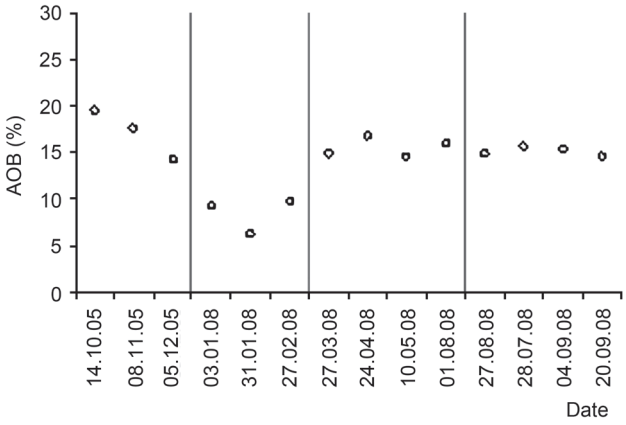

Fig. 6. Percentage of AOB in activated sludge samples collected at given dates.
Grey vertical lines separate samples from different seasons of the year.

of summer. The values obtained are consistent with the observations of Okabe *et al.* (1999) who reported that ammonia-oxidising bacteria comprise from 5 to 20% of bacteria in domestic wastewater systems. We showed that there was a significant correlation ($R = 0.61$, $p = 0.02$) between the wastewater temperature and percentage of AOB in activated sludge (Fig. 7a). The temperature dropped below 12°C in February which caused a decrease in AOB participation in the activated sludge to 6.2%. Low temperatures generally have a drastic effect on bacterial process rates and this is especially important when nitrification processes are concerned: below 15°C, the nitrification rate drops sharply, and is reduced by 50% at 12°C (Focht and Verstraete, 1977). However, Rittmann and Snoeyink (1984) mentioned that nitrification processes have the potential to achieve a good ammonia removal even at low temperatures. The critical aspect seems to be the maintenance of slow-growing nitrifying bacteria in the system. In our research, SRT applied at the level of 21.6 ± 14.9 d guaranteed that these essential bacteria would not be washed out from the wastewater treatment plant.

Throughout all the seasons studied the activated sludge process showed evidence of stable nitrification with efficiency at the level of 73.3–91.4%. No relationship between nitrification efficiency and ammonia-oxidising bacteria contribution in activated sludge ($R = 0.06$, $p = 0.83$) was noted. This result is not consistent with previous studies conducted in full-scale nitrifying trickling filters, suggesting that the concentration of AOB has an impact on effluent quality (Dionisi *et al.*, 2002). In the research of Ebie *et al.* (2002), the FISH method with 16S rRNA-targeted oligonucleotide probes was used for quantitative estimation of ammonia-oxidising bacteria in an anaerobic-aerobic activated sludge process with membrane filtration. At the beginning of the experiment, the occupation ratio of AOB increased as nitrification progressed. Later, similarly to our results, the occupation ratio decreased, but good nitrification ability was, however, preserved.

The fact that organic carbon influences AOB populations in wastewater treatment systems is discussed in the literature (Lazarova *et al.*, 1998; Nogueira *et al.*, 2002). Organic carbon compounds in wastewater stimulate heterotrophic bacteria which compete for oxygen with nitrifiers and may produce intermediary metabolic byproducts, which inhibit AOB (Gieseke *et al.*, 2001). Xia *et al.* (2008) showed that the population of nitrifiers was inversely proportional to the C/N ratio in wastewater with an average fraction of AOB and nitrite-oxidising bacteria (NOB) to all bacteria of 5.4, 4.8, 3.1% and 4.6, 3.5, 2.7%, respectively, as the C/N ratio changed from 3:1, 5:1 to 10:1. Our research was conducted in a full-scale municipal wastewater treatment plant with a low organic load rate at a level of 0.15 ± 0.03 g COD × g $TSS^{-1} \times d^{-1}$. Even relatively low organic load rate, however, influenced the AOB population in activated sludge (Fig. 7b; $R = -0.55$, $p = 0.04$). In practice, the organic load rate in a wastewater treatment plant can be easily adjusted. On the basis of our research it may be concluded that the negative impact

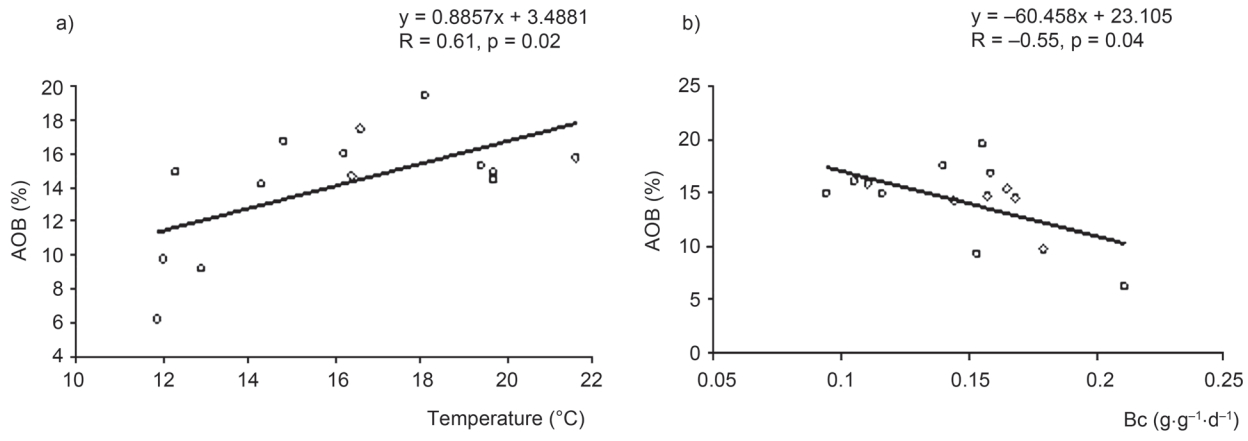

Fig. 7. Relationship between the participation of AOB in activated sludge and a) wastewater temperature, b) organic load rate

of low temperatures on AOB participation in activated sludge can be minimized by lowering the B_C value.

Conclusions. Presented research possesses utilitary values. It allows observing general trends in microorganisms' consortia during the year of plant operation in temperate climate, and characterizing the bacterial community, especially AOB, in activated sludge, in correlation with operational parameters of a full scale WWTP. The following conclusions may be drawn from the research:

1. The total bacterial community in activated sludge changed moderately with the passage of time. Bacterial communities from summer samples were the most similar to each other and differed significantly from consortia developed in other parts of the year.
2. In investigated range of values the total bacteria richness was correlated with the two parameters of wastewater treatment process that is the temperature ($R = -0.61$, $p = 0.02$) and organic load rate ($R = 0.57$, $p = 0.03$).
3. The percentage of AOB in activated sludge varied from 6.2 to 19.5% during the year of study and depended on temperature ($R = 0.61$, $p = 0.02$) and organic load rate ($R = -0.55$, $p = 0.04$).
4. The AOB community comprised of 4 different bacterial populations and did not change during the investigation period, with one dominant species closely related to *Nitrosospira sp.* REGAU.

Acknowledgements

The authors are grateful to WWTP staff for providing the samples and data from the activated sludge system.

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