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BIOL F342
F02

# Genomic and Physiological Identification of Bacterial Isolate from Canine Ear

## Introduction:

Microbes are the most abundant, diverse and widespread organisms on Earth. Just in the past decade, through environmental sampling and metagenomics, we have been able to identify species that have never been observed before, ranging from those living in subglacial antarctic lakes to volcanoes. We have also largely increased the understanding of the bacterial communities we share our bodies with (Hannigan and Grice 2013). One of the main advantages of this is the ability to distinguish between bacteria that are beneficial or commensal from those that are pathogenic. In the veterinary field, for instance, many studies (Perry et al 2017) are being conducted to determine which microbial populations are innocuous and which have the potential to cause otitis externa, one of the most common diseases in dogs (Dan et al 2014).

My intent was to culture a sample from a healthy canine ear cavity to explore which bacterial communities are present in it. The end goal of this project was to isolate and identify a bacterium living in a dog's ear. Once grown in pure culture, the isolate was identified through genome sequencing and physiological tests. The result was determined to belong to the genus *Kocuria*. These bacteria have been commonly found on mammalian skin and their physiological characteristics are consistent with the literature and the empirical evidence obtained in the lab.

## Methods:

The first step of my experiment was collecting samples from my dog's ear, using sterile cotton swabs and water. This dog is not in contact with other animals, lives indoors, and has no underlying diseases or lesions in the swabbed area. I inoculated a Tryptic Soy Agar (TSA) plate and kept it in semi-darkness and at room temperature (20 °C). One single colony grew on the plate.

In order to be sure to isolate a single bacterial colony, I inoculated another TSA plate following the directions provided in the Lab 2 Handout, and incubated it at 37 °C. This procedure was repeated three more times until the isolate was grown in pure culture. Following this, a series of physiological tests were conducted: Gram staining, as described in the Lab 4 Handout, to determine if the bacterium was Gram positive or negative; oxidase and catalase test (Lab 6 Handout), to establish whether the bacterium is able to produce cytochrome c oxidase and catalase, respectively. Fluid thioglycollate test (Lab 6

Handout), to determine oxygen class, and API 20E test strip, which contains 21 different tests to help distinguish between major strains of bacteria, and confirm some of the physiological abilities of the isolate.

The last step of the experiment consisted in extracting DNA from the isolate in order to sequence its genome. The protocol followed to obtain the purified DNA is described in Lab 5 Handout. The DNA samples were submitted to the DNA Core Lab for sequencing, using the Illumina MiSeq sequencer. Once the data had been processed, I analyzed it (as reported in Lab 7 Handout) using SPAdes Genome assembler - to determine what genes were present in the isolate - and Kraken metagenomics - to identify the bacterium to the species level. Lastly, Prokka Genome Annotation was used to annotate the genes and determine each of their functions.

In addition, the isolate was tested for antibiotic susceptibility, using Erythromycin, Piperacillin, Gentamycin, Clindamycin, Tetracycline, Cefazolin and Oxacillin. The procedure was conducted according to the instructions provided in Lab 9 Handout. As a final examination, the bacterium was cultured on selective media: MacConkey (MAC) and Eosyn Metylene Blue (EMB).

Results:

Upon observation, the colonies appear yellow in color, raised, with even margins and shiny (Fig. 1). The increased growth upon placement in the incubator indicates that the bacterium is a mesophile.

The Gram stain revealed that the bacterium is Gram positive, and under the microscope the cells are visibly coccoid, found in singles or tetrads, and in some cases clusters of irregular size (up to eight cells). They are non-motile and non-sporulating (Fig. 1).

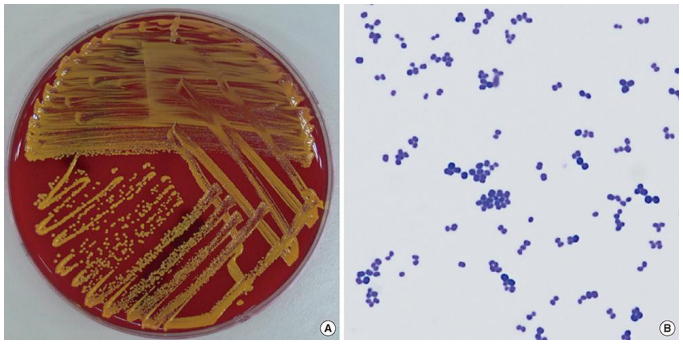


Figure 1: A quadrant streak of K. rhizophila (left) and the cells after Gram staining (right).

The bacterium was tested for oxidase and catalase, and resulted positive for both, meaning it is capable of respiring using cytochrome c oxidase, and to produce the enzyme catalase. In the fluid thioglycollate test, the growth pattern suggests a preferred aerobic metabolism, with tolerance for microaerophilic conditions and minimal growth in anaerobic conditions. The API 20E test strip included several tests, such as $H_2S$, which detects production of hydrogen sulfide, and GLU, which determines if the organism is capable of fermenting glucose. More tests to check for the fermentation of sugars include MAN (mannose), SAC (sucrose), SOR (sorbitol), etc. The API 20E test strip is designed for Gram positive bacteria specifically, and as a result yielded all

negatives.

The results of the genome sequencing were not optimal. The isolate was identified as 37.4% *Kocuria rhizophila*, and 63% unknown. The missed identification could be attributed to a relatively small database, and could have been resolved by running other metagenomic applications such as BLAST, but I felt confident the bacterial identity could be confirmed by the morphological and physiological data alone.

The number of contigs obtained was 114, and the tRNA number was 52. The Prokka application was able to find all 20 common amino acids among the annotated tRNAs, suggesting that the isolate is able to synthesize them independently. Some probable functional genes revealed by the annotation are a conjugal transfer protein (Rv3659c), a multidrug resistance protein EmrY, and a glutamine synthetase.

As the genes suggest, the bacterium identified as *K. rhizophila* is resistant to Cefazolin, Gentamycin and Oxacillin. Intermediate resistance was noted in the case of Piperacillin. Clindamycin was the drug that the bacterium proved to be most susceptible to; the remaining antibiotics did not have a standard on which to base their efficacy.

Culturing on selective media did not allow any growth on the EMB medium, and reduced growth on the MAC medium.

Discussion:

The low level of certainty for bacterial identification, caused by the low amount of DNA recovered during extraction (0.02 ng/l), required extensive literature background research. The colony and cell morphology of the isolate were consistent with the species of *K. rhizophila*, *K. marina* and *K. kristinae*.

*K. kristinae* is the only species confirmed to use cytochrome c oxidase (Stackebrandt et al 1995). For the other two species, a positive oxidase test is not consistent with the literature (Kim et al 2004), although this might be a different strain of *Kocuria* that respires with cytochrome c oxidase, which is transferrable by horizontal gene transfer (Cumsky et al 1983). Alternatively, contamination is a plausible explanation for the incongruence in the results.

The fluid thioglycollate result is also ambiguous. The growth pattern for my isolate in fluid thiogycollate suggests a preferred aerobic metabolism, with tolerance for microaerophilic conditions and minimal growth in anaerobic conditions *K. kristinae* has been shown to grow in anaerobic conditions, but some strains of *K. rhizophila* have also been documented to exist as facultative anaerobes (Savini et al 2010). Because of the variability present among the various species and strains, further attempts of identifying the isolate through physiological tests are futile. The only conclusion that can be supported by the genotypic and phenotypic data is that the isolate belongs to the genus *Kocuria*.

The genes recovered by annotation were significant; the presence of a conjugal transfer protein is an advantageous adaptation that allows for the horizontal transfer of genes by conjugation. This would support the hypothesis that the isolate might have received abilities not normally present in its species' genetic information. Another functional gene contained glutamine synthetase, used in nitrogen metabolism, and commonly present in *K. rhizophila*. Lastly, the multidrug resistance protein found in the bacterium's DNA would account for the reduced susceptibility to many of the antibiotics tested using the Kirby-Bauer method.

The culture inoculated on the EMB medium ulteriorly confirmed that the bacterium was Gram positive, since the dye used in this medium is toxic to this category of microbes. MacConkey's medium also inhibits the growth of Gram positive bacteria, because of the crystal violet and bile salts contained in the agar.

There are many questions left unanswered at the end of this project: the lack of identification of the isolate needs to be resolved - the species and strain must be found in order to resolve the contradictions between literature and physiological tests. Ideally, the DNA extraction would be repeated until it gives a better yield, and following that the sequencing could be done on a larger database than Illumina, such as BLAST.

Fortunately, the presence of any of these species of the genus *Kocuria* in the canine ear is not cause for concern, since they are part of the typical mammalian skin microbiome.

Sources:

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