

Phenotypic, molecular characterization, antimicrobial susceptibility and draft genome sequence of *Corynebacterium argenteratense* strains isolated from clinical samples

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Abstract

During a 12-year period we isolated five *Corynebacterium argenteratense* strains identified by phenotypic methods, including the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and 16S rRNA gene sequencing. In addition, antimicrobial susceptibility was determined, and genome sequencing for the detection of antibiotic resistance genes was performed. The organisms were isolated from blood and throat cultures and could be identified by all methods used. All strains were resistant to cotrimoxazole, and resistance to β -lactams was partly present. Two strains were resistant to erythromycin and clindamycin. The draft genome sequences of these isolates revealed the presence of the *erm(X)* resistance gene that is embedded in the genetic structure of the transposable element Tn5423. Although rarely reported as a human pathogen, *C. argenteratense* can be involved in bacteraemia and probably in other infections. Our results also show that horizontal transfer of genes responsible for antibiotic resistance is occurring in this species. New Microbes and New Infections © 2016 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases.

Keywords: Antimicrobial resistance, clinical infections, *Corynebacterium argenteratense*, MALDI-TOF, whole genome sequencing

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Introduction

Corynebacterium argenteratense was described by Riegel et al. [1] in 1995 after isolating this organism in Strasbourg, a city formerly known as Argentoratum. The taxonomic description of this new species was based on four strains isolated from throat specimens of patients with tonsillitis. The bacterium is nonlipophilic and has typical coryneform morphology, and the

cell wall contains meso-diaminopimelic acid, arabinose, galactose and mycolic acids of short chain lengths [1]. The whole genome sequence of the type strain *C. argenteratense* DSM 44202 (IBS B10697) has a deduced size of 2 031 902 bp with an average G+C content of 58.9%, revealing what is to date the smallest chromosome of a corynebacterial species associated with humans [2].

The same group of researchers from Strasbourg reported the isolation of *C. argenteratense* from eight respiratory tract specimens (seven from the upper respiratory tract and one from the lower) and another one an ear specimen [3]. This report probably includes the four isolates previously referred to in the taxonomic description of *C. argenteratense* [1]. In addition to these nine isolates, this organism has been isolated from blood cultures of patients in Canada [4] and Saudi Arabia

[5], from intravenous sites (catheter and blood) of cancer patients [6], from the conjunctiva of patients with bacterial conjunctivitis [7] and as part of a mucosal biofilm in the adenoid tissue of a child with otitis media [8]. Table 1 summarizes the sources, geographical origins and clinical data of the *C. argentoratense* isolates described so far.

Data on the natural habitat and the susceptibility of *C. argentoratense* to antimicrobials are limited. Metadata of the microbiota of 18 body sites in more than 200 individuals obtained in the course of the Human Microbiome Project indicated that *C. argentoratense* is mostly present in saliva and to a lesser extent on the hard palate [9]. An additional study including 19 gastritis patients with a typical greasy white or dense yellow tongue coating revealed that *C. argentoratense* is part of this characteristic tongue-coating microbiome [10]. The *in vitro* susceptibility of *C. argentoratense* to eight antibiotics (ampicillin, cefotaxime, ceftazidime, ciprofloxacin, erythromycin, fusidic acid, gentamicin and rifampin) has been determined by the disk diffusion method for nine isolates [3]. Only resistance to ceftazidime was broadly present (78%) in *C. argentoratense*.

Here we present data on the origin of *C. argentoratense* isolates, microbiologic profiles, including identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and 16S rRNA gene sequencing, as well as data on the minimum inhibitory concentrations for 28 antibiotics as determined by Etest. Moreover, draft genome sequences of two erythromycin- and clindamycin-resistant isolates are presented, leading to the detection of the *erm(X)* resistance gene.

Material and Methods

Strains

Two strains were isolated from blood cultures belonging to two different patients, and three other strains were recovered from throat specimens of three different patients with pharyngitis. All strains were obtained from patients who sought care at a hospital in the north of Spain (Complejo Asistencial Universitario de León, León) from January 2003 to December 2014.

Phenotypic identification

API Coryne 2.0 (bioMérieux, Marcy l'Etoile, France), API Strep (hippurate hydrolysis), API NH (fructose fermentation) and API NE (assimilation of maltose, *N*-acetyl-glucosamine and phenylacetic acid) were used for the phenotypic characterization of the corynebacterial isolates. In addition, catalase and oxidase reactions, lipophilia, Christie Atkins Munch-Peterson (CAMP)

TABLE 1. Data on 21 published *Corynebacterium argentoratense* isolates from human sources

Specimen (n)	Geographical source	Clinical data	Reference
Upper respiratory tract (7)	Strasbourg, France	Probably including 4 patients with tonsillitis	[3]
Lower respiratory tract (1)	Strasbourg, France	Not reported	[3]
Ear (1)	Strasbourg, France	Not reported	[3]
Blood culture (1)	Ontario, Canada	Not reported	[4]
Blood culture (1)	Riyadh, Saudi Arabia	Patient with tonsillitis	[5]
Intravenous sites (2)	Rio de Janeiro, Brazil	Cancer patients	[6]
Conjunctival fornix (2)	US and/or Asian clinical sites	Patients with bacterial conjunctivitis	[7]
Adenoid tissue (1)	Leiden, The Netherlands	Child with otitis media	[8]
Pharyngeal (3)	León, Spain	3 patients with pharyngitis	This study
Blood culture (2)	León, Spain	2 febrile patients (upper respiratory tract infection and ischemic colitis)	This study

reaction, glucose fermentation at 42°C, growth on blood agar at 20°C and susceptibility to vibriostatic factor O/129 were tested following previously described methods [11].

MALDI-TOF was carried out with a Bruker Biotyper MALDI-TOF system (Bruker Daltonics, Leipzig, Germany). Software and library version 3.1 was used for the bacterial identification. The direct colony method, including spotting onto a MALDI-TOF target plate covered with 1 µL of formic acid (100%) and 1 µL of matrix, was performed as previously described [12]. Scores of ≥ 1.5 and ≥ 1.7 were used for genus and species identification respectively [13].

Genotypic identification

The amplification of the 16S rRNA gene and the DNA sequencing were performed according to previously described methods [14].

Antimicrobial susceptibility

Antimicrobial susceptibility testing to 28 antimicrobials was determined by Etest on Mueller-Hinton agar with 5% sheep's blood, incubated in air at 35°C and read after 48 hours. Susceptibility to antibiotics was interpreted following the 2014 recommended criteria for coryneform organisms [15].

Genome sequencing and search for antibiotic resistance genes

Technical details of genome sequencing, assembly and annotation of two antibiotic-resistant *C. argentoratense* isolates have been announced previously [16]. Both genome sequences were annotated using the RAST genome annotation server [17].

BLAST (Basic Local Alignment Search Tool) was used for the search for antibiotic resistance genes in the Antibiotic Resistance Genes Database [18] and in published genome information of *Corynebacterium* strains. Comparative genomic analysis was performed with the EDGAR software [19]. All-against-all comparisons were based on the BLASTP algorithm with the standard scoring matrix BLOSUM62 and an initial *E*-value cutoff of 1×10^{-5} . The final score ratio value was calculated and set to 32. Two genes were considered orthologous when revealing a bidirectional best BLAST hit with a single score ratio value exceeding the precalculated cutoff.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences corresponding to the five isolated strains have been deposited in the GenBank nucleotide database under accession numbers KP230551 (CNM463/05), KP230549 (CNM629/14), KP230548 (CNM630/14), KP230547 (CNM631/14) and KP230550 (CNM601/08).

Results

Isolation of *C. argentoratense* from clinical sources

C. argentoratense was obtained from a single blood culture in a 3-year-old boy with an upper respiratory tract infection (strain CNM463/05) and from two blood cultures in an 85-year-old woman with ischemic colitis and high fever (strain CNM601/08). In three other patients, aged 7 months, 4 years and 50 years and with tonsillitis, *C. argentoratense* was isolated in mixed culture with normal pharyngeal flora (strains CNM629/14, CNM630/14 and CNM631/14 respectively).

Species identification by microbiologic and molecular methods

Microbiologic and genetic methods and MALDI-TOF were used to assign the five isolates to a corynebacterial species. All isolates showed identical microbiologic phenotypic characteristics. After 48 hours' incubation in air at 35°C, colonies on blood agar were nonhaemolytic, slightly rough and cream colored, and 2 mm in diameter. All isolates presented the same API profiles (2100104 at 24 hours and 2100304 at 48 hours), suggesting *C. argentoratense* (79.2%, *T* = 1) or *C. jeikeium* (93.6%, *T* = 1). The latter profile was doubtful because the isolates were nonlipophilic. Positive reactions were observed for catalase, hippurate hydrolysis, pyrazinamidase and alkaline phosphatase. Acid was produced from glucose (24 hours), ribose (48 hours) and fructose (4 hours) but not from mannitol, sucrose, ribose, xylose, maltose, lactose and glycogen. On the other hand, the organisms were oxidase negative, urease negative and nitrate reductase negative. Negative reactions

were also observed for esculin and gelatine hydrolysis, for β -glucuronidase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase activities, for the CAMP test and for the assimilation of maltose, *N*-acetyl-glucosamine and phenylacetic acid. The organisms grew on blood agar at 20°C and fermented glucose at 42°C within 3 days. All isolates were inhibited by the O/129 factor (150 μ g) showing an inhibition diameter between 18 and 27 mm.

Using MALDI-TOF, as previously described, the isolates were tested five times recording the two values given by the system, so 50 score values were obtained ranging from 2.166 to 2.378 (mean, 2.293) and providing a reliable identification of *C. argentoratense*. For comparison, two *C. jeikeium* strains were also studied five times with two readings, obtaining score values ranging from 1.816 to 2.268 (mean, 1.965). The PCR fragments of the 16S rRNA gene obtained were 1200 bp (CNM463/05), 1411 bp (CNM629/14), 1399 bp (CNM630/14), 1425 bp (CNM631/14) and 1141 bp (CNM601/08) in length. The nucleotide sequence similarities with the 16S rRNA gene of the *C. argentoratense* type strain were in the range of 99.5 to 99.9%. All data clearly demonstrated that the five isolates are members of the species *C. argentoratense*.

Antimicrobial susceptibility profiling of

C. argentoratense isolates

The antimicrobial susceptibility data for the five isolates tested are presented in Table 2. Using the Etest method and applying the recommendations of the Clinical and Laboratory Standards Institute for antimicrobial susceptibility of coryneform organisms [15], the new *C. argentoratense* isolates were uniformly sensitive to vancomycin, linezolid, daptomycin, tetracycline, ciprofloxacin, quinupristin/dalfopristin, gentamicin and rifampin. Strain CNM631/14 showed very high minimum inhibitory concentration (MIC) values of benzylpenicillin (>256 mg/L), cefotaxime (8 mg/L) and imipenem (>32 mg/L), and MICs of cefixime were ≥ 64 mg/L for all isolates. All isolates were resistant to cotrimoxazole. Two strains (CNM463/05 and CNM601/08) were resistant to erythromycin and clindamycin.

Genome sequencing and search for antibiotic resistance genes

To identify the genes responsible for the detected erythromycin and clindamycin resistance of *C. argentoratense*, draft genome sequences of CNM463/05 and CNM601/08 were established [16]. Prominent features of the *C. argentoratense* genomes and of those from taxonomically related corynebacteria are summarized in Table 3. Both *C. argentoratense* isolates showed similar genome sizes of approximately 2.02 Mbp with a mean G+C content of 58.9% and similar numbers of predicted genes. These data supported the previous result

TABLE 2. Antimicrobial susceptibility (MIC, mg/L) of five *Corynebacterium argentoratense* strains, isolated from bacteraemia (strains 1 and 2) and pharyngotonsillitis (strains 3–5), to 28 antimicrobial agents

Antimicrobial agent	Strain 1 (CNM463/05)	Strain 2 (CNM601/08)	Strain 3 (CNM629/14)	Strain 4 (CNM630/14)	Strain 5 (CNM631/14)
Benzylpenicillin ^a	1.5	4	1.5	0.75 ^b	>256
Ampicillin	1.5	1.5	2	1	>256
Cefuroxime	3	2	2	1.5	4
Cefixime	> 256	>256	96	64	>256
Cefotaxime ^a	3	4	2	1.5	8
Imipenem ^a	0.75 ^b	0.5 ^b	0.75 ^b	0.38 ^b	>32
Vancomycin ^a	0.5 ^b	0.75 ^b	0.5 ^b	0.5 ^b	0.5 ^b
Teicoplanin	1	1	1	0.5	1
Linezolid ^a	0.38 ^b	0.25 ^b	0.5 ^b	0.38 ^b	0.38 ^b
Daptomycin ^a	0.016 ^b	0.016 ^b	0.016 ^b	<0.016 ^b	0.032 ^b
Tetracycline ^a	0.5 ^b	0.38 ^b	0.25 ^b	0.38 ^b	0.5 ^b
Tigecycline	0.64	0.023	0.094	0.094	0.094
Chloramphenicol	1.5	1	1	1	1
Ciprofloxacin ^a	0.125 ^b	0.25 ^b	0.125 ^b	0.125 ^b	0.125 ^b
Moxifloxacin	0.047	0.064	0.064	0.064	0.064
Levofloxacin	0.125	0.5	0.125	0.125	0.125
Erythromycin ^a	>256	12	<0.016 ^b	<0.016 ^b	<0.016 ^b
Clarithromycin	>256	16	0.016	0.016	0.016
Azithromycin	>256	>256	0.125	0.094	0.125
Clindamycin ^a	>256	>256	0.094 ^b	0.094 ^b	0.094 ^b
Quinupristin-dalfopristin ^a	0.038 ^b	0.094 ^b	0.038 ^b	0.038 ^b	0.038 ^b
Streptomycin	3	16	2	1	3
Kanamycin	12	64	8	4	12
Gentamicin ^a	1.5 ^b	1 ^b	1.5 ^b	1 ^b	2 ^b
Tobramycin	4	6	4	2	6
Amikacin	4	4	4	2	8
Rifampin ^a	0.032 ^b	0.023 ^b	0.023 ^b	0.023 ^b	0.032 ^b
Cotrimoxazole ^a	>32	>32	>32	>32	>32

MIC, minimum inhibitory concentration.

^aAntibiotics proposed for testing coryneform organisms.

^bMIC values within the range of susceptibility (based on Clinical and Laboratory Standards Institute [15]).

from the type strain sequencing project that *C. argentoratense* has the smallest genome of a corynebacterium associated with humans [2]. The genomes of species assigned to the same taxonomic clade as *C. argentoratense* are in the size range from 2.6 to 2.7 Mbp with a G+C content ranging from 58.1 to 68.6% (Table 3). These variations are indicative of the diversity of the genus *Corynebacterium* in terms of lifestyle and habitat, including human, animal, terrestrial, marine and technical environments [20]. The draft genome sequences of both isolates revealed a high grade of similarity between them (>99.9%) as well as with the genome sequence of the type strain *C. argentoratense* DSM 44202 (>95.0%). A comparison of the predicted proteomes with the EDGAR software revealed that both clinical isolates and the type strain DSM 44202 share a common set of 1582 proteins. An all-against-all comparison with the proteomes of four taxonomically related species reduced this number to 1078 proteins that represent the conserved core of this diverse clade.

The antibiotic resistance gene *erm(X)* coding for macrolide, lincosamide and streptogramin B (MLSb) resistance was detected in the genome of both isolates, CNM 463/05 and CNM 601/08. This gene is absent in the genome of the type strain DSM 44202. It was allocated to a specific genomic region with 100% similarity to the transposable element Tn5432, initially found in the R-plasmid pTPI0 of *Corynebacterium striatum* M82B [21,22], indicating the horizontal

transfer of an antibiotic resistance region to the clinical strains from human sources. Tn5432 is composed of two IS1249 sequences, the *erm(X)* gene and the transposase gene *tnpCX* and was previously also detected in *Corynebacterium urealyticum* [23], in *Propionibacterium acnes* [24] and in some *Bifidobacterium* species [25,26].

Discussion

The pathogenicity of *C. argentoratense* in humans is not well understood. Two cases of bacteraemia by this organism have been reported previously, one in a patient with tonsillitis [5] and another from a patient in whom no clinical data were provided [4]. Here we add two additional cases of bacteraemia, one of them in a patient from whom the organism was isolated from two different blood cultures.

Although *C. argentoratense* is not presently recognized as a cause of pharyngotonsillitis, this organism has been isolated from seven patients with tonsillitis [1] (present study); its possible role in this condition thus merits further study. Moreover, if this organism is responsible for pharyngotonsillitis, and if patients are to be empirically treated with penicillins based on a suspicion of a *Streptococcus pyogenes* infection, such a treatment could be ineffective in some cases, as inferred by the antimicrobial susceptibility data obtained in this study. The

TABLE 3. Features of *Corynebacterium argenteratense* genomes and taxonomically related species

Corynebacterium species	Strain	Source	Genome						
			Status	Size (bp)	G+C%	No. of genes	Pseudogenes	No. of proteins	GenBank ID
<i>C. argenteratense</i>	DSM 44202	Human; respiratory tract	Complete	2031902	58.9	1890	55	1772	CP006365
<i>C. argenteratense</i>	CNM 463/05	Human; respiratory tract	Draft	2020912	58.9	1871	Not applicable	1643	JZEZ00000000
<i>C. argenteratense</i>	CNM 601/08	Human; blood	Draft	2014822	58.9	1878	Not applicable	1656	JZFA00000000
<i>C. epidemidicanis</i>	DSM 45586	Dog; skin	Complete	2692072	58.1	2541	Not detected	2465	CP011541
<i>C. humireducens</i>	DSM 45392	Microbial fuel cell	Complete	2681312	68.6	2595	46	2482	CP005286
<i>C. marinum</i>	DSM 44953	Coastal sediment	Complete	2607268	68.1	2457	50	2341	CP007790
<i>C. testudinoris</i>	DSM 44614	Tortoise; mouth	Complete	2721226	63.1	2590	40	2485	CP011545

isolation of *C. argenteratense* from intravenous sites in patients with cancer [6] and from the conjunctival fornix in patients with conjunctivitis [7] is of interest and also merits further investigation.

C. argenteratense is a nonlipophilic, fermentative, nitrate reductase-, urease- and oxidase-negative corynebacterium producing acid from glucose but not from maltose and sucrose [20]. It can be identified by API-Coryne, and some additional biochemical tests may be of great help for a phenotypic identification. MALDI-TOF scores of ≥ 2.166 were obtained for the five isolates, which can be considered as reliable values for a significant species identification [13]. To our knowledge, there is just one published article reporting the identification of two *C. argenteratense* isolates by MALDI-TOF [27]. As occurs with many other *Corynebacterium* species, 16S rRNA sequencing was also a useful tool to identify *C. argenteratense* [20].

The antimicrobial susceptibility of nine *C. argenteratense* strains, isolated between 1995 and 1996, has been determined by a disk diffusion method [3]. Using the interpretative categories recommended by the Antibiogram Committee of the French Society for Microbiology [28], the organisms were considered to be fully sensitive to ampicillin, cefotaxime, fusidic acid and rifampin. Only one of nine isolates was resistant to erythromycin and ciprofloxacin [3]. This unusual pattern of antimicrobial susceptibility, described approximately 15 years ago, agrees with the lack of typical corynebacterial antibiotics resistance genes in the genome of the type strain of *C. argenteratense* [2]. In our strains, isolated from 2003 to 2014, resistance to β -lactams, erythromycin and clindamycin was found. One isolate (CNM631/14) presented high MIC values for penicillins and cephalosporins. Isolate CNM463/05 was highly resistant to erythromycin and clindamycin, and isolate CNM601/08 was moderately resistant to erythromycin and highly resistant to clindamycin. Resistance to erythromycin, clindamycin and other MLSb antibiotics is often associated with the presence of the *erm(X)* gene in corynebacteria [20]. Interestingly, CNM463/05 and CNM601/08 presented the MLSb resistance gene *erm(X)*, located in the specific transposon Tn5432 previously described in *C. striatum* [22], as well as

C. urealyticum [23] and in the other actinobacterial genera *Propionibacterium* [24] and *Bifidobacterium* [25,26].

In summary, further studies on the pathogenicity, habitat and epidemiology of *C. argenteratense* should be carried out, including investigation of its possible role as a cause of pharyngotonsillitis. Moreover, as observed in a growing number of members of the *Corynebacterium* genus [20], it seems that horizontal transfer of genes responsible for antibiotic resistance is occurring in this species, which will in future make the treatment of corynebacterial infections a real clinical challenge.

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Conflict of Interest

None declared.

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