Antimicrobial Activities of *Bacillus* Strains containing biosynthetic genes against *Legionella pneumophila*

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Legionella pneumophila has been known to cause Legionnaires Disease and Pontiac fever. These bacteria live in natural waters and colonize at man-made water systems where is *Legionella* bacteria interact with other microorganisms such as *Bacillus*. It is known that there are several antimicrobial substances produced by *Bacillus* strains. Therefore, anti – *Legionella* activity of 27 *Bacillus* strains that are isolated from man-made water systems and one standard *Bacillus subtilis* (ATCC 6633) was investigated by agar well diffusion method in this study. In addition, we performed a screening for the presence of biosynthetic genes for antimicrobial lipopeptides, bacillomycin D, surfactin, iturin and fengycin in used isolates of *Bacillus* strains. It was found that 26 *Bacillus* strains produced at least one lipopeptide type and mostly these strains exhibited strong antagonistic activities against *L. pneumophila* serogroup 1 strains. Consequently, the results obtained from this study showed that some *Bacillus* strains could be used as biological control of *Legionella* bacteria in water systems.

Keywords: *L. pneumophila*, *Bacillus*, antimicrobial lipopeptides, biological control

1. Introduction

Legionella pneumophila which is known as an etiologic agent of Legionnaires Disease and Pontiac fever, lives in natural and man-made water systems (municipal water system, cooling tower)[1]. Also, there have been several reports indicating that non-*Legionella* heterotrophic bacteria sharing the same environment with *Legionella* can support or inhibit the growth of *L. pneumophila* [2-5].

Members of the genus *Bacillus* produce a wide range of secondary metabolites such as polyketides, cyclic lipopeptides, and bacteriocins that exhibit a broad spectrum of antimicrobial activity and very diverse structures [6]. It is well known that *Bacillus* species have long been used for antimicrobial activity and industrial processes [7-11]. However, the antagonistic activity of *Bacillus* against *L. pneumophila* is not well documented.

The best-known class of such compounds is cyclic lipopeptides that are synthesized nonribosomally, including iturin, surfactin and fengycin which exhibit antibiotic and surface active properties [10-15].

The iturin family, including the related lipopeptide iturin, mycosubtilin, and bacillomycin, are composed of heptapeptides interlinked with a β-amino fatty acid chain, consisting of 14-17 carbons [12,14]. Members of the fengycin family, represented by plipastatin, are cyclic lipodecapeptides with a β-hydroxy fatty acid having side chain length of 16-19 carbon atoms [13]. The surfactin family consists of heptapeptides containing a β-hydroxy fatty acid with a number of carbon atoms between 13 and 15 [16].

The aims of the present study were (1) to isolate the *Bacillus* strains from municipal waters and to evaluate their inhibitory activity against *L. pneumophila*; and (2) to perform a screening for the presence of biosynthetic genes intended for antimicrobial lipopeptides, bacillomycin D, surfactin, iturin and fengycin in isolates of *Bacillus.*

2. Materials and Methods

20 water samples collected from municipal water systems in different buildings in Istanbul city were investigated for *L. pneumophila* and *Bacillus* strains. Water samples were concentrated using filtration method and then filters were resuspended in 20 ml sterile tap water.

2.1 Isolation and identification of *Legionella*

2 ml of concentrated water samples were incubated at 50°C for 30 min and 10 ml of each concentrated water sample was treated with 1:1 HCl-KCl (pH: 2.2). 0.1 ml of each of these processed water samples were streaked onto buffered charcoal yeast extract agar (BCYEA) plates supplemented with glycine, vancomycin, polymixin and natamycin (GVPN) and incubated at 37°C for 10-14 days [17]. Colonies consistent with *Legionella* morphology were subcultured onto tryptone soy agar. Serological identifications were performed by using *Legionella* latex test kit (Oxoid).

2.2 Isolation and identification of *Bacillus* strains

For the detection of *Bacillus* strains, serial dilutions (10^{-1} to 10^{-3}) were then prepared with sterile tap water. 100 µl volumes of each dilution were spread onto Plate Count Agar plates. After the incubation period (24-48 h, 30°C) strains

determined as Gram-positive rods were selected. Identification of the strains was carried out by standard taxonomic descriptions with biochemical tests and by the API 50 CHB rapid identification system (Biomeriux) [18]. The standard *Bacillus subtilis* strain (ATCC 6633) was used in this study.

2.3 Assay of ability to inhibit *L. pneumophila* growth by *Bacillus* strains

Agar diffusion test was used for the detection of inhibitor effect of *Bacillus* strains against *L. pneumophila* strains. This test was a modification of that described by Toze et al. [2]. Each strain of *L. pneumophila* include standard *L. pneumophila* was suspended in sterile tap water to obtain the concentration of $3x10^8$ CFU.ml^{-1} and then 0.1 ml of this suspension was inoculated on the entire surface of the BCYEA plate. After this inoculation, each *Bacillus* strain which had been grown on PCA was spotted onto this plate. Sterile potable water was used as negative control in our experiments. Also, for seeing inhibition zone of *L. pneumophila*, cephalothin and tobramycin were used as antibiotics. Used standard *L. pneumophila* serogroup (SG)1 strain coded L3 (ATCC 33152) was provided from Hertfordshire University Biodeterioration Center.

2.4 DNA Extraction for *Bacillus* strains, Quantitation and Gel Electrophoresis

Genomic DNA was isolated from *Bacillus* strains by EZ-10 Spin Column Genomic DNA Kit according to the operation instructions of the Kit. The concentration of 2 µl DNA sample was determined using a NanoDrop 1000 (Thermo Scientific). Moreover, unamplified DNA (5 µl) was loaded on a 1% pure agarose gel in 0.5 x TBE buffer and ethidium bromide.

2.5 PCR identification of the lipopeptide genes for *Bacillus* strains

Lipopeptides genes were amplified from the extracted DNA by PCR using EmeraldAmp Max HS PCR Mastermix (Takara, Shuzo, Shiga, Japan). In order to perform screening of biosynthetic genes for antimicrobial lipopeptides, bacillomycin D, *sfp*, iturin and *fen* D,in all 27 isolates of *Bacillus* species and standard, primers specific for those genes were employed in PCR assays [19-21]. Four sets of primers were employed in the PCR reactions (Table 1). Primer pairs FEND1F/FEND1R, BACC1F/BACC1R, ITUP1F/ITUP2R and P17/P18 were employed to screen the presence of iturin, *fen* D, bacillomycin D and *sfp* genes, respectively.

Table 1 Specific primers for PCR detection of biosynthetic genes for antimicrobial lipopeptides

Lipopeptide	Primer name	Position in GenBank sequence	Sequence
Bacillomycin D	BACC1F	34274-34293	5'- GAAGGACACGGCAGAGAGTC -3'
	BACC1R	35148-35129	5'- CGCTGATGACTGTTCATGCT -3'
Surfactin	P17	167-186	5'-ATGAAGATTTACGGAATTTA-3'
	P18	841-822	5'-TTATAAAAGCTCTTCGTACG-3'
Iturin	ITUP1-F	15353-15383	5'-AGCTTAGGGAACAATTGTCATCGGGGCTTC-3'
	ITUP2-R	17326-17355	5'-TCAGATAGGCCGCCATATCGGAATGATTCG-3'
Fengycin	FEND1F	3687-3706	5'- TTTGGCAGCAGGAGAAGTT -3'
	FEND1R	4650-4631	5'- GCTGTCCGTTCTGCTTTTTC-3'

PCR reaction using FEND1F and FEND1R primers consisted of 45 cycles of 98°C for 15 sec (denaturation), 62°C for 1 min (annealing) and 72°C for 1 min, 45 sec (extension). PCR reaction condition for the primers BACC1F/BACC1R were 40 cycles of 98°C for 15 sec, 60°C for 30 sec and 72°C for 1 min,45 sec. The initial denaturation for the *fen* D and bacillomycin D gene was performed at 98°C for 3 min and final extension was carried out at 72°C for 6 min. On the other hand, a cycling condition consisting of 35 cycles of 98°C for 15 sec, 60°C for 30 sec and 72°C for 2 min, 30 sec was used in the PCR reaction using ITUP1-F/ ITUP2-R primers. PCR cycling condition for the primers P17 /P18 were 30 cycles of 98°C for 15 sec, 46°C for 30 sec and 72°C for 1 min. The initial denaturation for the iturin and the *sfp* gene was performed at 98°C for 3 min and final extension was carried out at 72°C for 10 min. All PCRs were performed in triplicate. Following PCR, the amplified product was examined by electrophoresis on a 1% pure agarose gel in 0,5 x TBE buffer and ethidium bromide.

3. Results

In this study, two *L. pneumophila* SG1 strains (L1, L2), two *L. pneumophila* SG 2-14 strains (L4, L5) and 27 *Bacillus* strains were isolated from municipal water in different buildings in vicinity of Istanbul. *Bacillus* strains were named as *B. pumilis* (15 strains), *B. circulans* (1strain), *B. amyloliquefaciens* (1 strain), *B. stearothermophilus* (2 strains), *Brevibacillus brevis* (3 strains), *B. megaterium* (1 strain), *B. subtilis* (3 strains) and *B. licheniformis* (1 strain) (Table 2). The inhibitory activity of each of the *Bacillus* strains tested against 5 strains of *L. pneumophila* and the presence of biosynthetic genes for the antimicrobial lipopeptides in *Bacillus* strains were provided in Table 2. Also the results of PCR screening revealed amplification of bacillomycin D gene fragments in 28 *Bacillus* strains (Figure 1).

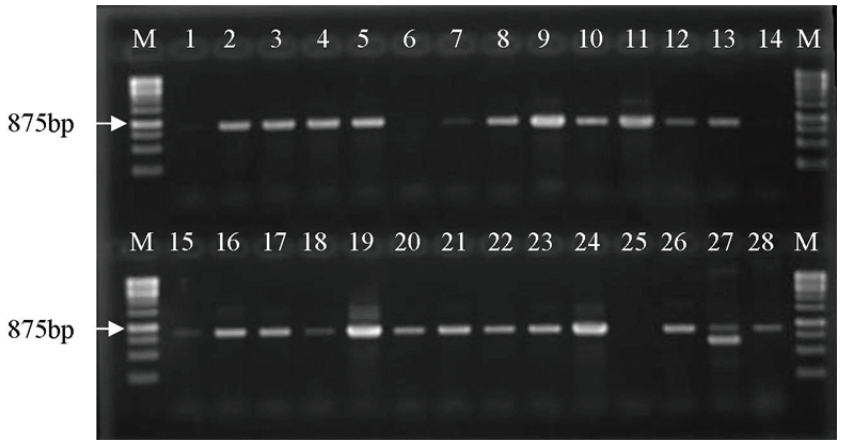

Fig. 1 Detection of bam C gene of the bacillomycin D synthetase biosynthetic operon. Lane 1-28: *Bacillus* strains, M: DNA Ladder

The size of the inhibition zones was found to change between 5 to 60 mm. The average of the inhibition zone of *L. pneumophila* strains against cephalothine and tobramycin were found as 34 mm and 26 mm, respectively. Therefore, measures of ≥34mm were evaluated as inhibition effect of *Bacillus* strains. In this study, 12 of 28 *Bacillus* strains (43%) inhibited all tested *L. pneumophila* strains. However, 2 (7%) of tested *Bacillus* strains inhibited none of *L. pneumophila* strains.

The five isolates (B2, B3, B4, B8, B18) were positive to specific primers of the iturin, fen D, bacillomycin D and sfp genes. In this study, results showed that 93% of the isolates have the genes for bacillomycin D production, 64% for surfactin, 43% for iturin and 25% for fengycin. In addition 26 strains produced at least one lipopeptide type.

Table 2 Listing of *Bacillus* species with an antimicrobial activity on *L. pneumophila* biosynthetic genes for antimicrobial lipopeptides

Code	Bacillus species	Inhibition Zones (mm)					Lipopeptides			
		L1	**L2**	**L3**	**L4**	**L5**	**Bacillomycin D**	**Surfactin**	**Iturin**	**Fengycin**
B1	*Bacillus pumilis*	40	36	35	9	30	+	-	-	-
B2	*Bacillus circulans*	45	37	30	-	38	+	+	+	+
B3	*B. amyloliquefaciens*	43	44	29	-	34	+	+	+	+
B4	*B. stearothermophilus*	45	37	33	-	40	+	+	+	+
B5	*B. stearothermophilus*	45	39	33	-	45	+	-	+	+
B6	*Brevibacillus brevis*	-	10	9	-	12	-	-	-	-
B7	*Bacillus megaterium*	-	-	-	-	-	+	-	+	-
B8	*Brevibacillus brevis*	-	-	-	-	-	+	+	+	+
B9	*Bacillus pumilis*	17	45	45	-	35	+	+	+	-
B10	*Bacillus pumilis*	14	40	32	8	31	+	+	+	-
B11	*Bacillus pumilis*	15	32	30	-	34	+	-	-	-
B12	*Bacillus pumilis*	12	25	20	10	32	+	+	+	-
B13	*Bacillus subtilis*	-	45	35	-	35	+	+	-	-
B14	*Bacillus pumilis*	20	60	40	11	37	+	-	-	-
B15	*Bacillus subtilis*	-	45	42	-	32	+	+	-	-
B16	*Bacillus pumilis*	20	32	32	10	35	+	+	+	-

B17	*Bacillus pumilis*	20	40	30	8	39	+	+	-	-
B18	*Bacillus licheniformis*	15	30	28	8	34	+	+	+	+
B19	*Bacillus pumilis*	25	24	-	-	30	+	+	-	-
B20	*Bacillus subtilis*	8	10	-	-	12	+	+	-	-
B21	*Bacillus pumilis*	18	8	20	8	37	+	-	-	-
B22	*Bacillus pumilis*	17	25	30	12	30	+	+	+	-
B23	*Bacillus pumilis*	17	30	32	5	40	+	-	-	+
B24	*Bacillus pumilis*	17	20	33	1	40	+	+	-	-
B25	*Bacillus pumilis*	16	25	35	7	40	-	-	-	-
B26	*Bacillus pumilis*	15	35	32	-	30	+	+	-	-
B27	*Brevibacillus brevis*	7	10	8	-	11	+	+	-	-
B28	*B. subtilis*(ATCC 6633)	-	-	-	-	11	+	-	-	-

- no zone and no gene; + PCR product of expected size was seen

4. Discussion

Legionnaires Disease caused by *L. pneumophila* is a very important health problem in the world [22]. *L. pneumophila* living in aquatic environments such as natural and man-made water systems can infect people through the inhalation of contaminated aerosols. *Legionella* infections in man can be treated with antibiotics [23]. Certainly, the prevention of infections are more important everytime than the treatment of them, because the use of antibiotics have some disadvantages such as economic concerns, possibility to cause endotoxic shock, side effects, and multiple resistance. The establishment of disinfection procedures such as chemical and physical ones may not prevent the occurrence of *Legionella* in the water systems [22,24]. Also, disinfection procedures have some disadvantages such as the incorrect selection and application of biocide, the build up of bacterial resistance and the corrosion of metals [25]. At the same time, it is known that *Bacillus* species secrete numerous proteins, that are produced industrially, into the environment and they produce some antimicrobial lipopeptide compounds such as bacilysin and iturin [26,27]. Therefore, we assume that, due to the disadvantages of antibiotics use and shortcoming disinfection procedures, the members of natural environments such as *Bacillus* strains can be used for prevention of *L. pneumophila* infections.

In this study we performed the screening of 27 isolates of *Bacillus* for antagonism against *L. pneumophila* and examined for the presence of genes for biosynthesis of antimicrobial lipopeptides, bacillomycin D, surfactin, iturin and fengycin. Moreover, these study methods could be applied to screen *Bacillus* strains producing bacillomycin D, surfactin, iturin and fengycin in commercial formulations and used in vivo against on *L. pneumophila*.

The most active *Bacillus* strain was B14 (*Bacillus pumilis*) against to L2 (60mm). 11 of 15 *B. pumilis* inhibited all tested *L. pneumophila* strains. Also, other *Bacillus* species, except B7 (*B. megaterium*) and B8 (*Brevibacillus brevis*), inhibited at least one of the tested *L. pneumophila* strains (Table 2). According to our findings, *L. pneumophila* SG 2-14 strain (L4) was the most resistant and L5 was the most sensitive strain against *Bacillus* bacteria.

Eight different *Bacillus* species were named in this study. 15 out of 27 *Bacillus* strains were *B. pumilis.* Other *Bacillus* species were fewer in number (Table 2). For that reason, it is not easy to determine the most effective *Bacillus* species against *L. pneumophila* strains. Therefore, further studies are needed to be performed with more *Bacillus* species except for *B. pumilis*. There are a small number of studies about the inhibition effect of some *Bacillus* species against *Legionella* [28-29]. However, to our knowledge, this is the first study in view of the determinations of the inhibitory effects of *B. circulans, B. amyloliquefaciens, B.stearothermophilus* and *B. licheniformis* against *Legionella*. Thereby, we think that the inhibition action of *Bacillus* strains against *L. pneumophila* strain is strain specific. In fact, we found that *B. circulans* inhibited *L. pneumophila.* On the other hand, Kimiran Erdem and Yazıcı [29] reported that *B. circulans* did not inhibit *L. pneumophila*. It was found that *Brevibacillus brevis* was able to inhibit the growth of *Legionella* species, similarly our results [29].

Lipopeptide profile was showed to vary greatly even among the same species. 26 *Bacillus* strains produced at least one lipopeptide type and mostly these strains exhibited strong antagonistic activities against *L. pneumophila* strains. Similar to results obtained from our study, Stankovic et al. determined that the presence of genes responsible for lipopeptide production in Bacillus strains as bacillomycin D (81%), surfactin (54%), iturin (38%) and fengycin (25%), respectively [30].

According to the results of our experiments, B6 strain (*Brevibacillus brevis*) inhibited the production of *L. pneumophila* strains, however, fen D, bacillomycin D, iturin A and sfp genes were not determined in this strain. In contrast to this result, while iturin, fen D, bacillomycin D and sfp genes were determined in B7 (*B. megaterium*) and B8 (*Brevibacillus brevis)* strains, no effect on the production of *L. pneumophila* strains was detected. This may be due to the low concentration of lipopeptides synthesized by B7 and B8 strains. The fact that B6 strain has antagonistic effects

on *L.pneumophila* strains, even without iturin, fen D, bacillomycin D and sfp genes, can be due to the antimicrobial lipopeptides that were not investigated in this study, or may point to the fact that the antimicrobial lipopeptides investigated in *Bacillus* strains may not be the only cause of the antagonistic effect on *L.pneumophila* strains. Moreover, it is clear the enzymes need to be studied as well in order to fully analyze the effects of *Bacillus* strains. There are studies demonstrating the antimicrobial effects of *Bacillus* enzymes as well [31,32]. In addition, we showed that the lipopeptide types of *Bacillus* strains are strain specific. Also, several studies reported that presence of surfactin and fengycin genes differ in different *Bacillus subtilis* ATCC strains [20,33].

An interesting result of this work is the highly strong antagonistic effect of *Bacillus* isolates against *L. pneumophila*. According to the obtained results, we do not state that this activity is only the result of the production of cyclic lipopeptides from bacillomycin D, surfactin, iturin and fengycin in most strains. Consequently, the results obtained from this study showed that some *Bacillus* strains could be used as biological control of *Legionella* bacteria in water systems. Further studies are required to isolation and characterization of secretory proteins (lipopeptides, bacteriocin and enzym) of *Bacillus* strains which we used in this study and then effective concentration of these proteins should be examined against *L. pneumophila* strains. Afterwards, the results of these studies can be evaluated with the results of this study and mechanism of action can be determined.

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