



Genetic markers for detection of *Escherichia coli* K-12 harboring ampicillin-resistance plasmid from an industrial wastewater treatment effluent pond

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ABSTRACT. Biotechnology industries that use recombinant DNA technology are potential sources for release of genetically modified organisms to the environment. Antibiotic-resistance marker genes are commonly used for recombinant bacteria selection. One example is the marker gene coding for β -lactamase (*bla*) in plasmids found in *Escherichia coli* K-12. The aim of this study was to provide an approach to develop a molecular method for genetic marker detection in *E. coli* K-12 harboring *bla* genes from an industrial wastewater treatment effluent pond (IWTEP). For the detection of *bla* and *Achromobacter lyticus protease I (api)* genes in samples from IWTEP, we employed multiplex polymerase chain reaction (PCR) using *E. coli* K-12 genetic marker detection primers, previously described in the literature, and primers designed in our laboratory. The microbiological screening

method resulted in 22 bacterial colony-forming units isolated from three different IWTEP harvesting points. The multiplex PCR amplicons showed that five isolates were positive for the *bla* gene marker and negative for the *E. coli* K-12 and *api* genes. The 16S rRNA regions of positive microorganisms carrying the *bla* gene were genotyped by the MicroSeq[®]500 system. The bacteria found were *Escherichia* spp (3/5), *Chromobacterium* spp (1/5), and *Aeromonas* spp (1/5). None of the 22 isolated microorganisms presented the molecular pattern of *E. coli* K-12 harboring the *bla* gene. The presence of microorganisms positive for the *bla* gene and negative for *E. coli* K-12 harboring *bla* genes at IWTEP suggests that the ampicillin resistance found in the isolated bacteria could be from microorganisms other than the *E. coli* K-12 strain harboring plasmid.

Key words: Wastewater treatment effluent; Genetic detection; *E. coli* K-12; Plasmid; *bla* gene; Ampicillin resistance

INTRODUCTION

Wastewater treatment plants receive waste from several sources, including residential, industrial, and recreational. Wastewaters generated from these sources contain liquids and human fecal discharge, other animals, household waste, industry-specific materials, and runoff from storm water channels. These materials are removed by primary, secondary, and tertiary sedimentation stages. The effluent is usually clarified; it can be recycled as nonpotable water and is normally disposed in receiving supplies of water (Kay et al., 2008; Anastasi et al., 2010). To minimize the risk of environmental contamination from release of fecal bacteria, the effluents are also disinfected using oxidative processes to destroy and disable these microorganisms (Anastasi et al., 2012). Inadequate procedures or precarious operations in effluent treatment stations may result in the survival of several microorganisms that may contaminate the surrounding environment and generate a public-health problem, especially if they are pathogenic (Anastasi et al., 2012; Frigon et al., 2013).

Strains of *Escherichia coli* have been used as indicators of fecal contamination in aquatic environments where they are normally considered to be nonpathogenic, although there are pathogenic strains of *E. coli* (Bower et al., 2005; Frigon et al., 2013). Quantification of *E. coli* in surface waters is used to evaluate the performance of wastewater treatment plants in reducing microbial load. However, many enteric pathogens, such as viruses, protozoa, and bacteria, have different survival rates, and they are known as indicators of fecal contamination. In addition, the existence of microbial lineages transporting virulence genes in wastewater treatment and their potential environmental release have not been investigated (Anastasi et al., 2012).

A large number of *E. coli* and different lineages derived from *E. coli* K-12 are emerging for use in biotechnology research and development, as well as for industrial production. *E. coli* K-12 is the most common host used in gene cloning experiments (Kuhnert et al., 1995). Although strains of *E. coli* K-12 are considered environmentally safe for activities involving recombinant DNA technology, the intact plasmids could be discarded into the environment, potentially becoming an environmental and public health problem. The vast majority of these plasmids contains antibiotic resistance genes for selection and is kept under selective pressure inside the bacteria.

There is considerable concern about antibiotic resistance in bacteria in humans and farm animals; however, the spread of antibiotic resistance in aquatic ecosystems has received less attention (Livermore et al., 2001, Literak et al., 2010). *E. coli* isolated from normal human intestinal bioburden and diverse populations of wildlife has presented resistance to a range of antibiotics (Wallace et al., 1997; Literak et al., 2010). The emergence of antibiotic-resistant bacteria poses a major threat to public health because it reduces the effectiveness of antimicrobial treatment, leading to an increase in morbidity, mortality, and health costs (Smith and Coast, 2002; Collignon et al., 2009; Amaya et al., 2012). The main determining factor in this process is the selective pressure generated by the use of antimicrobials in human and animal medicine, as well as in aquaculture and agriculture (Collignon et al., 2009; Kümmerer, 2009; Amaya et al., 2012), and potentially from genetic handling at in laboratories or industries. Therefore, the spread of antibiotic-resistant bacteria occurs in any environment (Baquero et al., 2008; Martinez, 2009; Amaya et al., 2012).

In Brazil, the National Biosafety Policy, with regard to genetically modified organisms (GMOs; including *E. coli* K-12 strains) is advised by the National Technical Biosafety Commission (CTNBio) through the establishment of technical standards of safety and technical advice regarding the use of GMOs and their derivatives (Conceição et al., 2006; Neves et al., 2007). The main aspects to be considered in the risk assessment of GMOs are the possible effects on other organisms upon their release to the environment and a possible transfer and expression of the new gene inserted, or transgene, in other species (Kuhnert et al., 1995). Thus, it is essential that the biotechnology industries develop methods for the detection of GMOs in their multiple production process steps in order to prevent release of GMOs to the environment (Miralles et al., 2009; Costa et al., 2011). The industrial effluent treatment station (IETS) is used to reduce the impacts of the release of toxic chemicals to the environment. However, many microorganisms, such as fecal coliforms that can acquire resistance to antibiotics present in IETS, are recorded in bodies of water without prior environmental risk assessment. These bacteria can transfer resistance to other organisms and cause various diseases; the development of bacteria increasingly resistant to new antibiotics is of great concern in the medical field (Cases and de Lozenzo, 2005; Passamano and Pighini, 2006).

Proteases, or peptidases, constitute the largest group of enzymes in bioindustry and have an array of uses. They play a decisive role in industrial biotechnology, especially in detergent, food, and pharmaceuticals (Deng et al., 2010; Jisha et al., 2013).

Proteases obtained from the microbial community are preferred for the large-scale production of proteases due to their fast growth and simple life cycle for the generation of new recombinant enzymes with desired properties (Kumar and Takagi, 1999; Jisha et al., 2013). *Achromobacter* protease I (API) is a lysine-specific serine protease that specifically hydrolyzes the lysyl peptide bond (Ohara et al., 1989). *E. coli* carrying a recombinant plasmid containing the *api* gene overproduced and secreted the protein into the periplasm (Rao et al., 1998). The biotechnology industry where our work was conducted produces *Achromobacter* protease I (API) enzyme on a large scale. Methodologies that allow the detection of host bacteria and/or their recombinant plasmids provide the potential to track the spread of these factors in IETS and other environments in case of accidental discharge. The aim of this study was to provide an approach to develop a molecular method for genetic marker detection of *E. coli* K-12 containing plasmids with the *bla* and *api* genes from an industrial wastewater treatment effluent pond (IWTEP) in southeastern Brazil.

MATERIAL AND METHODS

Water sampling from IWTEP

Two samples of wastewater from three distinct regions of a final wastewater treatment station pond were collected in July 2013. The samples were collected by rapidly submerging sterile 250-mL bottles 20 cm below the surface in regions defined and mapped as P1, P2, and P3 at IWTEP (Figure 1A, B, C, and D). After sampling, they were immediately packed into a box containing ice and transported to the laboratory where they were stored at 4°C until analysis.



Figure 1. Location of sampling points in the industrial wastewater treatment effluent pond (IWTEP) of a biotechnological industry. **A.** General view of IWTEP indicating sampling points P1, P2, and P3. **B.** P1 sampling point located at the effluent discharge into the pond. **C.** P2 sampling point at the central pond margin. **D.** P3 sampling point located at the pond margin opposite of the P1 side.

Isolation of ampicillin-resistant microorganisms from IWTEP

Water samples diluted 10^{-1} were inoculated directly onto three Petri dishes containing tryptic soy agar (TSA) medium, prepared according to the manufacturer's instructions (BD Difco™), and supplemented with MUG (4-methylumbelliferil 1 β -D-galactopyranoside, 70 mg/L) and ampicillin (100 μ g/mL). The plates were incubated for 48 h at 32°C, followed by exposure to UV light at the wavelength of 266 nm (UV_{266nm}). The colonies that showed fluorescence, as well as characteristic and morphological aspects of *E. coli*, were regarded as

presumptive *E. coli* (N = 3). All the colonies grown on TSA plates (N = 22) were analyzed using the multiplex polymerase chain reaction (PCR) to detect markers for *E. coli* K-12 strains, *bla* and *api* genes that code for resistance to ampicillin and API, respectively.

Design of genetic markers for *E. coli* K-12 harboring *bla* and *api* genes

We used three pairs of specific primers for multiplex PCR: 1) K12L/K12R (IS5 mutation detection inserted into the *rhamnose transferase* gene), which is a marker for strains of *E. coli* K-12 designed and tested by Kuhnert et al. (1995); 2) BLA523F/BLA708R marker to partial *bla* gene sequence of resistance to ampicillin (GenBank access No. NC006671, gene ID: 3244915); and 3) API83F/API549R marker to partial *api* gene sequence of API (GenBank access No. JO 5128.1) designed by the authors (Table 1). All primers were synthesized by IDT Integrated DNA Technologies, Inc. (Coralville, USA). In the experiments, the *E. coli* K-12 strain W3110 harboring pUC18 plasmid containing *api* and *bla* genes was used as a positive control.

Table 1. Primers used to detect genetic markers in *Escherichia coli* K-12 harboring plasmids containing *bla* and *api* genes.

Primer pair	DNA Sequence	Amplicon size (bp)	Target gene	Marker	Reference
K12L K12R	5'-TTCCACGGACATGAAGACTACA-3' 5'-ATCCTGGCGACCAATCAACAA-3'	488	ORF* Rhamnose transferase without IS5 insertion	<i>E. coli</i> serovar O16	Kuhnert et al. (1995)
K12L K12R	5'-TTCCACGGACATGAAGACTACA-3' 5'-ATCCTGGCGACCAATCAACAA-3'	1687	ORF* Rhamnose transferase with IS5 insertion	<i>E. coli</i> K-12	
API83F API549R	5'-CGTTCGATTACGCCAATCTTTC-3' 5'-CCGACGTAGTCGTGGTTGACC-3'	466	ORF* partial <i>api</i> 83-549	<i>E. coli</i> W 3110 harboring <i>api</i> gene inserted in a plasmid	This work
BLA523F BLA708R	5'-AAGTTGGCCGAGTGTATC-3' 5'-GCTATGTGGCGCGTATTAT-3'	186	ORF* partial <i>bla</i> 523-708	Ampicillin resistance partial gene	This work

*ORF = open-reading frame.

Multiplex PCR assay

The colonies grown in TSA medium supplemented with MUG and ampicillin were transferred directly to microtubes properly tagged containing 100 µL sterile saline (NaCl 0.9%) and centrifuged at 8000 g for 5 min. The supernatant was discarded and sediment resuspended in 100 µL 0.9% sterile saline solution.

Multiplex PCR was conducted as described as following. The suspension containing microbial cells was washed and resuspended in sterile 0.9% saline at a dilution of 1:10; two µL of this dilution was used as DNA template. All samples were subjected to amplification with primers K12L/K12R, BLA523F/BLA708R, and API83F/API549R. The positive control was DNA from *E. coli* strain W3110 with pUC18 containing the cloned *api* gene. The negative control was DNA from *Bacillus subtilis* ATCC 6633 and *E. coli* ATCC 8739.

The PCR total volume was 50 µl and consisted of Promega GoTaq® Green Master Mix (Green Go Taq® Flexi buffer 1X, 1.25 U Taq enzyme, 4 mM MgCl₂, and 20 µM of each deoxynucleotide triphosphates) and each primer at the final concentration of 20 µM. PCR was performed using the thermal cycler model GeneAmp PCR System 9700 from Applied Biosystems®. PCR conditions were as follows: predenaturation at 94°C for 4 min, annealing at 55°C for 30 s, and extension at 72°C for 5 min, followed by a final extension for 10 min at 72°C. The amplicons were separated on 2% agarose gels in 1X TBE buffer containing 0.25 mg/mL ethidium bromide and photographed under UV light. The Perfect DNA™ 1-kb ladder (EMD Chemicals, Inc., San Diego, California, USA) was used as molecular marker mass (MMM).

Microorganism genotype identification

The genomic DNA from bacteria colonies shown to be positive by multiplex PCR was isolated using the extraction reagent PrepMan™ Ultra sample, according to manufacturer's instructions (Applied Biosystems®). The 500 base pairs that corresponds to 16S rRNA universal bacterial gene regions was amplified by PCR in the GeneAmp 9600 thermal cycler (Applied Biosystems®), according to manufacturer's instructions. DNA from the bacterial strain *E. coli* ATCC 53503 was used as a positive control. The amplicons were subjected to electrophoresis and sequencing reactions in Applied Biosystems Genetic Analyzer® using the kit MicroSeq®500 16S rRNA Sequencing, according to the manufacturer's instructions. The sequenced amplicons were submitted to computational analysis using the MicroSeq® ID Analysis Software (Applied Biosystems®). A list of percentages of similarity of the comparison between the consensus sequences of sequenced amplicons and the MicroSeq library (16S rRNA ID MicroSeq 500 V 2.2 library) was generated. The genus level was determined, but the species was only considered when the percentage of similarity of the amplicon with the sequences deposited in the library of the MicroSeq was equal to or greater than 99% identity.

RESULTS

The primers for detection of the *bla* and *api* genes in plasmids of *E. coli* K-12 strains (Table 1) were tested in single PCR using DNA from the *E. coli* W3110 strain with the pUC18 plasmid containing cloned *bla* and *api* genes. Subsequently, the primers K12L-K12R, API83F-API549R, and BLA523F-BLA708R were grouped and tested in a multiplex PCR. The expected sizes of the amplified DNA fragments, 1687 bp (K12L- K12R), 466 bp (API83F-API549R), and 186 bp (BLA523F-BLA708R), were present on a 2% agarose gel after optimization (Figure 2A).

The rationale for the design and inclusion of primers BLA523F-BLA708R in multiplex PCR is based on the frequent insertion of ampicillin resistance genes into cloning and expression plasmids, including pUC18. The nucleotide sequence corresponding to the *bla* gene of a strain of *E. coli* (nucleotide position from 523 to 708) available in GenBank® has been accessed and used for the design of primers in the virtual program Perfect Oligo Design of Life technology (Table 1).

The partial nucleotide sequence of the *api* gene (nucleotide position from 83 to 549) available in GenBank® was also used for the design of these primers and in the multiplex PCR assay. The primers API83F-API549R were used because the biotechnology industry where this study was held produces the API encoded by that gene.

After multiplex PCR optimization using microorganisms maintained in the laboratory (positive and negative controls as shown in Figure 2A, B, and C), water samples were collected at three different IWTEP points as shown in Figure 1A, B, C, and D. Twenty-two isolated colonies were obtained from those points collected; five colonies tested positive for the *bla* gene and negative for the *E. coli* K-12 markers and *api* gene (Table 2; Figure 2B and C). The remaining 17 isolated colonies were negative for all gene markers tested by PCR.

The five isolated microorganisms that tested positive for the *bla* gene were submitted to genotypic identification by the MicroSeq® system using the universal bacteria 16S rRNA gene. We classified at the species-level if similarity of the amplicon was equal to or greater

than 99% compared to the sequence from the MicroSeq® databank and other parameters considered by the MicroSeq® software. Considering all parameters, only three samples (3/5) could be identified at the species level as *E. coli* and others were identified at the gender level (Table 2). The colony identified as *Chromobacterium* spp had 95.03% identity with the *Chromobacterium violaceum* 16S rRNA sequence. Samples identified as *Aeromonas* spp have been considered by submitting the same identity value to two different species (99.8%), namely: *Aeromonas caviae* and *Aeromonas enteropelogenes*.

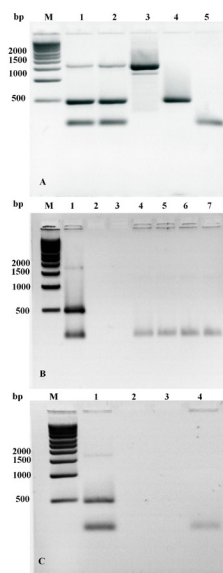


Figure 2. Results from the molecular method developed to detect *Escherichia coli* K-12 harboring *bla* and *api* genes in an industrial wastewater treatment effluent pond (IWTEP). M, molecular weight marker Perfect DNA™ 1-kbp ladder. **A.** PCR multiplex optimization results from *E. coli* W3110 harboring pUC18 containing *bla* and *api* cloned genes maintained in the laboratory. Lanes 1 and 2 show, respectively, 1687-bp, 466-bp, and 186-bp amplicons obtained with KL12L-KL12R, API83F-API549R, and BLA523F-BLA708R primers (PCR multiplex assay) as positive control. Lanes 3, 4, and 5 show amplicon results from primers K12L-K12R, API83F-API549R, and BLA523F-BLA708R, respectively, tested by single PCR. **B.** PCR multiplex results from water samples collected at IWTEP water samples. Lane 1 shows the positive control; lanes 2 and 3 show *Bacillus subtilis* ATCC 6633 and *E. coli* ATCC 8739, respectively, utilized as the negative control. Lanes 4 to 7 show PCR multiplex results for positive colonies 1 to 4 grown on an ampicillin TSA plate. **C.** PCR multiplex results from water samples collected at IWTEP water samples. Lane 1 shows the positive control; lanes 2 and 3 show *B. subtilis* ATCC 6633 and *E. coli* ATCC 8739, respectively, utilized as the negative control. Lane 4 shows PCR multiplex results for positive colony 5 grown on ampicillin TSA.

Table 2. Occurrence of genetic markers detected in microorganisms isolated from industrial wastewater treatment effluent pond (IWTEP).

Local (Samples)	PCR (Number of positive samples/Total sample number)			Microorganism identified by MicroSeq system
	K12L-K12R	BLA523F-BLA708R	API83F-API549R	
P1- IWTEP (N = 2)	0/6	2/6	0/6	<i>Escherichia coli</i> , <i>Chromobacterium</i> spp
P2 - IWTEP (N = 2)	0/10	3/10	0/10	<i>Escherichia coli</i> <i>Escherichia coli</i> <i>Aeromonas</i> spp
P3 - IWTEP (N = 2)	0/6	0/6	0/6	-
Total	0/22	5/22	0/22	-

DISCUSSION

The industrial wastewater treatment plants aim to reduce the impact of the toxic products discharged directly to the environment by removing the toxic organic and inorganic contaminants; reducing the concentration of dissolved organic carbon, nitrogen, and phosphorous; and eliminating the viable pathogens contained in wastewater that originated from the industrial factory (Rahube and Yost, 2010).

Due to the presence of biofilms and activated sludge in wastewater treatment plants, different antibiotic resistance genes can accumulate in the environment (Li et al., 2010). These activated sludge and biofilms are rich in nutrients with a high load of organic matter and bacterial density, thus creating an environment conducive to cell contact and exchange of plasmids that contain resistance genes to multiple antibiotics (Martinez, 2009).

It is recognized that broad range hosts harboring plasmids encoding antibiotic resistance genes may accidentally escape the wastewater treatment (Takabatake, 2011). Having acquired antibiotic resistance, these microorganisms can represent a threat to public health if they are easily disseminated in the environment and ultimately transmit resistance to pathogenic bacteria (Li et al., 2010). Rivers and lakes are major sources of water for human and animal consumption, and they are typically recipients of the flow from final wastewater ponds. Consequently, antibiotic-resistant pathogens may flourish and spread in water sources used in many human activities. Such microorganisms with antibiotic-resistant plasmids from industrial effluents can mediate bacterial diseases transmitted through contaminated water and food (Avşar and Berber, 2014).

In this work, we have developed a methodology to detect host lineages derived from *E. coli* K-12 carrying plasmids containing *bla* gene at IWTEP. In the multiplex PCR, positive control *E. coli* W3110 with pUC18 showed that the electrophoresis pattern corresponds to the expected amplification (Figure 2A). In addition, the individual amplifications using each pair of primers, K12L-K12R, API83F-API549R, and BLA523F-BLA708R, confirmed the specificity for the expected size amplicon for the single PCR (Figure 2A). During the amplification of the negative control *E. coli* ATCC 8739, the expected fragment size of 488 bp corresponding to the amplification with primers K12L-K12R was not detected (Figure 2B and C). The lack of amplified fragment suggests a problem with the PCR; however, there are cases reported in the literature regarding the amplification of some *E. coli* K-12 strains and non-K-12 strains. Kunhert et al. (1995) showed that *E. coli* K-12 and its derivatives and serovar O16 have the *orf264* and could be amplified by primer pair K12R-K12L; however, all other non-K-12 strains and other *E. coli*, *Salmonella*, and *Shigella* strains isolated from a broad range of sources, including humans, animals, and the environment, could not be amplified. Thus, the negative control *E. coli* ATCC 8739 is not related to K-12 strain derivatives or the serovar O16 strain, resulting in no amplification of the expected fragment size of 488 bp.

Twenty-two colonies were isolated from the P1 (6/22), P2 (10/22), and P3 (6/22) samples from IWTEP. All colonies underwent PCR analyses, and five of them showed an amplicon of 186 bp corresponding to the *bla* gene. All others were negative for all primer pairs (Figure 2B and C). This confirms the absence of *E. coli* K-12 or serovar O16 strains and their derivatives in the sampled locale, but the ampicillin resistance gene is present. Furthermore, for those five microorganisms, 16S rRNA was amplified, sequenced, and compared to the MicroSeq library. The result showed these to be *Escherichia coli* strains (3/5), *Aeromonas* spp (1/5),

and *Chromobacterium* spp (1/5) (Table 2). The presence of ampicillin-resistant *E. coli* (3/5) supports the studies that have demonstrated the presence of bacteria from genus *Escherichia* that is antibiotic-resistant in the surface water of wastewater treatment plants (Watkinson et al., 2007a,b). In addition, this work contributes to our understanding of antibiotic resistance in aquatic environments and the public health risks due to exposure to microorganisms in these environments. However, less attention is given to the aquatic environments than to the management and monitoring of the use of antibiotics (Watkinson et al., 2007a,b; Peixoto et al., 2012). In relation to the presence of *Aeromonas* spp, which are opportunistic pathogens, there are reports related to multi-resistance to antibiotics, among them from aquatic environments (Peixoto et al., 2012). Another bacterial genus identified was *Chromobacterium* spp, from which the most important species is the *C. violaceum*. Gram-negative coccobacilli, which is facultative anaerobic and non-sporulated, is part of the bioburden of the water and soil in tropical and subtropical regions of the world and can occasionally be found in foods (Koburger and May, 1982; Lima-Bittencourt et al., 2007).

In aquatic environments, it is important to consider that those bacteria can alter their physiological characteristics depending on the availability of nutrients. The diversity of microbial communities in an industrial effluent station can be enormous and depends on the compounds present in the environment that can contribute to the growth of microorganisms (Martinez, 2009; Takabatake, 2011). The microbial communities in a water system can be influenced by many factors, including salt concentration, geographical location, season, and rainfall, among others (Jiao et al., 2010; Jakobsson et al., 2010; Wang et al., 2011; Wang et al., 2014). The main representatives of these microorganisms include Proteobacteria, Firmicutes, Chloroflexi, Spirochetes, and Bacteroidetes, which represent phyla of a variety of Gram-positive and negative bacteria, including some opportunists and clinically significant bacterial pathogens (Li et al., 2010). A microbial community very close to the composition described above was found in a municipal wastewater system in China. The predominant phylum found in China was Proteobacteria, followed by Bacteroidetes and Firmicutes. Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria represented 90% or more of all Proteobacteria (Wang et al., 2014).

Furthermore, whether there is an accidental or deliberate release of GMOs into the environment, via plants, animals, or microorganisms that contain recombinant DNA, they need to be distinguished from the many other organisms that are resistant to antibiotics such as ampicillin, tetracycline, and chloramphenicol (Martinez, 2009; Li et al., 2010; Avşar and Berber, 2014).

The presence of antibiotic-resistant microorganisms in aquatic environments is an important indicator, representing a threat to public health when these waters end up in streams that are intended for human use. For this reason, the molecular methods for the detection of genetic markers of bacteria may be promising, because they are a sensible way to measure local pollution, easy to interpret, and specific to the host (Bower et al., 2005).

Wastewater treatment plants (WWTPs) are also thought to be important reservoirs for different types of antibiotic resistance genes that are associated with human pathogens. Therefore, microbial communities in WWTP exist as model systems to explore various plasmids carrying genes with important functions for their hosts, including antibiotic resistance, pathogenicity, virulence, biodegradation, and heavy metal resistance (Li et al., 2015). Several studies have reported the presence of immense varieties of plasmids containing antibiotic resistance genes in WWTP (Li et al., 2015).

In this study, we validated a molecular method for the detection of *E. coli* K-12 strains containing *bla* and *api* genes in IWTEP. This is the first study proposing the identification of cell host, the plasmid antibiotic gene marker, and the recombinant protein gene cloned for differentiation of GMO protein-producers originating from the biotechnology industry rather than a natural source. Since the identification is now possible, it can be used to monitor an accidental discharge or gene transference among the bacteria. In the future, studies of the prevalence of microorganisms harboring antibiotic resistance genes at IWTEPs using a metagenomic approach will be conducted.

Conflicts of interest

The authors declare no conflict of interest.

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