Isolation and identification of *Lysinibacillus boronitolerans* from automotive cabin air.

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Automotive interiors may appear safe, but many hazardous microbes can reside in biofilms within the ducts of air conditioning systems, and remain viable for over two years. Potentially pathogenic organisms in the genera *Acinetobacter*, *Bacillus*, *Pseudomonas*, and *Stenotrophomonas* have been identified in automobile cabin air, so it is likely that an air sample obtained from a twenty-year-old passenger car will contain one or more of these organism types. A nutrient agar plate was exposed to air inside the cabin of a frequently used automobile, and one organism type designated strain TEG998, was selected for identification. Chromosomal DNA was extracted from a pure culture of TEG998 and the polymerase chain reaction was used to amplify the 16S rRNA genes present. Observation of cell and colony morphology, nucleotide sequencing and enzymatic tests were used to characterize strain TEG998. Specific results including a positive urea hydrolysis (formation of urease) and positive oxidase test (formation of cytochrome C oxidase) were used to distinguish between different *Lysinibacillus* species. Strain TEG998 was found to be a type of Gram-positive bacillus with terminal endospores. Based on BLAST results and confirmatory enzymatic testing, strain TEG998 was tentatively identified as *Lysinibacillus boronitolerans*. While *L. boronitolerans* is not one of the organism types previously isolated from vehicle ventilation, it is closely related to *Bacillus*, and those are many *Bacillus* species, as well as other members of the family Bacillaceae, can be pathogenic by producing exotoxins and by exacerbating asthma symptoms. *Lysinibacillus* species are not generally considered to be pathogenic, but some can be opportunistic pathogens in those individuals with weakened immune systems. Also, due to the continued overuse of antibiotics, these organisms might become pathogenic in the future. Additional tests could be run to more completely verify the identity of *L. boronitolerans*, and to identify the multiple other organism types cultured on the original automotive cabin air plate. This study did not conclusively rule out the presence of potential pathogens.

INTRODUCTION

Personal vehicles are the main sources of transportation for most people in North America, with around 80% of US commuters using their private vehicles and another 5.6% riding as passengers (Sattar, 2016). Although most people think of automobiles as safe and secured from the hazards of the outside world, automotive air conditioning systems are not as beneficial as those within buildings, due to smaller size ducting and sharp airflow changes that can allow for particle and microbe deposits along the interior (Vonberg, 2010). Bacterial and fungal contaminants can grow within ducts and eventually make their way into the cabin. In addition, the cabin air filter is frequently overlooked during routine vehicle maintenance (Sattar, 2016). This air contamination can lead to odor problems, or to the more alarming “sick car syndrome” that causes flu-like symptoms in human inhabitants (Daly, 2006). More importantly, breathing contaminated air can cause serious health issues, such as chronic obstructive pulmonary disease COPD and asthma (Muller, 2011). Testing of automobile air conditioning systems has shown as many as 1,400 colony forming units (airborne fungal spores) per cubic meter (CFU/m³) under humid conditions, and 200 CFU/m³ of total microbes with 20 CFU/m³ fungal spores detected from an air conditioning system running with an old filter (Vonberg, 2010). Viable bacteria and fungi were recovered from the ventilation systems of vehicles even after storage for 27 months (Simmons, 1999). Overall, studies have revealed the presence of over 400 species of bacteria and 18 species of fungi associated with automobile cabin air. Opportunistic pathogens in the genera *Acinetobacter*, *Bacillus*, *Pseudomonas*, and *Stenotrophomonas*, were identified (Sattar, 2016). Given the abundance of microorganisms present in automobile cabin air, and that members of the genera *Acinetobacter*, *Bacillus*, *Pseudomonas*, and
Stenotrophomonas have been identified, an air sample collected inside a 20-year-old passenger vehicle is likely to contain bacteria from one or more of these genera.

MATERIALS AND METHODS

A nutrient agar plate was exposed to ambient air for one hour inside a 1998 Acura Integra with the air ventilation system running on low and the windows up. The sample was incubated at room temperature for one week and then observed for colony formation. One colony, designated strain TEG98, was selected and replated on nutrient agar for isolation and to obtain colony morphology. A Gram stain and nigrosin indirect stain were prepared using cells from the bacteria type isolated. These were observed under 1000X magnification to determine cell wall type and cellular morphology. Three percent potassium hydroxide (3% KOH) was used to determine if the culture had thick or thin cell walls.

Chromosomal DNA was extracted from strain TEG98 using a boiling and beating with glass beads method as described in the Microbiology Laboratory Manual (Wilson, 2016). The 16S rRNA genes (rDNA) were amplified using the Polymerase Chain Reaction (PCR) with the primers Bacteria 8-Forward and Enteric 1530-Reverse. Agarose gel electrophoresis was used to confirm proper DNA extraction and amplification, and then DNA from strain TEG98 was cut from the gels and purified using a QIAquick Gel Purification Kit (Qiagen). The purified DNA was then taken to the UC Davis, DNA sequencing facility (Storer Hall) for nucleotide sequencing using the Basic Local Alignment Search Tool (BLAST) algorithm available on the National Center for Biotechnology Information (NCBI) website. The organism type with a nucleotide sequence most similar to strain TEG98 was selected from the results by looking at the highest query coverage values and identity scores.

Using procedures described in the laboratory manual (Wilson, 2016), a series of enzymatic tests were performed. Two tubes of oxidation/fermentation (O/F) test media were inoculated to determine if the isolate had a respiratory or a fermentative type of metabolism. A catalase test was performed to determine the ability of the culture to produce catalase enzymes and catabolize hydrogen peroxide. An oxidase test was performed to identify the production of cytochrome C oxidase enzymes, allowing for oxidative phosphorylation. A tube of urea agar was inoculated to determine if the isolate produced the urease enzymes needed to break down urea and form ammonia. Lastly, a Methyl Red-Voges-Proskauer (MR-VP) test was performed to determine if or not strain TEG98 could produce mixed acids and/or acetoin.

RESULTS

Strain TEG98 grown on nutrient agar formed colonies 2-3mm in diameter after 48 hours. These were circular, entire to undulate, flat to raised, opaque, smooth, glossy, wet-looking, and cream-colored. Older colonies swarmed over the plate surface, darkened to a light tan color, and became filamentous along the border. There was a slight odor of vinegar associated with the culture in. A Gram stain showed purple bacilli (though in some areas the cells appeared pinkish), indicating a Gram positive culture with thick cell walls.

Figure 1. Gram stain of strain TEG98, showing Gram-positive bacilli.
The nigrosin stain revealed bacilli 1-1.5 microns in diameter and 4-5.5 microns in length, with some having a club-shaped end measuring up to 2 microns in diameter. A sample of culture exposed to 3% KOH yielded no change in viscosity so was a negative result.

Figure 2. Nigrosin indirect stain of strain TEG98, showing bacilli, some with clubbed ends.

Results obtained with the NCBI BLAST indicated the 16S rRNA gene nucleotide sequence from strain TEG98 was most similar to that from *Lysinibacillus boronitolerans* strain NBRC 103108, accession number NR_114207.1. This sequence showed 96% identity matching nucleotides pairwise (1395/1450) with 97% query coverage and a bit score of 2459. The taxonomic lineage was: Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; *Lysinibacillus*.

Results obtained from the O/F test with strain TEG98, showed an open tube with a blue surface and green base, indicating an alkaline product was formed in the presence of oxygen. The vaspar-sealed tube remained green throughout, showing no growth and no glucose utilization with acid or gas formation. This is a negative result for fermentation but a positive result for respiration. The catalase test was positive, as bubbles were produced with addition of 3% hydrogen peroxide. A fresh sample of strain TEG98 culture turned purple on the oxidase test paper, indicating a positive result for the presence of cytochrome C oxidase. The urea agar slant turned bright pink, while the bottom remained peach in color. Bright pink is positive for ammonia production, so was considered positive. The MR broth was yellow in color and had a pH of 6.8 when tested with a pH paper strip, indicating negative for large amounts of acid. The VP broth remained tan in color immediately following the addition of Barrit’s reagents A and B and vortex mixing, but after 2.5 hours a slight pink hue developed, indicating a very weak positive for reaction for acetoin production.

**DISCUSSION**

The colony morphology of strain TEG98 changed greatly with age. Young cultures formed isolated, small colonies with a very defined border; however, over time, the colonies melded together seamlessly as strain TEG98 swarmed the plate, indicating motility. The border became uneven and filamentous as nutrient resources ran thin, and the color changed from cream to light tan. Endospores were formed by older cultures. The Gram stain was deceiving, as some areas appeared more like Gram-negative bacilli (pink in color). This may have been caused by over decolorizing, but the color did darken when adjusting the focus. It was because of this original data that an O/F test was performed. Once the culture was confirmed as being Gram-positive, an MR-VP was also performed and both confirmed the culture had a respiratory metabolism. Based on the results of the Gram stain and KOH test, strain TEG98 cells had thick peptidoglycan walls. Many spores were free-floating as exospores, as the aged cells degraded when nutrients were sparse. Colony morphology, cell morphology, and the presence of terminal endospores and swollen sporangia were consistent with descriptions of *Lycinibacillus* species (Coorevits, 2012).

The nucleotide sequence initially obtained from strain TEG98 contained multiple wobble bases in the first section (up to 489 bases) suggesting that the sequencing primer 8-forward did not anneal well or that the multiple rRNA genes within this organism type are not identical in that region. The 16S rRNA sequence from isolate TEG98 was nearly identical with that from *Lysinibacillus boronitolerans* for the 533F and 1530R primer sequences; however, similarity overall was only 96% and genes from *Lycinibacillus marcroides* strain LMG 18474 also showed 96% identity with 98% query coverage. The bacteria 8-forward sequence was
corrected based on electropherogram results by converting the wobble bases to the nucleotide type showing the highest peak when discernable. Results then showed a 98% identity and 98% query coverage match between genes from strain TEG98 and those from *L. boronitolerans*, with 1443/1467 bases matching pairwise. The corrected sequence still showed high similarity with *L. macroides*, so enzymatic test results were compared in order to more accurately identify strain TEG98.

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<tr>
<th></th>
<th><em>L. boronitolerans</em></th>
<th><em>L. macroides</em></th>
<th>TEG98</th>
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<tr>
<td>Thick Cell Wall</td>
<td>+</td>
<td>V</td>
<td>+ (V)</td>
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<tr>
<td>Forms Terminal Endospores</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Motile (Swarm agar)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Aerobic (does not ferment)</td>
<td>+</td>
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<tr>
<td>Produces Catalase Enzyme</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Produces Urease Enzyme</td>
<td>+</td>
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<td>Is Heterofermentative (produces mixed acids)</td>
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<td>Produces Acetoin</td>
<td>+</td>
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<td>(+)</td>
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<tr>
<td>Produces Cytochrome C (respiratory)</td>
<td>+</td>
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Table 1. Comparison of characteristics of strain TEG98 with *L. boronitolerans* and *L. macroides* (Coorevits, 2012).

The blue surface on the unsealed O/F medium showed an alkaline product was formed in an aerobic environment by catalyzing peptone in the medium. This indicated strain TEG98 was obligately aerobic with a respiratory, non-fermentative type of metabolism. The oxidase test confirmed respiratory function as the positive oxidase test indicated the presence of cytochrome C oxidase, an enzyme necessary for oxidative phosphorylation. *L. boronitolerans* produces cytochrome C oxidase, whereas *L. macroides* does not. Because the catalase test was positive, strain TEG98 produced catalase, an enzyme that catalyzes hydrogen peroxide (H₂O₂) into water and oxygen gas, causing bubbles. The negative MR result indicated strain TEG98 did not ferment glucose and form large amounts of mixed acid. The pH was 6.8, so was very close to neutral (the initial pH in the test medium). There was not enough acid produced to decrease the pH to 4.4 or below; therefore, strain TEG98 was not heterofermentative. The VP test yielded a slight positive result, indicating strain TEG98 did produce a small amount of acetoin. The MR-VP broth was only slightly turbid, therefore a longer, warmer incubation time may have produced a stronger result. Isolate strain TEG98 produced the urease enzyme needed to catalyze urea, as indicated by the positive urea hydrolysis result, and *L. boronitolerans* produces urease, whereas *L. macroides* does not.

Based on the colony morphology, cell morphology, DNA sequencing results, and enzymatic test results, isolate TEG98 was identified as *Lysinibacillus boronitolerans*. The corrected DNA sequence indicated this was the case, but the positive urea hydrolysis and oxidase test results confirmed it. All other tests run on strain TEG98 matched the results shown for *L. boronitolerans*; however, more testing such as cell wall amino acid content and cell membrane fatty acid analysis could be run to ensure the identity of isolate strain TEG98. When grown in broth culture, *L. macroides* produces long, filamentous bacilli, while *L. boronitolerans* does not; therefore, growing isolate TEG98 in broth culture could help confirm the identity. An N-Acetyl-D-glucosamine or D-Fructose 6-phosphate utilization test could further distinguish the two, as *L. macroides* can utilize both but *L. boronitolerans* cannot utilize either (Coorevits, 2012).

The identification of strain TEG98 as *Lysinibacillus boronitolerans* agreed with previous studies that found *Bacillus* organisms in the ventilation systems of vehicles, as they are a related genus. This is significant because some strains in the Bacilleae Family are known to be pathogenic e.g., *Bacillus anthracis*. The thick peptidoglycan cell walls and endospore features of these organisms makes treatment difficult because they can survive many antibiotics and survive in spore form for long periods of time. While *Lysinibacillus* is not generally pathogenic, it can be harmful in certain cases of reduced immunity (Wenzler, 2015). In addition, the
overuse of antibiotics may lead to more pathogenicity in the future. The genus *Bacillus*, a close relative, has many pathogenic strains, usually acquired by ingesting spores in contaminated foods. Once germinated, *Bacillus* produces exotoxins that can cause diarrhea, and in severe cases, death (Logan, 2011). These exotoxins can also adversely affect asthmatics, and have been found in issue-causing levels in automotive cabin air quality studies (Sattar, 2016). The formation of biofilms within automotive air ducts have been shown to house members of the Bacillales Order, releasing spores when the airflow is turned on (Sattar, 2016). *L. macroides* has been isolated from cow dung, which integrates into the soil reservoir over time (Coorevits, et al 2012). So it is not surprising to find *L. boronitolerans* in a vehicle frequently in contact with soiled shoes. Further studies should include identification of the other organisms cultured on the original vehicle cabin air plate to see if other potential pathogens may be present.

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