Acinetobacter septicus sp. nov, Associated with a Nosocomial Outbreak of Bacteremia in a Neonatal Intensive Care Unit

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Abstract

*Acinetobacter* species, other than *A. baumannii*, have rarely been reported to be associated with nosocomial outbreaks of bloodstream infections. Within a period of one week, seven *Acinetobacter*-like isolates were recovered from peripheral blood and catheter specimens of five patients at a neonatal intensive care unit (NICU) in a tertiary hospital in Turkey. All five patients had placement of central venous catheters and had received total parenteral nutrition before the onset of bacteremia. Two of the five patients died. Medical devices, tap water, aerators, water samples, various surfaces, intravenous fluids, and the hands of health care workers in the NICU were sampled and were culture-negative for the bacterium. All seven of the isolates had identical biochemical reactions, antimicrobial susceptibility results, and pulsed field gel electrophoresis patterns, indicating a clonal nosocomial outbreak. A panel of standard biochemical reaction profiles and three phenotypic commercial identification systems failed to identify these isolates. Phenotypically, the isolate differed from *A. ursingii* by its hemolysis on sheep blood agar and its negative citrate utilization. Sequences of the full 16S rRNA gene, which contained at least three different gene copies with polymorphic sequences between nucleotide positions 70 and 206, were determined from the first recovered isolate. The complete 1,529-1,531 base-pair 16S rRNA gene sequences and partial, 801 base-pair *rpoB* gene sequences had similarities of 99.5% and 97.2%, respectively, to an *Acinetobacter ursingii* isolate. The DNA-DNA similarity of the strain against the type strain of *A. ursingii* was 64.7 and 68.7%, which was lower than the recommended threshold value of 70% for the definition of bacterial species. These data indicate that a novel *Acinetobacter* organism caused the nosocomial outbreak of bacteremia in the NICU unit. We propose the designation of *Acinetobacter septicus* sp. nov. for these isolates, with isolate AK001 as the type strain.
Introduction

Genus *Acinetobacter* was proposed by Bouvet and Grimont in 1986 (3) and recently was expanded to 32 genomic species, including 17 with a validated name (37). The *Acinetobacter* species are non-motile, non-fermentative, aerobic gram-negative bacilli widely distributed in nature (2). Only 10 nomenspecies have been isolated in human specimens including *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. parvus*, *A. radioresistens*, *A. schindleri*, and *A. ursingii* (12). The most frequently isolated species from humans is *A. baumannii*, which appears also to be the *Acinetobacter* species of greatest clinical importance.

Nosocomial infections are one of the most important causes of mortality and morbidity in hospitals, and numerous nosocomial outbreaks due to *Acinetobacter* species have been described. Risk factors associated with these infections include antibiotic exposure, length of stay in intensive care units, mechanical ventilations, and severity of underlying illness (1, 7, 9, 11, 21, 23, 39). Hospitalized patients can become “colonized” with *Acinetobacter* species, which may cause endemic problems in the hospital setting due to cross-transmission between patients (7, 11, 23). *Acinetobacter* species have been isolated from pneumonia (6.9%), bloodstream infections (2.4%), surgical site infections (2.1%) and urinary tract infections (1.6%) according to the U.S. National Nosocomial Infections Surveillance data from hospitals in 2003 (15). *A. baumannii* remains the main species associated with outbreaks of nosocomial infection. A genotypic analysis of *Acinetobacter* bloodstream infection isolates in a Turkish university hospital indicated that 80.5% were *A. baumannii* (1). Nosocomial infections caused by other *Acinetobacter* species have rarely been reported; although an outbreak of infections with *A. calcoaceticus* in burn patients has been reported (39). *Acinetobacter* genomic species 3 and
13TU also have been implicated in nosocomial infections (10), while A. johnsonii has been associated with catheter-related bacteremia (38). A sporadic case of bacteremia caused by A. ursingii has been reported (32). Most non A. baumannii caused nosocomial Acinetobacter infections are seen in patients who are already suffering from severe underlying diseases and their clinical significance remains defined (14, 22, 26, 42).

Here we are reporting a novel Acinetobacter species associated with a nosocomial outbreak of bacteremia in a neonatal intensive care unit (NICU). Within a period of one week, seven Acinetobacter-like isolates were recovered from peripheral blood and catheter specimens of five patients at an NICU in the Gulhane Military Medical Academy Hospital in Turkey. All seven isolates had identical biochemical reactions, antimicrobial susceptibility results, and pulsed field gel electrophoresis (PFGE) patterns, indicating a clonal nosocomial outbreak. Identification systems based on biochemical reactions failed to identify these isolates. Sequence analysis of full 16S rRNA and partial rpoB genes and DNA-DNA hybridization suggests that these isolates are new Acinetobacter species and the designation of A. septicus sp. nov is proposed.
Material and Methods

Patients and participants. Gulhane Military Medical Academy Hospital is a 1,500-bed teaching hospital in Ankara, Turkey. Three doctors, four nurses and one nurse trainee work in the 15-bed NICU. Patients with low birth weight or respiratory dysfunction related to prematurity are generally admitted to this NICU. During one week in March 2006, a total of seven *Acinetobacter* isolates were recovered from five of eight newborn patients’ blood specimens. Informed consent was obtained from the parents of all five patients. Clinical specimens were collected, and medical records were reviewed. Medical devices, tap water, aerators, water samples, various surface, intravenous fluids, and the hands of health care workers were collected and cultured to evaluate potential risk factors and to find a source of the bacteremia.

Specimen processing and bacterial isolation. Patients’ blood samples were processed with the Bactec 9240 non-radiometric blood culture system (Becton Dickinson, Sparks, MD). When the positive blood cultures were confirmed to be gram-negative bacilli by Gram stain, they were subcultured onto MacConkey and 5% sheep blood agar for up to 3 days at 37°C. Four related and well-characterized *Acinetobacter* strains, including an *Acinetobacter* species (ATCC 410000, American Type Culture Collection (ATCC), Manassas, Va.), *A. lwofii* (ATCC 19002), *A. ursingii* (DSM 16037, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany) and one *A. baumannii* strain isolated at Vanderbilt University Medical Center (7), were used as reference controls. Clinical isolates and reference strains were collected and saved in brain heart infusion (BHI) containing 7.5% glycerol at –80°C for further study.

Phenotypic identification. All isolates were presumptively identified by conventional methods including hemolysis on sheep blood agar, sugar fermentation, motility, catalase, oxidase, citrate,
urease, indol and H₂S production and by the API 20E (bioMerieux Inc., Durham, NC), the Rapid NF Plus (Remel Inc., Lenexa, KS) and the Biolog GN2 (Biolog Inc., Hayward, Ca.) (24, 25, 41). The proposed type strain, AK001, was further identified based on a panel of standard biochemical methods at the DSMZ according to protocols published previously (3).

Antimicrobial Susceptibility Testing. Susceptibilities of the isolates were determined by a disk diffusion method. The following antimicrobial agents (Oxoid, Basingstoke, Hampshire, UK) were tested: ciprofloxacin, trimethoprim-sulfamethoxazole, imipenem, meropenem, ceftazidime, ampicillin-sulbactam, amikacin, cefotaxime, gentamicin, piperacillin-tazobactam, and cefepime. Results were expressed as susceptible or resistant according to the criteria recommended by the Clinical Laboratory Standards Institute (5).

Genomic DNA analysis by PFGE. PFGE typing of SmaI-digested DNA was performed by a modification of a previously described method (7). Electrophoresis was performed with a run time of 18.5 h under 1 to 17 sec linear ramped pulse times by the contour-clamped homogeneous electric field method with a Bio-Rad CHEF DR II system (Bio-Rad, Hercule, CA). After PFGE, the gels were stained with ethidium bromide (0.5 µg/ml) and analyzed under UV transillumination using the Quantity One software (Bio-Rad).

16S rRNA gene amplification, cloning, and sequencing. A loopful of each purified bacterial isolate was put into 1 ml of distilled water. The suspension was vortexed, heated for 7 min at 95°C, centrifuged at 8,000 ×g for 15 s, and 1 µl of supernatant was used for PCR amplification. A highly conserved primer set (5'-TGG AGA GTT TGA TCC TGG CTC AG-3' and 5'-AAG GAG GTG ATC CAR CCG CA-3') spanning 5-1,553 nucleotide positions of the 16S rRNA gene was used to amplify the DNA fragment by PCR (41). The PCR products were directly used for
sequence determination on an ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster City, CA), as previously described (41). Furthermore, the PCR products were cloned in a pCR2.1 vector (TA Cloning Kits, Invitrogen Corp., Carlsbad, CA) according to the manufacturers’ instructions. M13 forward and reverse universal primers and several additional 16S rRNA gene internal primers were used for sequencing the cloned rDNA on the same DNA sequencer as previously described (41).

**rpoB gene amplification and sequencing.** Amplification of the partial ribosomal polymerase B subunit (rpoB) gene (902 bp) was performed by using primers Ac696F and Ac1598R, as previously described (29). In addition to the PCR primers, two internal primers, Ac1093R and Ac1055F, were used for sequencing (29). The sequencing products were purified using CentriSep Spin Columns (Princeton Separations, Adelphia, NJ) and were analyzed on the ABI Prism 3130 Avant Genetic Analyzer, according to the manufacturer’s instructions (Applied Biosystems).

**Phylogenetic analysis.** Full sequences of the 16S rRNA gene were included for analysis. For the rpoB gene, the region from base positions 2900 to 3700 (corresponding to A. baumannii rpoB gene positions of the GenBank entry DQ207471) of the rpoB gene was used. Both full 16S rRNA and partial rpoB gene sequences were analyzed using Ridom TraceEditPro software (version 1.1; Ridom GmbH, Würzburg, Germany). Multiple alignment, sequence similarities of the 16S rRNA and partial rpoB sequences, and neighbor-joining trees with bootstrap values were calculated using MEGA 3.1 software (27). Both full 16S rRNA and partial rpoB gene sequence trees were outgroups rooted with *Pseudomonas aeruginosa* strain PA01 sequences.
**DNA-DNA Hybridization.** DNA-DNA hybridization of strain AK001 against *A. ursingii* (DSM 16037) was performed at the DSMZ. Bacterial DNA was isolated from logarithmic-phase cultures (Thermo Spectronic, Madison, WI) and was purified by chromatograph on hydroxyapatite, as described previously (4). DNA-DNA hybridization was carried out in 2×SSC at 67°C (8) under consideration of the modifications described previously (18) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with in-situ temperature probe (Varian Inc., Palo Alto, CA). A threshold value of 70% DNA-DNA similarity was used for the definition of bacterial species according to the ad hoc committee recommendations (44).
Results

There were eight patients during the study period in the NICU. A total of seven unusual *Acinetobacter* isolates – from five blood and two catheter tip samples – were recovered from a total of five newborns. All seven isolates had the same antibiotic susceptibility patterns and were susceptible to the antibiotics that were used in this study except for ceftazidime and cefotaxime. Two of the five neonates died; the others recovered after a week’s course of intravenous amikacin and meropenem (Table 1). The same *Acinetobacter* strain was not isolated from environmental and personnel samples, which were sampled at the same time period.

All seven strains had the same biochemical test results, including hemolysis on sheep blood agar, non-fermentative, non-motile, catalase-positive, oxidase-negative, citrate-negative, urease-negative, and were indol and H2S production negative. A full panel of standard biochemical reactions were performed on the first isolate AK001, which was a Gram stain-negative rod with a width and length of 0.7-0.9 and 1.0-2.5 µm. It produced hemolysis on the sheep blood agar and was lysed by 3% KOH. It was positive for aminopeptidase and ADH, but negative for conversion from NO₃ to NO₂. Other key biochemical reactions are listed in Table 2. This unusual *Acinetobacter* isolate was differentiated from *A. ursingii* by its hemolysis on sheep blood agar and negative citrate reaction (34, 36). Biochemical reactions were the same for all seven isolates in bioMerieux API 20E, Remel Rapid NF Plus and Biolog GN2 systems: all three systems failed to make an identification with acceptable similarity scores. The epidemiologic relatedness of the seven isolates was further analyzed by PFGE. For comparison, one *A. baumannii* strain, which was isolated from the burn unit at Vanderbilt University Hospital multiple times, was included (7). The PFGE patterns of the seven isolates were identical, suggesting that the isolates were epidemiologically related and clonal in origin (Fig. 1).
The 16S rRNA gene for all seven isolates was amplified by PCR, and the direct sequence
determined on amplicon was performed. Mixed sequences around nucleotide position 100 were
repeatedly produced. Therefore, amplicons of strain AK001 were sub-cloned and a total of 12
clones were selected and subjected to DNA sequencing. Sequencing resulted in three different
operons of the 16S rRNA gene, named TAAK0106, TAAK0110, and TAAK0112, respectively,
ranging from 1,529 to 1,531 nucleotides. The three full 16S rRNA gene sequences had a 99.98%
similarity among them and were closely related to an *A. ursingii* (DSM 16037) (34, 36) at a
99.5% similarity (Fig. 2). To further characterize the seven unknown *Acinetobacter* strains,
partial 801 bp *rpoB* direct gene sequencing was applied and the sequences were compared to the
sequences of all 17 type strains. All seven unknown *Acinetobacter* strains showed identical *rpoB*
sequences. Therefore, only the AK001 was included in Fig. 3. The sequence of AK001 showed a
similarity of 97.2% to *A. ursingii* (DSM 16037), and 76.6 to 81.2% to other *Acinetobacter*
species (Fig. 3).

The DNA-DNA hybridization of strain AK001 against *A. ursingii* (DSM 16037) was performed
in duplicate, and the resulting DNA-DNA similarity was 64.7 and 68.7%, which were both lower
than the recommended threshold value of 70% for the definition of bacterial species (44). Based
on all physiological and chemotaxonomical data, we propose the designation of *Acinetobacter
septicus* sp. nov for the novel species in *Acinetobacter* genus with strain AK001 as the type
strain.
Discussion

In this report, we have described a cluster of five cases of bacteremia within a week in an NICU that were caused by a novel *Acinetobacter* species. Seven isolates were recovered from peripheral blood and catheter specimens of these five patients. Two of five patients died; the others recovered after receiving a week’s course of intravenous amikacin and meropenem. All the seven isolates presented identical biochemical reactions, antimicrobial susceptibility profiles, and pulsed field gel electrophoresis patterns, indicating a clonal nosocomial outbreak.

Bacteremia among newborns in NICUs cause considerable mortality and morbidity and account for approximately 30% of hospital-acquired infections in this population (30). The incidence of bacterial infection in NICUs is estimated to be about 1 to 8 newborns per every 1000 live births and 160 to 300 per 1000 in very low-birth-weight newborns. Coagulase-negative staphylococci and *Enterobacter* species are the most commonly isolated pathogens from the NICU (31).

*Acinetobacter* spp. are usually considered non-pathogenic to healthy individuals; however, especially in debilitated individuals and patients in ICUs, they cause nosocomial infections (13). Premature and low-birth-weight infants, as well as the length of hospital stay cause significant risk for developing infections (33). In this study, three patients were premature and had low birth weight.

*Acinetobacter* species are widely distributed in nature and in the hospital environment. It has been shown that the digestive tract of intensive care unit patients is an important epidemiologic reservoir in hospital outbreaks (6). Environmental contamination of various hospital items has been often identified, ranging from suctioning equipment to pillows and mattresses (13). Foreign bodies such as catheters play an important role in the pathogenic occurrence of *A. lwofi*
bacteremia (40, 43). Hand organism carriage by health care workers has been implicated in
outbreaks of *Acinetobacter* infections (2). During an outbreak of *A. baumannii* bacteremia in a
NICU in Taiwan, multiple *A. baumannii* isolates were recovered from hand washing samples and
some of them were epidemiologically related to those recovered from patients’ blood, suggesting
that the hospital environment was the potential reservoir, and that transmission was possibly via
the hands of health care workers (17). Higher device-associated infection rates and higher device
utilization ratios in an ICU were reported in Turkey (20). In our event, all five patients had
placement of central venous catheters and had received total parenteral nutrition and before the
onset of bacteremia. We suspected that cross-contamination of *Acinetobacter* via the hands of
staff members was the likely source of this outbreak. Immediately after the cluster of bacteremia
cases were observed, medical devices, tap water, aerators, water samples, various surfaces,
intravenous fluids, and the hands of health care workers in the NICU were sampled and were
culture-negative for the bacterium. The transmission of this *Acinetobacter* bacteremia outbreak
remains unknown.

Identification of *Acinetobacter* isolates to the species level has been problematic in clinical
microbiology services. The majority of genospecies cannot be reliably separated by phenotypic
tests (37). Some of *Acinetobacter* species present inert biochemical reactions, which make
accurate identification difficult, based on phenotypic profiles. In our study, standard biochemical
reactions and three phenotypic identification systems, including API 20E, RapID NF Plus, and
Biolog GN2 (24, 25, 41), were unable to discriminate the unusual *Acinetobacter* isolates to the
species level. The 16S rRNA gene sequencing determinations have been widely used to identify
gram-negative bacilli, including *Acinetobacter* species (16, 41). However, this technique may
fail to distinguish closely related genomic species of *Acinetobacter* (19, 28). Several studies have
demonstrated the usefulness of *rpoB* gene sequences for identification and taxonomic
classification of various bacterial species including *Acinetobacter* (29, 35). Our data based on both 16S rRNA and *rpoB* gene amplification and sequencing, indicated that the unusual *Acinetobacter* species was most closely related to *A. ursingii* at similarities of 99.5% and 97.2%, respectively. Considering significant differences in several key biochemical reactions, a standard DNA-DNA hybridization method was used to further characterize and contrast the unusual *Acinetobacter* isolate from the *A. ursingii* type strain. The DNA-DNA hybridization of strain AK001 against the type strain of *A. ursingii* resulted in similarities that were below the recommended threshold value of 70% for the definition of bacterial species (44). It is worthwhile to point out that *Acinetobacter* species are a group of organisms which include naturally competent species and published genomes that reveal a large amount of mobile genes - DNA/DNA hybridization can be expected to vary significantly within bacterial species of this type.

**Description of *A. septicus* sp. nov.** *Acinetobacter septicus* was named to indicate its clinical relevance as an isolate causing sepsis in humans, especially newborns. The bacteria are gram-negative bacilli with inert biochemical activities. Two key phenotypic characteristics, hemolysis on sheep blood agar and negative citrate utilization, were the only differences between *A. septicus* and *A. ursingii*. Commercial biochemical identification systems were not useful to identify the bacterium. Genotypically, *A. septicus* is related most closely to *A. ursingii*, based on nucleotide sequence analysis of both the 16S rRNA and *rpoB* genes. DNA-DNA hybridization against *A. ursingii* gave results below the recommended species delineation threshold. *A. septicus* has been found only in human blood and is considered to be a pathogen that causes nosocomial sepsis outbreak in NICU.
Description of the type strain. The type strain of Acinetobacter septicus is AK001 (DSM 19415). The sequences of its full 16S rRNA (3 operons) and partial rpoB genes were deposited in the GenBank database under accession numbers EF611418-EF611420 and EF611383. It was isolated from the blood of a newborn boy in Ankara, Turkey.
Acknowledgements

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References


Figure legend

Figure 1. Pulsed-field gel electrophoresis patterns of Smal-digested genomic DNA of the Acinetobacter isolates. Lanes 1 to 8 are isolates of A. baumannii, AK001, AK002, AK003, AK004, AK005, AK006 and AK007, respectively. Molecular sizes are in kilobases.

Figure 2. Rooted neighbor-joining tree based on almost complete 16S rRNA gene sequence (1,346 bp) showing the phylogenetic relationship among all type strains of the genus Acinetobacter (n = 17) and three sequences from different clones of one representative strain (AK001) of the unusual Acinetobacter isolates. The scale bar indicates the evolutionary distance between sequences, determined by measuring the lengths of the horizontal lines connecting two organisms. Numbers at nodes (shown if ≥ 50% within the consensus phylogenetic tree) indicate percentages of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets. Pseudomonas aeruginosa strain PA01 was used for outgroup rooting. In parenthesis are GenBank accession numbers of downloaded sequences. Bar, 0.2% sequence divergence. T: type strain.

Figure 3. Rooted neighbor-joining tree based on partial rpoB gene sequences (801 bp) showing the phylogenetic relationship of strain AK001 among all type strains of the genus Acinetobacter (n = 17) and further Acinetobacter culture collection strains exhibiting unique 16S rRNA gene sequences. The scale bar indicates the evolutionary distance between sequences, determined by measuring the lengths of the horizontal lines connecting two organisms. Numbers at nodes (shown if ≥ 50% within the consensus phylogenetic tree) indicate percentages of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets. Pseudomonas aeruginosa strain PA01 was used for outgroup rooting. In parenthesis are GenBank accession
numbers of submitted sequences. Bar, 5% sequence divergence. T: type strain. CIP: Collection de l’Institut Pasteur, Paris, France; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen; LMG: Belgian Coordinated Collections of Microorganisms, Gent, Belgium.
Table 1. Demographic and clinical characteristics of five patients with bacteremia in NICU

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Diagnosis on admission</th>
<th>Gestational age (wk)</th>
<th>Weight at birth (g)</th>
<th>Antibiotics</th>
<th>Procedures</th>
<th>Method of Delivering</th>
<th>Prognosis</th>
<th>Dates blood culture performed (results)</th>
</tr>
</thead>
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Abbreviations: TPN, total parenteral nutrition; PC, peripheral catheter; ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation; N: Negative; P, Positive.
Table 2. Contrast of phenotypic characteristics of unusual *Acinetobacter* isolate AK001 and *A. ursingii*.

<table>
<thead>
<tr>
<th>Phenotypic profiles</th>
<th>AK001</th>
<th><em>A. ursingii</em> $^b$</th>
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<tr>
<td>Growth at</td>
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<td>–</td>
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<tr>
<td>$41^{\circ}$C</td>
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<td>–</td>
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<tr>
<td>$37^{\circ}$C</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Acid from D-glucose</td>
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<td>–</td>
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<tr>
<td>Gelatinase</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Hemolysis of sheep blood</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Utilization of:</td>
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<tr>
<td>DL-Lactate</td>
<td>+</td>
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<tr>
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<td>–</td>
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<td>Trans-Aconitate</td>
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<td>L-Ornithin</td>
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$^a$ Indexes used: “+”, positive; “−”, negative; ND, not done. Numbers are percentages of positive strains.

$^b$ Biochemical reactions were recorded from references (34, 36).