TECHNICAL NOTE

Larry S. Davidson,[1] *M.S.; William S. Best,*[2] *B.S.; and James D. Oliver,*[3] *Ph.D.*

Use of *Bacillus subtilis* var. *aterrimus* in a New Method of Tagging

REFERENCE: Davidson, L. S., Best, W. S., and Oliver, J. D., **"Use of *Bacillus subtilis* var. *aterrimus* in a New Method of Tagging,"** *Journal of Forensic Sciences*, JFSCA, Vol. 30, No. 2, April 1985, pp. 531-534.

ABSTRACT: This study describes a new method of tagging in which a nonpathogenic, black pigmented bacterium, *Bacillus subtilis* var. *aterrimus*, is employed as the tagging agent. Articles composed of various materials, including paper, glass, metal, and plastic, were sprayed with cell suspensions of the bacterium. Detection of the tagging agent was accomplished through recovery of black pigmented bacterial colonies from tagged articles. This procedure has advantages over conventional tagging methods in that the bacteria are not visible after application and are easily detected without special equipment. The procedure is inexpensive to perform and does not require specially trained personnel.

KEYWORDS: forensic science, bacteria, tagging

Current methods universally employed in forensic tagging involve the application of organic dyes, rare earth metals, or fluorescent compounds. *Bacillus subtilis* var. *aterrimus*, originally described by Biel and Lund [*1,2*], may be easily and safely used as a tagging agent.

Materials and Methods

Selection of Bacterium

American Type Culture Collection (ATCC) Strain 6460, *B. subtilis* var. *aterrimus*, was selected as the tagging agent. It is a rare bacterium that is not normal flora to any part of the human body. In the course of this study, approximately 100 environmental samples (that is, soil, plants, and water) were monitored for the presence of this bacterium. In no case was the bacterium recovered on the selective Spizizen Potato Agar. *B. subtilis* var. *aterrimus* is capable of

Received for publication 6 June 1984; revised manuscript received 2 July 1984; accepted for publication 3 July 1984.

[1]Graduate student, Department of Biology, University of North Carolina at Charlotte, Charlotte, NC and medical student, East Carolina University of Medicine, Greenville, NC.

[2]Assistant lab director, Charlotte Police Crime Laboratory, Charlotte, NC.

[3]Professor of biology, Department of Biology, University of North Carolina at Charlotte, Charlotte, NC.

forming spores that are able to survive harsh environmental factors such as heat, cold, low nutrient availability, and dehydration. Bacterial spores will survive for an indefinite period of time. When reintroduced into suitable environmental conditions (such as a petri plate of media), spores revert to a more typical bacterial cell state. Only intense, prolonged heat (100°C+), pressure, or specific sporicidal chemical agents are effective in destroying bacterial spores.

ATCC Medium 423, Spizizen Potato Agar, was used to detect the tagging agent. The medium is prepared as described by Ref *3*, and is summarized here. Peeled, diced potatoes, 200 g, were boiled for 30 min in 1 L of tap water. Manganese sulfate, 5 mg, was added to the filtered potato soup. The pH of the soup was adjusted to 6.8 with 1.0*M* sodium hydroxide and the volume brought to 1 L with tap water (distilled water is not necessary). Fifteen grams of agar (Sigma Chemical, St. Louis) were added as a gelling agent. The soup-agar suspension was autoclaved at 121°C for 15 min, cooled to 50°C in a water bath, and aseptically dispensed into sterile petri plates. The potato agar plates were stored at 4°C until needed.

Preparation of Tagging Suspension

The tagging suspensions were prepared by suspending ATCC Strain 6460 grown on potato agar in 10 to 20 mL of sterile saline. (These suspensions may be kept in sterile test tubes and stored at room temperature, refrigerated at 4°C, or frozen at −15°C.)

Application of Tagging Suspension

Using a common aerosolizer, the tagging suspension was sprayed for a few seconds onto five articles including a plastic pen, glass beaker, white paper envelope, metal cup, and a one-dollar bill. All tagged articles were maintained at room temperature. (The tagging suspension may be further diluted in saline or water so as to increase its volume to facilitate the tagging of a large number of articles. Cell concentration in the tagging suspension is not a crucial factor. Preparation of the suspension, as described in the Materials and Methods section, will provide a more than ample concentration sufficient for tagging. Additional dilutions have been conducted without compromising the sufficiency of the suspension.)

Detection of Tagging Agent

Detection of the tagging bacteria was accomplished through brief swabbing of a tagged article with a saline- or water-moistened cotton swab and subsequent inoculation onto a potato agar petri plate. Inoculated plates were incubated at 25°C for 36 h or 37°C for 24 h and observed periodically. The temperature of 37°C seems to be a somewhat better choice. Detection and recovery of the tagging bacterial was confirmed by the growth of colonies exhibiting a black pigment on the potato agar medium. All tagged articles were monitored for recovery of the tagging bacterium over a four-month period.

Examination of Cross Transfer of Tagging Agent

The cross transfer of ATCC Strain 6460 from tagged articles to skin was examined. A group comprised of five people was assigned to each tagged article. Each article was touched in an appropriate manner by one person in the group. A series of successive handshakes to the other members of the group was initiated by the person initially touching the tagged article. Cross transfer of the tagging bacteria was monitored through swabbing of each person's hands with a moistened cotton swab and inoculation onto potato agar.

The cross transfer of ATCC Strain 6460 from tagged articles to clothing was also examined. A tagged article was touched in an appropriate manner and the hand previously making con-

tact with the article was then placed into a coat pocket. Cross transfer of the tagging bacteria was monitored through swabbing of the inside lining of the pocket and inoculation onto potato agar.

Note that conducting bacterial counts for the purpose of calculating the recovery of this tagging bacterium is not necessary. This tagging method is based on qualitative interpretation (presence or absence of the bacteria) rather than quantitative interpretation (the rate or level of recovery). If an object is tagged, even with low concentrations of the bacteria, the detection method is capable of indicating the presence of the bacteria.

Pathogencity Study

The inability of ATCC Strain 6460 to cause infection was examined. A 24-h-old 5-mL nutrient broth culture of ATCC Strain 6460 was centrifuged at 10 000 *g* for 10 min and the pellet resuspended in a 5 mL of sterile saline solution. Three laboratory mice each received one 0.5-mL intraperitoneal injective of the bacterial suspension (an inoculum of approximately 3.5×10^6 cells). The mice were observed over a one-week period.

Results

Detection and Recovery of Tagging Bacterium

The applied suspension of tagging bacteria is not visible on tagged articles. However, brief swabbing of these articles with cotton swabs and subsequent inoculation onto Spizizen Potato Agar, previously described under the bacterial medium, resulted in growth of colonies exhibiting a black pigment. Because only ATCC Strain 6460 exhibits such a pigment on Spizizen Potato Agar, development of black pigmented colonies served as a positive marker of recovery of the tagging agent. This tagging bacterium was recovered from all articles experimentally tagged over the entire monitoring period of five months.

Cross Transfer of Tagging Agent

It was found that ATCC Strain 6460 is easily transferred from tagged article to skin. The tagging bacterium is also easily transferred between persons via skin contact as it was recovered from the hands of all persons of each group assigned to a tagged article.

It was also found that the tagging bacteria is transferred from tagged articles to clothing via skin contact. The tagging bacteria were recovered from the inside of the coat pocket previously touched by a tagged hand.

Pathogenicity Study

Laboratory mice receiving intraperitoneal injections of ATCC Strain 6460 were observed for one week. None of the mice exhibited signs of physical distress.

Conclusion

Obvious disadvantages exist with current methods of forensic tagging. Organic dyes afford a convenient method of tagging, but as they are visible, they are not necessarily the most practical choice. Rhodamine B supposedly remains invisible on tagged articles while fluorescing under ultraviolet light, however, in moist environments this compound may exhibit a red to purple color.

Safety considerations as well as expensive detection instruments are required with the use of rare earth metals in tagging procedures.

The use of bacteria as tagging agents avoids most of the problems often encountered with other procedures. For instance, *B. subtilis* var. *aterrimus* is undetectable on tagged articles except through the specific detection procedure that uses Spizizen Potato Agar. This detection procedure is convenient as it requires no extensive expertise in bacteriology. This tagging procedure may be performed at a minimal cost compared to some conventional procedures. It is important to note that because ATCC Strain 6460 is nonpathogenic, this procedure requires minimal safety considerations.

Several properties of *B. subtilis* var. *aterrimus*, allow this bacterium to be a successful tagging agent. This organism exhibits a unique black pigment when grown on potato agar thus providing a means to differentiate the tagging bacteria from other microorganisms. This pigment is best observed with isolated colonies which may be obtained by streaking away from the initial inoculum with an inoculating loop in a zigzag manner over the remainder of the potato agar plate at the time of inoculation. Also, being a member of the genus *Bacillus*, this organism is capable of sporulation, a process rendering the organism extremely resistent to harsh environmental factors such as high and low temperatures and low nutrient availability. Thus, this biological tagging agent would survive environmental conditions normally encountered in tagging operations. Use of *B. subtilis* var. *aterrimus* is an initial step in the introduction of safe bacterial tagging agents.

References

[1] Biel, W., "Uber einen schwarzes Pigment bildenden Kartoffelbacillus," Abt. 2, No. 2, 1896, pp. 137-140.

[2] Lunt, J., "On *Bacillus mesentemicos niger* (a new Potato Bacillus)," *Centralblatt Fuer Bakteriologie, Parasitenkunde Und Infektionskrankheiten*, Abt. 2, No. 2, 1896, pp. 572-573.

[3] *American Type Culture Collection Catalogue of Strains I*, 15th ed., 1982, p. 618.

Address requests for reprints or additional information to
William S. Best
Charlotte Police Crime Laboratory
825 E. 4th St.
Charlotte, NC 28202-2889