Microbiological Characterization of *Actinotignum schaalii* Strains Causing Invasive Infections during a Multiyear Period in a Large Canadian Health Care Region

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**ABSTRACT** *Actinotignum schaalii* is an underrecognized Gram-positive bacillus that is associated with urinary tract infections and cutaneous abscesses. The role of *A. schaalii* in invasive infections continues to be unappreciated because the bacteria can be isolated from a diverse spectrum of clinical specimens, ranging from being a single pathogen in urine and blood cultures to being deemed a colonizer in polymicrobial anaerobic cultures of sterile fluids and tissues. We conducted a microbiological analysis of clinical isolates obtained from 2012 through 2019. A total of 86 isolates were analyzed; 37 (43%) were from blood cultures, 35 (41%) were from deep wounds and abscesses, 6 (7%) were from urine samples, and the rest were recovered from peritoneal, kidney, and scrotal fluid samples. Urinary tract infections were clinically identified as the source of most cases of bacteremia, although no simultaneous urine cultures yielded positive results. The 16S rRNA gene sequences were available for 32 isolates (37%). Phylogenetic analysis revealed that *AS.1/AS.2* strains caused a larger proportion of bloodstream infections (BSIs) (100% versus 52% \(P = 0.01\)) and trended toward a higher rate of hospitalization (91% versus 76% \(P = 0.18\)) but had a lower clindamycin MIC\(_{90}\) (0.12 versus >256 \(\mu g/mL\)). Our study emphasizes the emergence of *A. schaalii* as a pathogen in human urine samples, BSIs, and skin and soft tissue infections. It highlights the pitfalls of current laboratory methods in recovering and identifying this organism from clinical specimens, particularly urine samples. Phylogenetic analysis showed unique genotypic sequences for *A. schaalii* *AS.1/AS.2* strains causing urosepsis, which requires further study to identify potential virulence factors.

**IMPORTANCE** *Actinotignum schaalii* is an underrecognized Gram-positive bacillus due to its special growth requirements and prior phenotypic identification methods, and it is often mistaken as a contaminant. It has been associated with various clinical syndromes, from urinary tract infections to cutaneous infections. The widespread use of molecular diagnostic methods allowed for improved detection. However, its role in invasive infections remains underappreciated. We conducted a detailed microbiological analysis to improve our understanding of this organism's genotypic and phenotypic characteristics. Our results highlight the pitfalls of clinical laboratory recovery, particularly from urine cultures. Although most BSIs were caused by urinary tract infections, no simultaneous urine cultures identified *A. schaalii*, largely due to the failure of phenotypic methods to reliably isolate and identify this organism. Additionally, this is the first study demonstrating *A. schaalii* strains with differences in clinical and microbiological characteristics, raising the possibility of potential bacterial virulence factors contributing to invasive infections.

**KEYWORDS** *Actinotignum schaalii*, *Actinobaculum schaalii*, MALDI-TOF MS, 16S rRNA gene sequencing, phylogenetic analysis, uropathogen, emerging infection, urine culture, anaerobic infection, bloodstream infection, skin and soft tissue infection
Actinotignum schaalii (formerly designated Actinobaculum schaalii) is an opportunistic pathogen that is emerging as an uncommon cause of invasive infections; it primarily causes urinary tract infections and cutaneous abscesses (1). Currently, the Actinotignum genus includes A. schaalii along with Actinotignum urinale and Actinotignum sanguinis (2). More broadly, it is within the order Actinomycetales, which encompasses the genera Actinomyces, Arcanobacterium, Trueperella, and Actinotignum (3).

In invasive infections, accurate organism identification of anaerobic bacteria is required to inform optimal clinical management, initiate appropriate empirical antibiotic therapy, and optimize clinical outcomes (4). A. schaalii is a fastidious bacterium that grows best under anaerobic conditions or in a 5% carbon dioxide (CO₂) atmosphere (1). For these reasons, it is often missed by routine culture techniques, which favor aerobic conditions and shorter incubation periods, particularly for nonsterile specimens (1, 5–7). The pathogenic potential of A. schaalii alone or contributing to polymicrobial invasive infections has also been underrecognized since clinical microbiology laboratories rely on phenotypic methods for identification, dismissing A. schaalii as a coryneform bacillus, a commensal, or a contaminant (8, 9).

The widespread adoption of advanced proteomic approaches, such as matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and genomic methods (i.e., 16S rRNA gene sequencing), have led to increased identification of this organism (6–9). However, commercial biochemical identification panels for aerobic and anaerobic Gram-positive bacilli, such as the Vitek ANC cards (bioMérieux, Marcy l’Étoile, France), and MALDI-TOF MS databases only recently included A. schaalii, as well as the Vitek ANC cards (bioMérieux, Marcy l’Étoile, France), and MALDI-TOF MS databases only recently included A. schaalii in 2016 (1). Our laboratory routinely uses fast partial sequencing of the 16S rRNA gene (~500 bp of the V1 to V3 region) to identify a wide variety of Gram-positive bacilli deemed clinically relevant and isolated from sterile clinical specimens (10). Limited data on methods of identification for A. schaalii have been reported (11). Currently, there is no standardized antimicrobrial susceptibility testing or reporting guidelines published for this organism; therefore, many clinical microbiology laboratories do not routinely perform antimicrobial susceptibility testing. Although disc diffusion and Etest methods are used for Gram-positive anaerobes, interpretation of results in the literature has been based on breakpoints for Streptococcus spp. or Staphylococcus spp. (1, 12, 13). We previously performed a comprehensive study of the clinical manifestations and population-based epidemiology of clinically relevant infections due to A. schaalii in our health care region (14). In this study, we conducted a detailed microbiological analysis of the previously described isolates to improve our understanding of the genotypic and phenotypic characteristics of this organism. Additionally, in constructing a phylogenetic tree using available sequencing data from a subset of clinical isolates, we aimed to determine whether distinct A. schaalii strains have clinical, phenotypic, or genotypic differences that potentially predict a unique virulence profile.

RESULTS
Patient demographic and clinical characteristics. Eighty-six unique patients with A. schaalii infections were analyzed during the study period. All patients except 1 were >18 years of age. According to clinical chart review, bloodstream infections (BSIs) were predominantly caused by urinary tract infections (35/37 cases [94.6%]), while non-BSIs were primarily caused by skin and soft tissue infections (36/49 cases [73.5%]); urinary tract infections were only the cause of 10.2% (5/49 cases) of non-BSIs. Most BSIs affected patients >65 years of age (32/37 cases [86.5%]), while patients with non-BSIs were primarily <65 years of age (37/49 cases [75.5%]). Most urinary tract infections were diagnosed clinically, and isolation of A. schaalii from urine cultures was rarely seen (5/40 cases [12.5%]). Chart review identified no infectious diagnosis for 5.4% of BSIs and 12.2% of non-BSIs. Most patients (77/86 patients [90.7%]) received antibiotic therapy for a median duration of 10 days (interquartile range [IQR], 7 to 15 days).

Microbiological characteristics. The distribution of A. schaalii in all clinical specimen sources is outlined in Fig. 1, with a total of 86 unique isolates identified. Most invasive A. schaalii infections were from blood cultures (37/86 cases [43.0%]), while the
The rest were isolated from a variety of other sources, i.e., 71.4% (35/49 cases) from deep wounds and abscesses, 12.2% (6/49 cases) from urine samples, of which 2 were collected by an invasive method, 6.1% (3/49 cases) from implantable devices, 4% (2/49 cases) from bone tissue, and the remaining from peritoneal, kidney, and scrotal fluid samples (1 each). Deep wound and abscess specimens were predominantly isolated from the following sources: perianal and genital, 31.4% (11/35 cases); breast, 22.9% (8/35 cases); inguinal, 20% (7/35 cases); lower extremities, 11.4% (4/35 cases).

Pure growth of *A. schaalii* was observed in 69.8% of all clinical specimens, and this rate decreased to 60.5% when findings were restricted to isolates without bacteria seen in Gram stains. Pure isolates were seen in larger proportions in blood specimens than in non-blood specimens (78.4% versus 63.3%), and this was more apparent when findings were restricted to specimens without other bacteria seen in Gram stains (78.4% versus 49.0%) (Table 1). Aerobic Gram-positive copathogens were commonly observed (28.0%), including skin flora (11.6%), *Enterococcus* spp. (5.5%), *Staphylococcus aureus* (3.5%), and *Streptococcus anginosus* group (3.5%), while *Escherichia coli* (2.3%) and *Pseudomonas aeruginosa* (2.3%) were the main Gram-negative bacteria observed. Anaerobic copathogens were commonly observed, being isolated from 12.7% of all specimens; in 9.3% of specimens, bacteria were seen in Gram stains but failed to grow in aerobic cultures. The latter were all observed from wound culture isolates. *Actinomyces* spp., *Prevotella* spp., and *Bacteroides fragilis* group were the most frequently isolated anaerobes; the rest were mixed anaerobes.

**Performance of MALDI-TOF MS for identification.** Prior to the MALDI-TOF MS Vitek MS v3.0 update (2016), *A. schaalii* was not included in the database, which resulted in 100% (n = 13) discordant results, compared to 16S rRNA gene sequencing, in the preceding study years. Five cases (38%) yielded no identification, and 8 (62%) discordant identifications, including *Actinomyces meyeri*, *Actinomyces radingae*, *Actinomyces turicensis*, *Actinomyces* spp., *Clostridium tyrobutyricum*, *Kocuria kistrianae*, *Listeria grayi*, *Streptococcus pseudopneumoniae*, and *Yersinia enterolitica*. Once the library was updated in 2016, MALDI-TOF MS had 100% (n = 11) agreement with 16S rRNA gene sequencing.

![Distribution of Actinotignum schaalii clinical specimens by source.](image)
and became the primary method for definitive identification. During the study period, Vitek 2 ANC and GPI cards were used 11 times for identification. Before 2017, all results were discordant (n = 10 [100%]) with 16S rRNA gene sequencing results, including 1 result with no organism identification. Discordant identifications included Actinomyces meyeri, Actinomyces spp., Granulicatella adiacens, Kocuria kristinae, Listeria grayi, Micrococcus luteus, Micrococcus lylae, and Yersinia enterolitica. Vitek 2 ANC card analysis was performed only once after 2016, with low discrimination (92%) for A. schaalii.

For blood cultures, the median incubation time was 42.0 h (IQR, 39.0 to 49.0 h). Fifty-four isolates (62.8%) had definitive identification with MALDI-TOF MS and 21 (24.4%) with 16S rRNA gene sequencing; 11 isolates (12.8%) underwent identification with both methods as part of our verification (Table 1).

**Antimicrobial susceptibility.** Susceptibility testing was performed for 48 invasive isolates (55.8%), including 34 blood culture isolates (70.8%), 8 abscess and wound culture isolates (16.7%), 5 urine culture isolates (10.4%), and 1 implantable device isolate (2.1%). Antimicrobial susceptibility results for testing of ≥8 A. schaalii isolates showed 100% susceptibility to penicillin (n = 50), ceftriaxone (n = 10), meropenem (n = 8), and vancomycin (n = 22), based on the Clinical and Laboratory Standards Institute (CLSI) M45 edition 3 MIC interpretative guidelines for Corynebacterium spp. (Table 2) (15). For β-lactams, MIC_{90} values were universally low at <0.06 µg/mL, except for piperacillin-tazobactam (23 isolates), with MIC_{90} values of 0.25 µg/mL. Based on the CLSI M45 ED3 interpretative guidelines for Corynebacterium spp. (15), high rates of resistance were found for clindamycin (n = 13 [45%]) and trimethoprim-sulfamethoxazole (TMP-SMX) (n = 9 [33%]). The MIC_{90} for clindamycin was >256 µg/mL, and that for TMP-SMX was 32 µg/mL. Tetracycline, erythromycin, gentamicin, ciprofloxacin, and moxifloxacin

| Parameter* | Data for: | All specimens (n = 86) | Blood specimens (n = 37 [43.0%]) | Non-blood specimens (n = 49 [57.0%]) |
|------------|-----------|------------------------|----------------------------------|------------------------------------|
| Pure A. schaalii growth (no. [%]) | 60 (69.8) | 29 (78.4) | 31 (63.3) |
| Pure A. schaalii growth without other bacteria seen on Gram stain (no. [%]) | 52 (60.5) | 29 (78.4) | 23 (49.0) |
| Mixed growth of ≥2 species (no. [%]) | 26 (30.2) | 8 (21.6) | 18 (36.7) |
| Mixed growth of ≥3 species (no. [%]) | 9 (10.5) | 0 | 9 (18.4) |
| Copathogen (no. [%]) | | | |
| Skin flora | 10 (11.6) | 3 (8.1) | 7 (14.3) |
| Staphylococcus aureus | 3 (3.5) | 0 | 3 (6.1) |
| Staphylococcus lugdunensis | 1 (1.2) | 0 | 1 (2.0) |
| Streptococcus agalactiae | 2 (2.3) | 0 | 2 (4.1) |
| Streptococcus anginosus group | 3 (3.5) | 1 (1.7) | 2 (4.1) |
| Enterococcus spp. | 5 (5.8) | 0 | 5 (10.2) |
| Escherichia coli | 2 (2.3) | 1 (2.7) | 1 (2.0) |
| Pseudomonas aeruginosa | 2 (2.3) | 1 (1.7) | 1 (2.0) |
| Other Gram-negative bacteria | 2 (2.3) | 0 | 2 (4.1) |
| Actinomycyes spp. | 3 (3.5) | 0 | 3 (6.1) |
| Prevotella spp. | 3 (3.5) | 0 | 3 (6.1) |
| Bacteroides fragilis group | 2 (2.3) | 0 | 2 (4.1) |
| Mixed anaerobic bacteria | 4 (5.8) | 0 | 4 (8.2) |
| Candida spp. | 1 (1.2) | 0 | 1 (2.0) |
| Mixed bacteria that were seen in Gram stain but failed to grow in aerobic culture (no. [%]) | 8 (9.3) | 0 | 8 (16.3) |

*ID, identification.
### TABLE 2  Antimicrobial susceptibility testing and MIC distribution for *A. schaalii* based on CLSI and EUCAST v12.0 breakpoints for *Corynebacterium* spp.

| Antimicrobial agent | No. of isolates with MICs of: | Susceptible (%) using*: | CLSI breakpoint | EUCAST breakpoint |
|--------------------|-------------------------------|-------------------------|-----------------|-------------------|
|                    | <0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | >256 | MIC<sub>50</sub> (μg/mL) | MIC<sub>90</sub> (μg/mL) |
| Penicillin         | 47    | 3    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | <0.06  | <0.06  | 100  | 100  |
| Piperacillin-tazobactam | 16    | 3    | 2    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | <0.06  | 0.25   | NA   | NA   |
| Ceftriaxone        | 9     | 1    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | <0.06  | <0.06  | 100  | NA   |
| Imipenem           | 9     | 0    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | <0.06  | <0.06  | NA   | NA   |
| Meropenem          | 8     | 0    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | <0.06  | <0.06  | 100  | NA   |
| Metronidazole      | 0     | 0    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 34 | >256  | >256   | NA   | NA   |
| Clindamycin        | 10    | 2    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0.12   | 0.25   | 55   | 55   |
| TMP-SMX            | 5     | 0    | 1    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 9  | <0.06  | 32     | 67   | NA   |
| Tetracycline       | 1     | 0    | 1    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | <0.06  | —      | —    | —    |
| Erythromycin       | 2     | 0    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 2  | —      | —      | —    | —    |
| Gentamicin         | 0     | 0    | 0    | 0   | 0  | 2  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | —      | —      | —    | —    |
| Ciprofloxacin      | 0     | 0    | 0    | 0   | 0  | 1  | 2  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | —      | —      | —    | —    |
| Moxifloxacin       | 1     | 0    | 0    | 0   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | —      | —      | —    | —    |
| Vancomycin         | 5     | 10   | 5    | 2   | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0.12 | 0.25   | 100  | 100  |

*Percentage of susceptible isolates based on CLSI and EUCAST v12.0 MIC breakpoints (15, 24).

*NA, not applicable (no established breakpoints).

*Antimicrobial agent was tested ≤5 times, and MIC<sub>50</sub>, MIC<sub>90</sub>, and susceptibility values were not calculated.*
susceptibility testing was performed for a few isolates, and results are presented in Table 2. When analyses were restricted to BSIs and non-BSIs, there were statistical differences between antibiotics. Lower resistance to clindamycin and higher resistance to TMP-SMX were observed in the BSI group, compared to the non-BSI group (20.0% versus 58.3% and 60.0% versus 0%, respectively).

Phylogenetic analysis of invasive Actinotignum schaalii strains. Figure 2 illustrates the phylogenetic relatedness of the sequences. The phylogenetic tree tips are labeled with the GenBank accession number and the clinical infection diagnosis. The clinical outcomes are illustrated by shape (star, outpatient; circle, inpatient) and are color-coded based on the source of infection, as in Fig. 1. The reference strains are bolded, and the red taxa represent death within 90 days. The scale bar represents the number of nucleotide substitutions per site.

![Phylogenetic tree of Actinotignum schaalii 16S rRNA gene-sequenced partial sequences.](image)

**FIG 2** Phylogenetic tree of Actinotignum schaalii 16S rRNA gene-sequenced partial sequences. The neighbor-joining tree illustrates the phylogenetic relatedness of the sequences. The phylogenetic tree tips are labeled with the GenBank accession number and the clinical infection diagnosis. The clinical outcomes are illustrated by shape (star, outpatient; circle, inpatient) and are color-coded based on the source of infection, as in Fig. 1. The reference strains are bolded, and the red taxa represent death within 90 days. The scale bar represents the number of nucleotide substitutions per site.

Our data illustrate unique strain AS.1 (GenBank accession numbers ON110313, ON110320, ON110326, ON110328, and ON110336) and unique strain AS.2 (GenBank accession numbers ON110312, ON110316, ON110321, ON110322, ON110332, and ON110333). Table 3 summarizes the clinical and phenotypic characteristics of 16S rRNA gene-sequenced A. schaalii clinical isolates by strain type.

**TABLE 3** Clinical and phenotypic characteristics of 16S rRNA gene-sequenced A. schaalii clinical isolates by strain type

| Parameter | All strains (n = 32) | AS.1/2 strains (n = 11) | Other strains (n = 21) | P |
|-----------|---------------------|------------------------|------------------------|---|
| Age (median [IQR]) (yr) | 80.5 (54.0–85.8) | 84.0 (57.0–87.0) | 82.0 (62.0–86.0) | 0.51 |
| Male sex (%) | 25 (78.1) | 9 (81.8) | 16 (76.2) | 0.72 |
| BSI (no., %) | 22 (68.8) | 11 (100) | 11 (52.4) | 0.01 |
| Urinary tract infection (no., %) | 23 (71.9) | 11 (100) | 12 (57.1) | 0.02 |
| Hospitalization (no., %) | 26 (81.3) | 10 (90.9) | 16 (76.2) | 0.18 |
| 90-day death (no., %) | 4 (13.0) | 3 (27.3) | 1 (4.8) | 0.08 |
| No. with resistance to clindamycin/total no. (%) | 4/15 (26.6) | 0/8 | 4/7 (57.1) | 0.12 |
| Clindamycin MIC50 (µg/mL) | <0.06 | <0.06 | >256 |
| Clindamycin MIC90 (µg/mL) | >256 | 0.12 | >256 |
pares the clinical and phenotypical characteristics of strains AS.1 and AS.2 (AS.1/2) with other sequenced strains. Demographic data were similar for the two groups, but the AS.1/2 strains had a significantly larger proportion of BSIs caused by urinary tract infections, with a trend for higher hospitalization and mortality rates. This unique strain also had no detected clindamycin resistance, compared with 57.1% for the other strains.

DISCUSSION

Our unique study of A. schaalii strains recovered from a large retrospective population-based cohort of patients with invasive infections provides the largest report of isolates’ microbiological characteristics in which 16S rRNA gene-sequenced specimens were correlated with the patients’ clinical profiles (11, 16, 17). Our study highlights the previous and current pitfalls of clinical laboratory recovery and definitive identification of this important emerging pathogen. Given the Gram-positive bacillus morphology and fastidious nature of this organism, A. schaalii might have been previously overlooked as a skin contaminant or nonpathogenic coryneform bacillus in Gram stains of non-BSI specimens or analyses using available phenotypic methods, which were unable to provide an identification. Once the MALDI-TOF MS library was updated to v3.0, our internal verification showed perfect agreement (11/11 cases) with 16S rRNA gene sequencing results, and this method of identification became our laboratory’s primary routine tool for the definitive identification of Gram-positive bacilli, such as A. schaalii, recovered from the range of clinical specimens analyzed, leading to an increase in its detection. However, the number of A. schaalii strains identified in urine cultures, despite this pathogen being a primary cause of urinary tract infections, was much lower than in other studies (11, 16). This is particularly important since most of our sequences were identified in blood specimens with infections whose suspected etiology was reported to be from urinary tract infections. Similarly, all identified BSIs had no urine cultures in which A. schaalii was simultaneously identified. This highlights pitfalls in our current urine culture-based methods, which rely on chromogenic agar and nonoptimal growth conditions that do not favor the recovery of this fastidious, Gram-positive bacillus. In addition, A. schaalii appears as a white or dusty colony on Uriselect 4 chromogenic agar, produces a negative catalase reaction, and typically appears as Gram-positive bacilli on microscopic examination, leading it to be dismissed as a nonpathogenic organism.

Given the potential of this species to cause invasive BSIs, most often from ascending urinary tract infections, MALDI-TOF MS and optimal growth conditions should be considered a standard identification method for at-risk populations, especially if phenotypic characteristics suggest a Gram-positive rod. Our results showed that 86.5% of all A. schaalii BSIs involved patients >65 years of age. Therefore, assuming that BSIs came from a urinary source and would yield a positive urine culture result, use of an age cutoff of 65 years to implement routine MALDI-TOF MS analysis, with optimal growth conditions for the urine samples, 48 h of incubation, and 5% CO2 or anaerobic conditions, would allow for improved detection of A. schaalii in this vulnerable patient population.

A. schaalii infections were routinely identified as a constituent of polymicrobial infections, most notably in the non-BSI cases. Because A. schaalii is commonly identified as a copathogen in polymicrobial infections from deep wound and abscess cultures, this finding suggests that this organism is found as part of the normal skin and genitourinary flora. A. schaalii has been previously described as part of the microbiota of the urogenital area; however, our isolation of this organism from a variety of body sites, including breast, abdominal wall, and lower extremities, suggests that this organism may also be a colonizer of other body sites where there is a low-oxygen environment (1, 17). BSIs occurred mainly in adults >65 years of age, an age group with multiple risk factors predisposing patients to urinary tract infections and disruption of the skin barrier, which may explain why they are more prone to invasive BSIs.

There has been only one small study that compared the sequences of clinical A. schaalii isolates to compare different strains of A. schaalii using phylogenetic trees;
however, the study was not correlated with clinical phenotypes (11). We demonstrated that all 11 isolates (AS.1/2) with 16S rRNA gene sequences most closely related to that of *A. sanguinis* and from a higher node than the reference *A. schaalii* isolates were all isolated from blood specimens and, according to chart review, were from a urinary tract infection, a significant finding compared to other strains. Phenotypic differences were also seen, because resistance to clindamycin was observed only for the non-AS.1/2 strains (57.1% versus 0%). In addition, from a clinical perspective, AS.1/2 strains had a greater propensity to cause BSIs, with a trend for higher hospitalizations and mortality rates, compared to the other strains. This is the first study showing potential *A. schaalii* strains with differences in phenotypic and clinical characteristics. Similarly, although all isolates showed low β-lactam MICs, BSI isolates had unique patterns of resistance because they tended to have lower resistance to TMP-SMX, whereas non-BSI isolates, such as isolates from abscess and wound infections, had higher resistance to clindamycin (5, 17). This finding must be interpreted with caution, given the small number of isolates tested with non-β-lactam medications. Nevertheless, this difference raises the possibility of a potential bacterial virulence factor or strains with a higher risk of BSIs (5, 16).

Although this study involved a sizeable microbiological cohort, some limitations must be considered. Given the study’s retrospective nature, the definitive identification methods were not consistently applied for all isolates. After the Vitek MALDI-TOF MS library update in 2016, MALDI-TOF MS was routinely utilized, leading to a detection bias depicted by the increase in case detection. In addition, a selection bias could have been introduced because 16S rRNA gene sequencing was performed only for an important subset of isolates causing BSIs or other types of serious life-threatening infections. Further genomic analyses are required, including whole-genome sequencing of a larger number of *A. schaalii* isolates to confirm the clinical relevance of the AS.1/2 strain. The total incidence rate is most likely underestimated because urine specimen isolates were not detected for the reasons outlined above.

In summary, this work further advances the understanding of *A. schaalii*, a slow-growing bacterium with a wide spectrum of presentation and pathogenesis. From a laboratory perspective, our study revealed that *A. schaalii* can still be missed by the clinical laboratory, particularly in a high-throughput laboratory with a heavy emphasis on phenotypic detection methods to rule out contaminants. In nonsterile settings, implementing MALDI-TOF MS as part of the routine diagnostic algorithm for groups at high risk for invasive *A. schaalii* infections would allow for better detection while limiting the increase in laboratory resources. Also, we identified strains of *A. schaalii* with greater propensity to cause invasive infections, suggesting genotypic factors that could influence the organism’s virulence. More extensive studies linking microbiological and clinical characteristics with outcomes are required to better understand this organism’s pathogenesis.

**MATERIALS AND METHODS**

**Study design.** We completed a retrospective population-based cohort study at the centralized regional microbiology laboratory serving Calgary, Canada, and surrounding areas from 1 January 2012 to 31 December 2019. Calgary Laboratory Services (CLS) [now Alberta Precision Laboratories (APL)] is a centralized regional microbiology laboratory providing ambulatory and hospital-based testing to Calgary and the Alberta Health Services (AHS) South Zone, covering a population of 2.0 million (18). Basic demographic data for *A. schaalii* isolates, such as patient age and sex, were derived from the previously completed clinical study for the purposes of microbiological characterization (14). Infectious diagnosis, hospitalization, and 90-day mortality rates were determined through a retrospective chart review of the patients’ electronic medical records, which was independently performed by two investigators (J.M. and A.L.).

**Microbiological methods.** All clinical specimens with *A. schaalii* isolates were obtained from the laboratory information system (Millennium; Cerner, Kansas City, MO, USA) and microbiology database used by CLS Calgary Zone during the study period. Clinical specimens were processed using standard CLSI methods for blood and sterile and nonsterile fluid cultures (19). All isolates were anaerobic or aerotolerant non-sporing Gram-positive bacilli that were catalase negative. These key microbiological characteristics were used to confirm definitive identification. Routine urine specimens were plated on chromogenic agar (UriSelect 4; Bio-Rad, Hercules, CA), while urine samples collected through invasive methods were plated on blood agar and chromogenic agar. Chromogenic agar plates were incubated under aerobic conditions for 18 to 24 h, and blood agar plates were incubated for 48 h. Urine specimens were plated on *Brucella* agar and incubated for 48 h under anaerobic conditions at the physician’s request. Definitive identification was performed with the Vitek MALDI-TOF MS system (bioMérieux, Laval, Quebec) and/or 16S rRNA gene
sequence. The Vitek MS database was updated in 2016 (v3.0) to include A. schaalii. Previously, 16S rRNA gene sequencing was performed for invasive isolates only if definitive identification was required after clinical review by the microbiologist on call. Isolates were identified using a commercial MS system (Vitek MS, with ACQ software R2 v1.4.2b; bioMérieux) using MYLA v3.2.0-4 according to the manufacturer’s instructions. α-Cyano-4-hydroxycinnamic acid (CHCA) extraction was performed for all isolates using the protocol provided by the manufacturer. An aliquot of 1 μL of the extracted supernatants was placed on the steel target plate, dried, and overlaid with 1 μL of the matrix. The target plate was then loaded into the Vitek MS instrument for analysis. Samples were repeated if no identified results or low discrimination (<80% for Enterobacteriaceae, <90% for Streptococcus species not Enterococcus, and <99% for all other organisms) was obtained; 16S rRNA gene sequencing was performed when repeat MALDI-TOF MS analysis gave no identification or low discrimination. Molecular identification was performed by fast partial sequencing of the 16S rRNA gene (523 bp) with MicroSeq 500 kits and an ABI Prism 3130 sequencer (Applied Biosystems, Foster City, CA) using standard methods (10, 20, 21). A BLAST search against the SmartGene Integrated Database Network System (IDNS) for bacteria indicated the most closely related species (22). The overall identity score for all isolates was 99.9%, with 0 to 2 mismatches (22, 23). Some isolates were also tested using the Vitek ANC card (bioMérieux) but required MALDI-TOF MS or 16S rRNA gene sequencing for definitive diagnosis. Antimicrobial susceptibility testing was performed with MIC gradient strips (Etest; bioMérieux) but required MALDI-TOF MS or 16S rRNA gene sequencing for definitive identification. The Vitek MS database was updated in 2016 (v3.0) to include Actinotignum schaalii isolate 16S rRNA gene sequence was conducted. The clinical strains (n = 32/37 strains [86.5%]) and reference sequences (n = 5) were aligned with MEGA v11.0.10 software (Pennsylvania State University). Four reference sequences from culture type strains were included in the phylogenetic analysis (Actinotignum schaalii [GenBank accession number CP008802], Actinotignum urinale [GenBank accession number AU439453], Actinotignum sanguinis [GenBank accession number HG798952], and Actinobaculum suis [GenBank accession number S38623]), all of which are in the Actinomycetales order and Arcanobacteriaceae family (3). A neighbor-joining tree was inferred with 100 bootstrap replicates, and evolutionary distances were computed using the Jukes-Cantor method in MEGA (25). The tree was manually rooted on a taxonomic outlier (Actinomyces israelii [GenBank accession number AF479270]) using FigTree (26). Branches corresponding to partitions reproduced in <50% of bootstrap replicates were collapsed. Phylogenetic analysis. Phylogenetic analysis of available A. schaalii isolate 16S rRNA gene sequences was conducted. The clinical strains (n = 32/37 strains [86.5%]) and reference sequences (n = 5) were aligned with MEGA v11.0.10 software (Pennsylvania State University). Four reference sequences from culture type strains were included in the phylogenetic analysis (Actinotignum schaalii [GenBank accession number CP008802], Actinotignum urinale [GenBank accession number AU439453], Actinotignum sanguinis [GenBank accession number HG798952], and Actinobaculum suis [GenBank accession number S38623]), all of which are in the Actinomycetales order and Arcanobacteriaceae family (3). A neighbor-joining tree was inferred with 100 bootstrap replicates, and evolutionary distances were computed using the Jukes-Cantor method in MEGA (25). The tree was manually rooted on a taxonomic outlier (Actinomyces israelii [GenBank accession number AF479270]) using FigTree (26). Branches corresponding to partitions reproduced in <50% of bootstrap replicates were collapsed. Data analyses. The cohort data were analyzed using standard descriptive statistics. Categorical data such as frequencies were compared using Fisher’s exact test; continuous data were summarized as medians with IQRs and compared using Wilcoxon’s rank-sum test (27). P values of <0.05 were considered statistically significant. SPSS v25 (IBM, Chicago, IL) was used to perform the analyses. Definitions. Urinary tract infections were defined as clinician-defined urinary tract infections from the problem list in a chart review (27). Copathogens were defined as other bacteria growing in the same culture specimen as A. schaalii. Skin flora was defined as nonpathogenic commensal bacteria and included coagulase-negative Staphylococcus, viridans group streptococci, Cutibacterium spp., Corynebacterium spp., and Propionibacterium spp. Ethics approval. A waiver of consent requirements was obtained, and the study was reviewed by the Conjoint Health Research Ethics Board (REB) and approved under certificate number REB-21-0239. Data availability. All sequences were submitted to GenBank under accession numbers ON110308 to ON110339. ACKNOWLEDGMENT We thank the Alberta Precision Laboratory - Calgary Zone Microbiology staff for their help with sample processing.

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