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Asaia sp., an Unusual Spoilage Organism of Fruit-Flavored Bottled Water

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A gram-negative bacillus was isolated from a batch of fruit-flavored bottled water, which had spoiled as a result of bacterial overgrowth (>10^6 CFU/ml). The spoilage organism was extremely difficult to identify phenotypically and was poorly identified as *Pasturella* sp. (78.7% identification profile) employing the API 20NE identification scheme, which gave the profile 5040000. Molecular identification through PCR amplification of a partial region of the 16S rRNA gene followed by direct automated sequencing of the PCR amplicon allowed identification of the organism. Due to the sequence identity (100%) between the spoilage organism and a reference strain in GenBank, the spoilage isolate was considered to be an *Asaia* sp., a recently described genus and member of the acetic acid bacteria. This is the first report of *Asaia* sp. causing spoilage of a foodstuff and highlights the benefits of molecular identification techniques based on 16S rRNA gene sequences in the identification of unusual spoilage organisms.

There has been a gradual increase in demand for bottled water in the United States over the past 20 years, which has been exacerbated by public health scares relating mainly to waterborne outbreaks of human cryptosporidiosis caused by *Cryptosporidium parvum*. Furthermore, a recent study has demonstrated a high consumption (40%) of bottled water by human immunodeficiency virus-positive patients (2). To date, there have been relatively few reports employing the use of molecular identification methods, particularly the use of rRNA identification techniques, to identify contaminating bacterial and fungal agents in bottled water. We report an occurrence of spoilage in fruit-flavored bottled drinking water, which was isolated during production and prior to distribution. Our aim was to employ such molecular techniques to identify the bacterial spoilage organism, as phenotypic methods were unable to identify the organism.

Microbiological examination was performed on a batch of fruit-flavored bottled water to determine the causal spoilage organism. The spoiled product was turbid (equivalent to McFarland standard 5) and had a characteristic sour odor, with a pH of 3.5. Quantitative microbiological examination on Plate Count Agar (Oxoid CM; Oxoid Ltd., Basingstoke, England) at 30°C for 48 h demonstrated the presence of a pure culture (>10^6 CFU/ml) consisting of a single and characteristic morphotype from which a single colony was purified, yielding an unidentified gram-negative rod, with no other bacteria or fungi cultured from the spoiled drink. The isolation of this morphotype from the spoiled fruit drink was repeated on further culture of the fruit drink. The spoilage isolate grew at 22 and 30°C but failed to grow at 37°C. It was catalase positive and oxidase negative, with translucent, pale pink, shiny, smooth colonies which were raised with an entire edge. The colonies were extremely small (approximately 1 to 2 mm in diameter), and the isolate was relatively resistant to antibiotics and specifically resistant to ceftazidime (30 μg), meropenem (10 μg), imipenem (10 μg), trimethoprim (5 μg), amikacin (30 μg), vancomycin (30 μg), aztreonam (30 μg), penicillin (2 μg), ampicillin (10 μg), on extended antibiogram testing, using standard disk diffusion assays, as previously described (4). The API 20NE identification scheme (Biomérieux, Les Halles, France) was unable to identify the isolate with confidence, giving a profile of 5040000 (78.7% identification for *Pasturella* sp.). The isolate was subsequently referred for molecular identification.

All DNA isolation procedures were performed in a class II biological safety cabinet in a room geographically separate from that used to set up reaction mixes and also from the room used for post-PCR procedures in order to minimize the production of false-positive results. DNA was extracted from a single colony employing the Roche High Purity PCR Template kit (Roche Diagnostics Ltd., Lewes, United Kingdom), in accordance with the manufacturer's instructions. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the room used for amplification and post-PCR procedures in order to minimize contamination. Reaction mixes (50 μl) included the following: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1.25 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer); and 0.2 μM (each) 16S rRNA primer, as previously described (1). The 16S rRNA primers were PSL (forward primer) (5′-AGG ATT AGA TAC CCT GGT AGT CCA-3′) (positions 783 to 806 relative to *Escherichia coli* [GenBank accession no. J01859]) and PSR (reverse primer) (5′-ACT TAA CCC AAC ATC TCA CGA CAC-3′) (positions 1094 to 1071 relative to *E. coli* [GenBank accession no. J01859]). The reaction mixtures following a hot start were subjected to the following thermal cycling parameters in a Perkin-Elmer 2400 thermocycler: (i) 3 min at 96°C; (ii) 40 cycles, with 1 cycle consisting of 1 min at 96°C, 1 min at 55°C, and 1 min at 72°C; and (iii) a final extension step of 10 min at

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72°C. During each run, molecular grade water was included randomly as negative controls and DNA template from *Staphylococcus aureus* was included as a positive control. PCR products were subsequently sequenced in the forward direction using the Cy-5′ labeled primer, with the ALF Express II (Amersham-Pharmacia Ltd., Amersham, Buckinghamshire, England) employing the Thermo Sequenase fluorescence-labeled primer cycle sequencing kit with 7-deaza-dGTP (catalog no. RPN 2438; Amersham Pharmacia Biotech, UK), as detailed previously (5). The resulting sequences obtained were compared with those stored in the GenBank Data system using BLAST alignment software (http://www.blast.genome.ad.jp/).

The phenotypic characteristics of the spoilage isolate were consistent with those described previously for the genus *Asaia* (8). Antibiotic susceptibility testing demonstrated the high natural resistance of this isolate to several medically important antibiotic agents, particularly those employed against the gram-negative bacterial flora, and it is interesting to note this high level of antibiotic resistance in an environmental isolate which has never been under antibiotic selective pressure. Direct sequencing of the PCR amplicon identified this organism as *Asaia* sp. with all 270 bases examined (100% homology) with *Asaia* sp. (GenBank accession no. AB025932). In addition, the isolate is archived at the Northern Ireland Public Health Laboratory, Belfast City Hospital, Belfast, Northern Ireland, United Kingdom. An alignment of the sequence from the 16S ribosomal DNA with two described species within the *Asaia* genus demonstrated that the organism was neither *Asaia siamensis* nor *Asaia bogorensis* and may represent a new and novel species within this genus. Hence, it was assigned the identification of *Asaia* sp.

Asaia is a recently described genus consisting of two members, i.e., *A. bogorensis* and *A. siamensis* (3, 8). This genus is included in the acetic acid bacterial lineage and is phylogenetically closely related to the genera *Acetobacter, Gluconobacter, Acidomonas,* and *Gluconacetobacter*. The natural habitats of *Asaia* spp. have been reported to be in flowers of the orchid tree (*Bauhinia purpurea*), plumbago (*Plumbago auriculate*), and fermented glutinous rice, all originating in hot tropical climates, particularly in Indonesia and Thailand. Because this organism cannot grow at 37°C, coupled with the absence of known cases relating to infection with this organism, this organism does not appear to present a significant human health risk based on this absence of epidemiological association. Acetic acid bacteria have historically played an important role in the production of vinegar (6), as well as in the production of commercial fermented drinks, such as teakwass, kwass, and waterkefir (7). However, this report serves to illustrate the potential for the proliferation of such organisms in drinks with a low pH, where fermentation is not required. It is difficult to ascertain the origin of this organism in the spoiled bottled water. However, given that the flavoring came from natural fruit juices and not synthetic analogues, it may be speculated that the natural fruit juices were possibly the origin of the spoilage organism. Overall, this report highlights problems associated with the correct identification of unusual environmental organisms involved in food spoilage. This is mainly due to the absence of such identification profiles in commercially available identification schemes, such as the API scheme. This report therefore highlights the growing role of molecular identification schemes based on PCR amplification and direct sequencing of the rRNA genes, either from bacterial or fungal spoilage organisms, which may be of benefit to the food industry in the identification of economically important spoilage organisms.

Nucleotide sequence accession number. The sequence from the spoilage organism has been deposited in GenBank under accession number AF440747.

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