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The Use of ¹⁴C-FIAU to Predict Bacterial Thymidine Kinase Presence: Implications for Radiolabeled FIAU Bacterial Imaging

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Abstract

Currently available infectious disease imaging techniques cannot differentiate between infection and sterile inflammation or between different types of infections. Recently, radiolabeled FIAU was found to be a substrate for the thymidine kinase (TK) enzyme of multiple pathogenic bacteria, leading to its translational use in the imaging of bacterial infections. Patients with immunodeficiencies, however, are susceptible to a different group of pathogenic bacteria when compared to immunocompetent subjects. In this study, we wanted to predict the usefulness of radiolabeled FIAU in the detection of bacterial infections commonly occurring in patients with immunodeficiencies, *in vitro*, prior to attempting *in vivo* imaging with ¹²⁴I-FIAU-PET.

Methods—We obtained representative strains of bacterial pathogens isolated from actual patients with genetic immunodeficiencies. We evaluated the bacterial susceptibility of different strains to the effect of incubation with FIAU, which would implicate the presence of the thymidine kinase (TK) enzyme. We also incubated the bacteria with ¹⁴C-FIAU and consequently measured its rate of incorporation in the bacterial DNA using a liquid scintillation counter.

Results—Unlike the other bacterial strains, the growth of *Pseudomonas aeruginosa* was not halted by FIAU at any concentration. All the tested clinical isolates demonstrated different levels of ¹⁴C-FIAU uptake, except for *P. aeruginosa*.

Conclusion—Radiolabeled FIAU has been successful in delineating bacterial infections, both in preclinical and pilot translational studies. In patients with immunodeficiencies, *Pseudomonas* infections are commonly encountered and are usually difficult to differentiate from fungal infections. The use of radiolabeled FIAU for *in vivo* imaging of those patients, however, would not be useful, considering the apparent lack of TK enzyme in *Pseudomonas*. One has to keep in mind that not all pathogenic bacteria possess the TK enzyme and as such will not all retain FIAU.

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Our technique is simple, and can be easily used to assess whether a certain bacterial strain of interest can or cannot be visualized using radiolabeled FIAU.

Keywords

Bacterial imaging; PET; FIAU; thymidine kinase

Introduction

Structural imaging techniques, such as Computed Tomography, Magnetic Resonance Imaging and ultrasound are often used in the initial diagnosis and follow-up of patients with suspected infection. Their use is limited, however, by their inherent inability to differentiate between infection and sterile inflammation, or between different types of infection, such as bacterial versus fungal. Even clinically used functional imaging techniques such as radiolabeled leukocytes and gallium citrate scans suffer from the same limitation, since their mechanism of uptake relies upon increased vascular permeability, which occurs in most infections as well as in sterile inflammation. These limitations have created an urgent need for more specific imaging techniques capable of differentiating infection from sterile inflammation, and potentially differentiating between different types of infections, which would provide early non-invasive diagnosis and allow accurate follow-up of the infectious burden.

One recently described probe that is thought to target bacteria directly is radiolabeled 1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil (FIAU). FIAU is a thymidine analog, known to act as a substrate for the thymidine kinase enzyme (TK) of Herpes simplex virus-1 (HSV1-TK), and as such is frequently used in genetic molecular imaging as a radiolabeled reporter probe. It was recently found that FIAU can also be a substrate for the TK of multiple pathogenic bacterial strains, as demonstrated both by *in vitro* and *in vivo* bacterial phosphorylation of FIAU and secondary entrapment of the radioactive compound. This preclinical discovery was soon successfully translated into a pilot human application for the evaluation of musculoskeletal infections.

The ability to distinguish bacterial infection from other opportunistic infections and sterile inflammation would be especially beneficial in immunodeficient patients. Immunodeficiency can be either innate such as with chronic granulomatous diseases (CGD) and Autosomal Dominant Hyper-IgE syndrome (AD-HIES) or can be acquired such as with bone marrow and solid organ transplants, treated hematologic malignancies and Human Immunodeficiency Virus. Depending on the type of immunodeficiency, different types and different rates of bacterial and fungal infections are seen. For example, CGD is characterized by abnormal NADPH oxidase activity which renders patients susceptible to catalase-positive bacteria and fungi, including *Staphylococcus aureus*, *Burkholderia cepacia*, *Serratia marcescens*, *Nocardia* and *Aspergillus* species. In AD-HIES (Job's syndrome), which is caused by STAT3 mutations and characterized by recurrent skin and lung infections, *Staphylococcus aureus* is the most frequent pathogen causing pneumonia. This often results in pneumatocele formation and bronchiectasis, which then become secondarily infected with Gram negative bacteria, namely *Pseudomonas*, and molds, resulting in significant morbidity and mortality. Similar infections are also common in patients with acquired immunodeficiencies. *Pseudomonas*, for example, frequently causes pneumonias in subjects whose immunodeficiency has led to bronchiectasis, and is a frequent blood stream infection in patients with neutropenia.

Due to the detrimental consequences of uncontrolled infection in immunocompromised hosts, obtaining a definitive diagnosis is of utmost importance. This frequently involves

invasive procedures such as lung biopsy or bronchoscopy, and results may not be available in time to guide clinical decisions. If an imaging agent, such as radiolabeled FIAU, can provide a diagnosis of the infectious burden early in the course of disease, it could save those vulnerable patients a lot of invasive procedures, and improve their prognosis.

The goal of our study was to predict the potential usefulness of radiolabeled FIAU in diagnosing bacterial infections in patients with genetic immunodeficiencies such as CGD and AD-HIES. Towards this goal, our study was designed to predict, *in vitro*, the presence or absence of the TK enzyme in specific pathogenic bacteria known to affect immunocompromised patients.

Materials and Methods

Bacterial Strains

We obtained representative strains of bacterial pathogens (*S. aureus*, *B. cepacia*, *S. marcescens*, *Nocardia* species and *Pseudomonas aeruginosa*) isolated from actual CGD and AD-HIES patients from the Department of Microbiology at the Clinical Center of the National Institutes of Health, Bethesda, Md. A wild type reference strain of *Escherichia coli* with intact TK (w3110 CGSC strain#4474, TK^+ *E. coli*) and a TK negative mutant strain (KY895 CGSC strain #4842, TK^- *E. coli*) were obtained from the Yale University *E. coli* Genetic Stock Center, New Haven, CT.

In Silico Analysis

We attempted to identify the TK amino acid sequences of clinically relevant microorganisms in immunodeficient patients, including the target organisms named above, in the gene bank. When we were able to identify the gene sequences, we compared the amino acid sequences of the catalytic domain of TK (the central 100 amino acids) to the TK sequence of a representative bacterial species (*E. coli W3110*) using *in silico* amino acid sequence alignments (BLAST). The reference microorganism (*E. coli W3110*) is the same one we used as a positive control bacterial strain (TK^+ *E. coli*) in incubation studies. We also queried the gene bank for the presence of TK sequences in multiple fungi known to infect immunocompromised hosts, including *Aspergillus fumigatus*, *Aspergillus nidulans* and *Paecilomyces* species. For those organisms where a TK gene sequence could be identified, we calculated the percentage match of the selected amino acid sequence to that of the representative *E. coli W3110* sequence.

Bacterial Susceptibility Assays

Clinical isolates and reference strains of TK^+ and TK^- *E. coli* were grown in 3mL Luria broth (LB) at 37°C without and with FIAU (Moravek Biochemicals, Brea, California) over a range of FIAU concentrations (0, 0.25, 0.49, 0.98, 1.95 and 100 µg/mL). Bacterial survival in the presence of FIAU was evaluated by measuring the optical density (OD, 600 nm) at roughly 2-hour intervals over 30 hours, or until stationary growth phase was established.

Uptake of Radiolabeled FIAU Assays

^{14}C -FIAU was purchased from Moravek Biochemicals, with reported radiochemical purity of 99.9%, specific activity of 55mCi/mmol (2.035 GBq/mmol) and concentration of 100 µCi/ml (3700 KBq/ml; 676.5 µg/ml). Bacterial isolates in 1 mL LB were then incubated either with 4 µl (2.72 µg, 0.4 µCi) ^{14}C -FIAU, or without ^{14}C -FIAU, for 24 hours. The pelleted cells were washed in 100µL 50mM EDTA TRIS HCl –10% glycerol, 2 mg lysozyme per mL and 10% Triton X-100 – to remove un-phosphorylated ^{14}C -FIAU. Bacteria were then lysed by two freeze-thaws, and the lysate was washed again to remove un-incorporated cytosolic phosphorylated ^{14}C -FIAU. Bacterial DNA was isolated using the

QIAamp DNA Mini Kit, and DNA concentrations from each isolate were determined using ultraviolet (UV) light absorbance spectroscopy and micro-volume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc). 1 μ g of DNA from each isolate was diluted and assessed for radioactivity in counts per minute (CPM) using a liquid scintillation counter (LSC). An additional 10 μ g sample of DNA from *S. aureus* was also assayed.

Statistics

We performed linear regression analysis to determine the correlation between the degree of incorporation of ^{14}C -FIAU in the DNA of assayed bacterial species and the percentage match of their amino acid sequence to the reference *E. coli W3110* sequence.

Results

In Silico Analysis

Significant homology was identified between the *E. coli W3110* reference species and *Serratia*, *Staphylococcus*, *Streptococcus*, *Burkholderia*, and *Haemophilus* species (Figure. 1). No TK gene sequence could be identified in the gene bank, however, for *Pseudomonas* or *Nocardia* species. Similarly, no TK gene sequence could be identified for fungi known to infect immunocompromised hosts, including *A. fumigatus*, *A. nidulans*, and *Paecilomyces* species.

Bacterial Susceptibility Assays

The OD values for TK^+ *E. coli* were decreased by increasing concentrations of FIAU, as expected (Figure. 2A). Although TK^- *E. coli* should exhibit no susceptibility to FIAU, there was in fact a moderate reduction in growth of the bacteria at the high concentrations of FIAU. Because the growth media were not selective for the TK^- *E. coli* mutant, we attributed this result to partial recovery of TK activity in the mutant strain (Figure. 2A). Among the other strains under investigation, *S. aureus*, *B. cepacia*, and *S. marcescens* exhibited decreased OD with increasing concentration of FIAU (Figure. 2B), suggesting that the thymidine analog (FIAU) was incorporated into replicating DNA and negatively affected the growth.

Optical Density values for *P. aeruginosa*, in contrast, were not affected by FIAU at any concentration (Figure. 2B). The OD values for *Nocardia* across all concentrations were inconclusive, and were also inconsistent with normal growth. We believe this was due to the inherent difficulty in growing *Nocardia* species using non-selective growth media. This is why we decided to exclude the results from the *Nocardia* experiments.

Uptake of Radiolabeled FIAU

TK^+ *E. coli* demonstrated remarkable levels of ^{14}C -FIAU uptake, estimated in CPM, in comparison with TK^- *E. coli* (Figure. 3A). The ^{14}C -FIAU uptake of TK^- *E. coli*, however, was not equal to background as we observed a modest 1.7 fold increased uptake with ^{14}C -FIAU, suggesting the possibility of partial recovery of TK activity in the TK^- *E. coli* strain. *Pseudomonas*, as expected from its growth in the presence of FIAU, showed CPM values equivalent to background at all concentrations of FIAU.

The other clinical isolates demonstrated levels of FIAU uptake intermediate between the TK^+ *E. coli* and *P. aeruginosa* values. In particular, *S. aureus* (10 μ g), *B. cepacia*, and *S. marcescens*, showed substantially greater CPM counts than background values, indicative of the presence of ^{14}C -FIAU in the DNA sample. Figure 3B shows CPM values in the bacterial isolates grown with and without ^{14}C -FIAU present.

Notably, 10 μ g *S. aureus* DNA were required to produce comparable results to 1 μ g of *S. marcescens* and *B. cepacia* DNA. When only 1 μ g of *S. aureus* DNA was analyzed for the incorporation of ¹⁴C-FIAU, CPM values were higher than background, but to a lesser extent (1.4-fold increase versus 8.8-fold increase corresponding to 10 μ g and 1 μ g of DNA, respectively). These results were expected from the *in silico* analysis, in which the match between *S. aureus* and our reference *TK⁺ E. coli W3110* was relatively lower (25%) than other species capable of phosphorylating FIAU. In fact, the degree of ¹⁴C-FIAU incorporation in the DNA of the different assayed microorganisms with respect to background values significantly correlated ($R^2=0.968$) with the degree of match of the TK sequence of those microorganisms with that of the reference *E. coli W3110* sequence (Figure 4).

Discussion

Accurate microbiologic diagnoses are essential for immunocompromised hosts with clinical evidence of infection. Identifying imaging techniques and probes that target the microorganism directly has been attempted repeatedly using a variety of approaches, including radiolabeled antibiotics or Ubiquitin, with variable but limited success.

Recently, it has been demonstrated that the TK enzyme of many bacterial strains, similar to the Herpes Simplex Virus type-1 TK enzyme (HSV-TK), is capable of phosphorylating the nucleoside analogue FIAU. This major preclinical discovery was demonstrated through successful *in vivo* imaging of bacterial infection in an animal mouse model using radiolabeled FIAU, and was translated shortly thereafter in successful imaging of human musculoskeletal infections using ¹²⁴I-FIAU.

The mechanism of retention of FIAU in the presence of the appropriate TK enzyme is similar in some ways to the mechanism of FDG accumulation. Once inside the cell, FIAU gets phosphorylated by TK, becomes trapped within the microorganisms and can then be imaged using Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT). An assessment of TK protein sequences in 53 pathogenic bacteria has previously revealed striking homology with a clear consensus within the TK catalytic domain. Each one of those bacteria contained at least 25 residues that were identical to those of the consensus while, at the same time, this consensus sequence was not found in mammalian TKs, presumably accounting for the differential capacities of the mammalian enzymes to phosphorylate substrates such as FIAU.

However, not all bacteria possess the TK enzyme and as a result not all bacterial infections can be visualized using radiolabeled FIAU. In our study, we wanted to evaluate the usefulness of FIAU as a bacterial TK substrate in the setting of genetic immunodeficiency. If FIAU entrapment is proven to occur in bacteria commonly affecting immunosuppressed patients, radiolabeled FIAU can then be used to differentiate sterile inflammation from infection, and possibly differentiate fungal from bacterial infections in this patient population.

Our prospective patient population consisted of subjects with genetic immunodeficiencies. Towards this goal we selected five representative clinical isolates that were cultured from actual patients at the NIH Clinical Center. Those included *Staphylococcus aureus*, *Burkholderia cepacia*, *Serratia marcescens*, *Nocardia* and *Pseudomonas aeruginosa*. Due to problems with suboptimal growth of *Nocardia*, however, we are not presenting the results from those cultures. As positive and negative controls we decided to use the wild type *TK⁺ E. coli (W3110)* along with a mutant *TK⁻ E. coli* strain generously provided to us by the Yale University *E. coli* Genetic Stock Center, New Haven, CT.

Our results suggested that FIAU is phosphorylated by *S. aureus*, *B. cepacia*, and *S. marcescens*, while it did not appear to be phosphorylated by *P. aeruginosa*. There was also lower and more delayed phosphorylation associated with TK^- *E. coli*. Although one would expect no effect of FIAU on TK^- *E. coli* growth and no uptake of ^{14}C -FIAU, we observed partial effect which we believe is due to reversed mutation (“leaky mutation”) of the single gene mutant TK^- *E. coli* into TK^+ *E. coli* during its 24-hour culture in nonselective media. A similar phenomenon was observed in a recently published paper where the same mutant TK^- *E. coli* strain was incubated with ^{125}I -FIAU. Rather than complete lack of radioactivity accumulation in association with the mutant strain as would be expected in the absence of TK, some accumulated radioactivity was detected over time.

The fact that FIAU does not seem to be a substrate for *P. aeruginosa* is of major importance for potential use of radiolabeled FIAU *in vivo* for detection of infectious foci. While FIAU accumulation in the lungs is minimal, providing low background activity, the lack of FIAU uptake by *Pseudomonas* would make imaging for bacterial/fungal distinction futile, since *Pseudomonas* is a frequent infectious organism in patients with bronchiectasis complicating immunodeficiency and those with neutropenia. Fungi in general do not have TK, and would not be expected to phosphorylate FIAU either.

While this does not invalidate the huge potential of radiolabeled FIAU as a specific bacterial imaging agent, it may preclude using it to visualize infections in patient populations where *Pseudomonas* infection is likely. A negative result in that case could be misinterpreted as sterile inflammation or non-bacterial infection if one has to rely solely on non-invasive PET diagnosis.

Conclusion

Among all clinically available infectious agents, radiolabeled FIAU promises to be one of the long-sought “specific” imaging agents, i.e. agents capable of differentiating infection from sterile inflammation, and potentially differentiating between various types of pathogens, such as bacteria versus fungi. One has to keep in mind however that not all bacteria possess the TK enzyme and as such, will not all retain FIAU. Our technique is simple, and can be easily adapted to assess whether TK is present in specific bacterial strains versus others.

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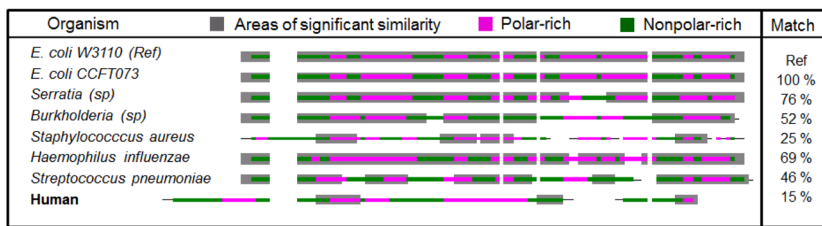
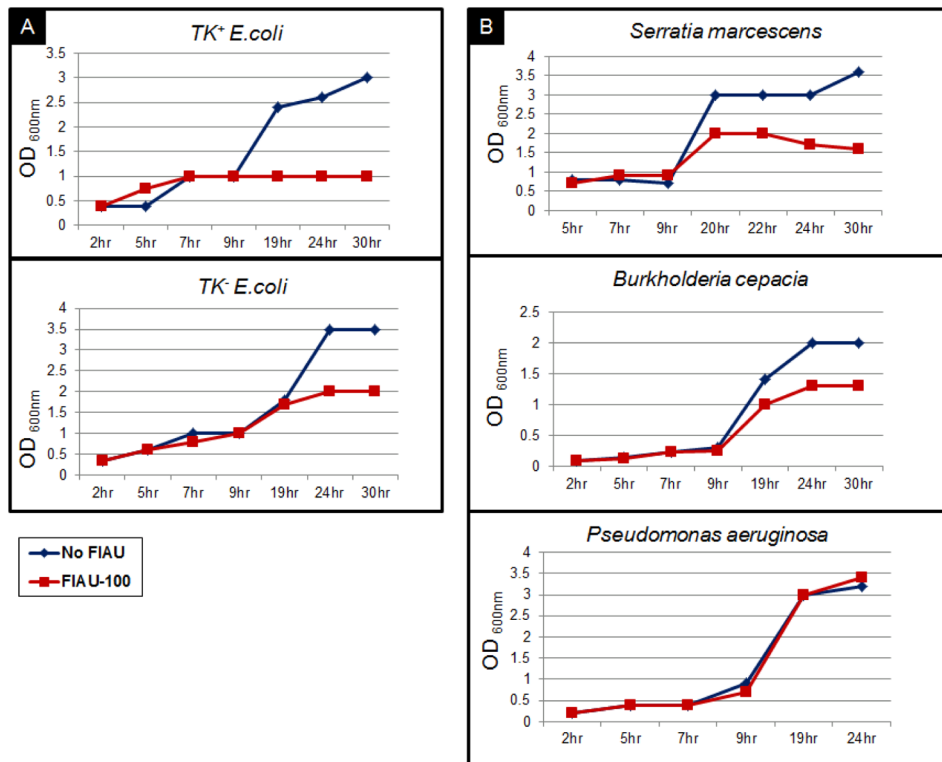


FIGURE 1. Amino acid sequences of the catalytic domain of the thymidine kinase of various microorganisms based on data extracted from the gene bank and analyzed using BLAST. The last column on the right shows the degree of match between the reference sequence (*E. coli* W3110) and the different bacterial species. Notice that there is only 15% match between the humanTK1 AA sequence and that of the reference microorganism.

**FIGURE 2.**

Bacterial survival in the presence of FIAU evaluated by measuring the optical density (OD, 600 nm) at roughly 2-hour intervals over 30 hours, or until stationary growth phase was established. OD values for **A.** *TK⁺E. coli* (*W3110*) and *TK⁻ E. coli* and **B.** *S. marcescens*, *B. cepacia* and *P. aeruginosa* in the absence (blue lozenges) and presence of 100 $\mu\text{g/mL}$ (red squares).

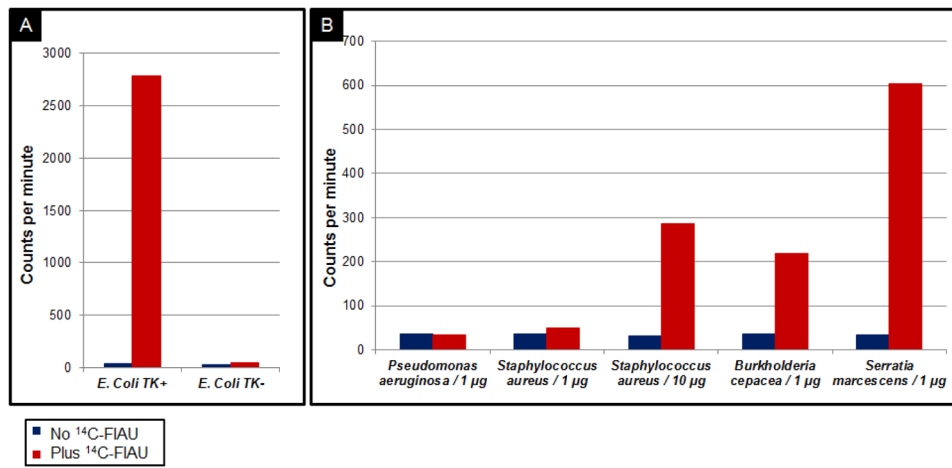


FIGURE 3. CPM values reflecting incorporation of ¹⁴C-FIAU in DNA extracts from **A.** *TK⁺E. coli* (*W3110*) and *TK⁻E. coli*, and **B.** *P. aeruginosa*, *S. aureus* (1µg and 10µg), *B. cepacia* and *S. marcescens*, with and without ¹⁴C-FIAU (background values) present.

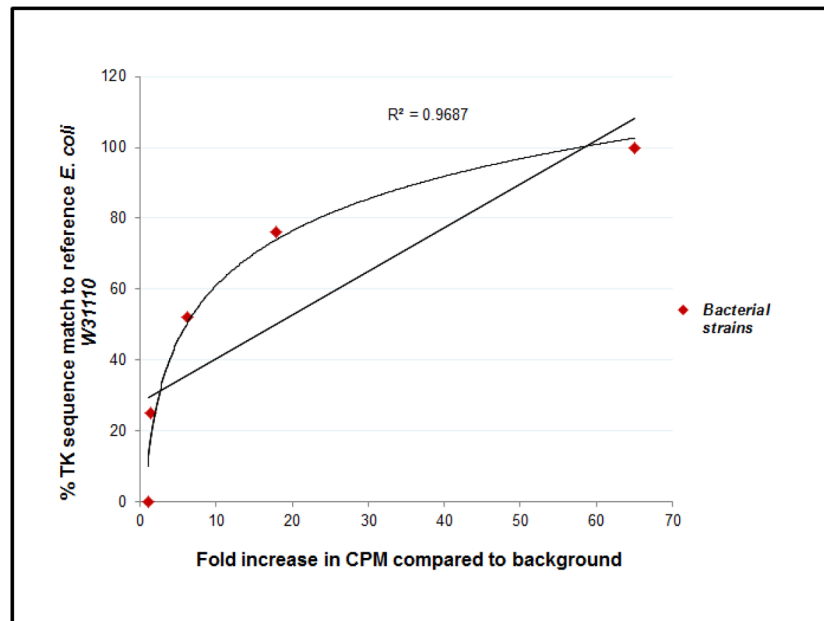


FIGURE 4.

Linear regression analysis shows significant correlation ($R^2=0.968$) between the degree of ^{14}C -FIAU incorporation in the DNA of the different assayed microorganisms (described in fold increased CPM compared to background values) and the degree of match of the TK sequence of those microorganisms with that of the reference *E. coli* W3110 sequence.