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Chromobacterium haemolyticum sp. nov., a strongly haemolytic species

Xiang Y. Han, Faye S. Han and Jonathan Segal

Section of Clinical Microbiology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

Correspondence
Xiang Y. Han
xhan@mdanderson.org

A Gram-negative bacterium, strain MDA0585^T, isolated from a sputum culture, was characterized by a polyphasic approach. The 16S rRNA gene and a conserved portion of the DNA gyrase A gene were sequenced and analysed phylogenetically. Strain MDA0585^T showed the closest relationships with *Chromobacterium violaceum* ATCC 12472^T and *Chromobacterium subtsugae* PRAA4-1^T (96.1 % and 96.3 % 16S rRNA gene sequence similarity, respectively). The cellular fatty acids of strain MDA0585^T consisted mainly of $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{16:1}\omega6c$ (summed feature 3) and $C_{18:1}\omega7c$ and $C_{18:1}\omega6c$ (summed feature 8), a profile that was similar to, but distinguishable from, those of *C. violaceum* ATCC 12472^T and *C. subtsugae* PRAA4-1^T. In culture, strain MDA0585^T differed from *C. violaceum* and *C. subtsugae* in several ways: lack of violet pigmentation, the ability to haemolyse sheep blood, differences in several biochemical reactions and higher resistance to antibiotics. The culture supernatant of strain MDA0585^T also caused remarkable haemolysis of human erythrocytes. These results suggest that strain MDA0585^T represents a novel species within the genus *Chromobacterium*, for which the name *Chromobacterium haemolyticum* sp. nov. is proposed. The type strain is MDA0585^T (=CCUG 53230^T=JCM 14163^T=DSM 19808^T).

Chromobacterium violaceum (Wooley, 1905) is a Gram-negative bacterium and is the type species of the genus *Chromobacterium*, which at the time of writing, comprises only three recognized species. One species, *Chromobacterium fluviatile* has been transferred to the genus *Iodobacter* as *Iodobacter fluviatilis* (Logan, 1989). The other member of the genus is *Chromobacterium subtsugae*, which was described recently by Martin *et al.* (2007). An organism with the non-validly published name of '*Chromobacterium viscosum*', known for its production of a lipase, is probably more likely to be a strain of *Burkholderia glumae*, when the identical amino acid sequences of the lipases are considered (Taipa *et al.*, 1995; Traub *et al.*, 2001). *C. violaceum* is an environmental organism with worldwide distribution, but is particularly abundant in the Brazilian Amazon basin [Brazilian National Genome Project Consortium (BNGPC), 2003; Hungria *et al.*, 2005]. *C. violaceum* is rarely pathogenic but may cause rapidly fatal sepsis (Teoh *et al.*, 2006). The most prominent feature of *C. violaceum* is the appearance of violet colonies due to the production of violacein, but non-pigmented organisms have also been documented. The genome of *C. violaceum* has been sequenced and many unique features have been revealed, including remarkable extracellular secretion pathways for various bacterial products, such as violacein (BNGPC, 2003).

There are considerable phylogenetic diversifications among strains of *C. violaceum* (Hungria *et al.*, 2005; Scholz *et al.*, 2005), suggesting the possibility of additional species, as exemplified by the species *C. subtsugae* that also produces violet pigment (Martin *et al.*, 2007). In this study, we report the characterization of a non-pigmented species of the genus *Chromobacterium* by the use of a polyphasic taxonomic approach. Strain MDA0585^T was isolated in June 2001 from a sputum culture (with doubtful clinical significance) in Texas, USA.

The almost full length 16S rRNA gene of MDA0585^T was amplified by PCR and sequenced according to previously published procedures (Han *et al.*, 2001, 2002). A conserved head portion of the DNA gyrase A gene (*gyrA*) was amplified and sequenced using primers 5′-TACGCGATGAG-CGTGATCGTC and 5′-GTTGTGCGGCGGGATGTTGGT (Han & Andrade, 2005). The fragment contained 489 base pairs that encoded 162 amino acids. Sequence analysis was performed through a query to the GenBank basic local

Abbreviation: RBC, red blood cells.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrA* gene sequences of strain MDA0585^T are DQ785104 and DQ785105, respectively.

Graphs showing the time course of the growth of strain MDA0585^T and *Chromobacterium violaceum* ATCC 12472^T and the lysis of human red blood cells by the culture supernatants are available as a supplementary figure with the online version of this paper. The cellular fatty acid contents of strain MDA0585^T and *C. violaceum* ATCC 12472^T are presented in a supplementary table.

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alignment search tool (Altschul *et al.*, 1997). Phylogenetic analysis was performed by using the Newick format of the DRAWTREE program of PHYLIP (www.molgen.mpg.de) and CLUSTAL W multiple alignments (www.ebi.ac.uk/clustalw) (Felsenstein, 1993).

The haemolysis assay was performed with modifications from a published procedure (Rowe & Welch, 1994). Human red blood cells (RBC) treated with anticoagulant were washed three times in phosphate buffered saline (PBS), pH 7.4, by centrifugation (1300 ***g*** × 5 min). Liquid bacterial cultures of strain MDA0585^T, *C. violaceum* ATCC 12472^T, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were grown in trypticase soy broth (BBL) at 37 °C with 2 % (v/v) seeding organisms and moderate aeration for varying or indicated incubation hours. Growth density was measured at OD_{580}. Culture supernatant was obtained by removing the cell pellet by centrifugation (15 800 ***g*** × 10 min). The assay was performed in PBS with or without added $CaCl_2$ (final concentration 0.5 mM) in a final volume of 1 ml, containing ~10^8 washed RBC (final ~1 % packed RBC) and 20 µl respective culture supernatants. The reaction mix was incubated for 1 hour at 37 °C, after which it was centrifuged (1300 ***g*** × 5 min) to remove cellular debris; the released deoxyhaemoglobin was measured at 558 nm (Voet & Voet, 1995). The assay included water and culture medium as positive and negative controls, respectively. The degree of haemolysis caused by a culture supernatant was measured as a percentage of the positive control.

Cellular fatty acids were analysed in a commercial laboratory using GLC, Sherlock Version 4.5 software and library RCLN505.00 (Microbial ID). The biochemical reactions were performed with several tube media (BBL), miniaturized API 20NE tests and a Vitek instrument (bioMérieux). The antibiotic susceptibility tests were performed using Etest (AB Biodisk) on Mueller–Hinton agar and the results were interpreted according to the breakpoints set by the Clinical and Laboratory Standards Institute (CLSI) for Gram-negative rods other than the family *Enterobacteriaceae* and *Pseudomonas aeruginosa* (CLSI, 2005).

A phylogenetic tree based on the 16S rRNA gene of strain MDA0585^T was constructed (Fig. 1). Strain MDA0585^T, *C. violaceum* and two chromobacterial sequences formed a distinct branch with the closest relationships. On the basis of 16S rRNA gene sequences, strain MDA0585^T shared 96.1–96.3 % similarity with those of *C. violaceum* ATCC 12472^T and *C. subtsugae* PRAA4-1^T, suggesting species-level phylogenetic distance. Other closer organisms, matching at levels of 90–89 %, without gaps, included *Ralstonia solanacearum* (GenBank accession no. AL646052, Salanoubat *et al.*, 2002), *Neisseria meningitidis* (AE002098, Tettelin *et al.*, 2000) and *Burkholderia glumae* BGR1 (AY224131, Jeong *et al.*, 2003). A previous study has found that the genera *Chromobacterium* and *Neisseria* are within the same family, the family *Neisseriaceae* (Dewhirst *et al.*, 1989).

The results of the *gyrA* gene sequence analysis were almost identical to the 16S rRNA gene sequence analysis: The *gyrA* gene sequence of the novel strain was closest, with 92 % similarity, to that of *C. violaceum* at both the nucleotide and translated amino acid level. The similarities with, in descending order, *Burkholderia glumae* M3 (AB121697, Maeda *et al.*, 2004), *Ralstonia solanacearum* (AL646052, Salanoubat *et al.*, 2002) and *Neisseria meningitidis* (AE002098, Tettelin *et al.*, 2000) were 85–76 % similarity for nucleotides and 87–80 % similarity for amino acids.

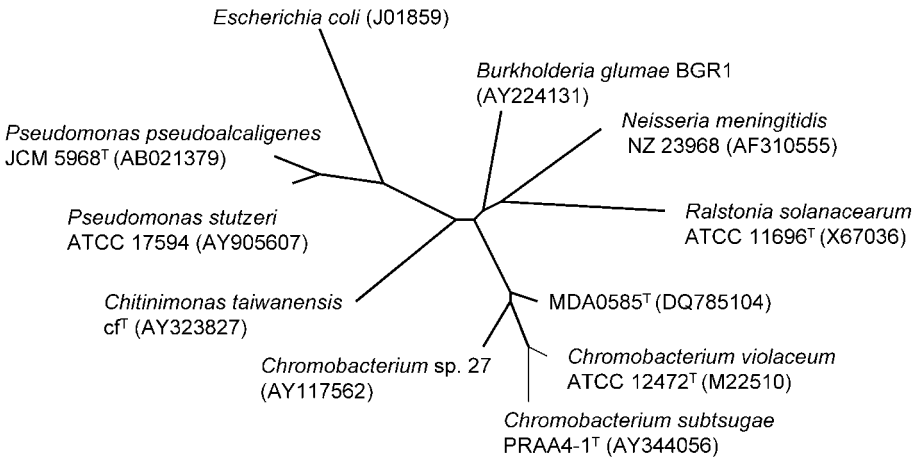

Fig. 1. A 16S rRNA gene-based phylogenetic tree showing strain MDA0585^T and other organisms. The sequence of *C. violaceum* ATCC 12472^T used was GenBank accession no. M22510 with 14 ambiguous nucleotides resolved. The genome sequences of *C. violaceum* (AE016825), *N. meningitidis* (AE002098) and *R. solanacearum* (AL646052) were not used for tree construction due to their large size.

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The 16S rRNA gene of strain MDA0585^T also matched those of several unnamed but probable chromobacteria, i.e. 99.5 % (1447/1454) with GenBank accession no. AY345393 (S. P. Donachie and others, unpublished), 98.9 % (1444/1460) with AB017489 (T. Hamada, unpublished) and 97.5 % (1421/1457) with AY117562 (Hungria *et al.*, 2005).

The most striking feature of strain MD0585^T was its strong haemolytic activity on sheep blood agar culture (Fig. 2a). The haemolysis began after several hours incubation in the heavily streaked area and reached 5 mm in diameter surrounding individual colonies at 24 h. To our knowledge, this degree of haemolysis is the fastest and strongest thus far seen in a clinical microbiology laboratory. There was no violet pigmentation, contrasting with the deep purple colour of non-haemolytic colonies of *C. violaceum* (Fig. 2b). Upon extended incubation (4 days), *C. violaceum* caused slight haemolysis. In a similar manner to *C. violaceum*, the newly described species *C. subtsugae* also exhibits non-haemolytic and deep purple colonies (Martin *et al.*, 2007).

Using human RBC, the haemolytic activity of culture supernatants from 14 h incubation was tested. Assayed with or without $CaCl_2$, strain MDA0585^T produced the strongest haemolysis, 45.5–70.1 % (mean 56.5 % in three culture experiments) (with $CaCl_2$) and 36.8–70.9 % (mean 51.2 %) (without $CaCl_2$), as compared with *C. violaceum*, which showed means of 18.6 % (15.8–20.5 % in three culture experiments) and 4.0 % (2.0–7.3 %) with or without $CaCl_2$. *Pseudomonas aeruginosa*, *E. coli* and *S. aureus* caused 0–9.5 % haemolysis in the presence or absence of $CaCl_2$. The culture densities for these organisms were similar (OD_{580} of 1.8–2.4). In a time-course study, strain MDA0585^T and *C. violaceum* grew in a similar manner, but caused varying degrees of haemolysis (see Supplementary Fig. S1 in IJSEM Online). Strain MDA0585 caused little haemolysis in 2 to 4 h cultures but remarkable haemolysis was seen in 7, 10 and 14 h cultures. The peak was at 7 h, corresponding to the mid- to late-exponential growth phase, and it reached 86.4 % in the presence of calcium. After the peak, haemolytic activities dropped steadily to 45.5 % (with $CaCl_2$) at 14 h. Overall, the presence of calcium slightly increased haemolytic activities. For *C. violaceum*, the cultures showed a much lower rate of haemolytic activity, which essentially plateaued after 7 h, with a small peak of 20.5 % (with $CaCl_2$) at 14 h. The presence of calcium substantially increased the rate of haemolytic activity of *C. violaceum*. As measured by the peak rates of haemolysis, strain MDA0585^T caused 4.2 times (86.4 % versus 20.5 %) more haemolysis than *C. violaceum*.

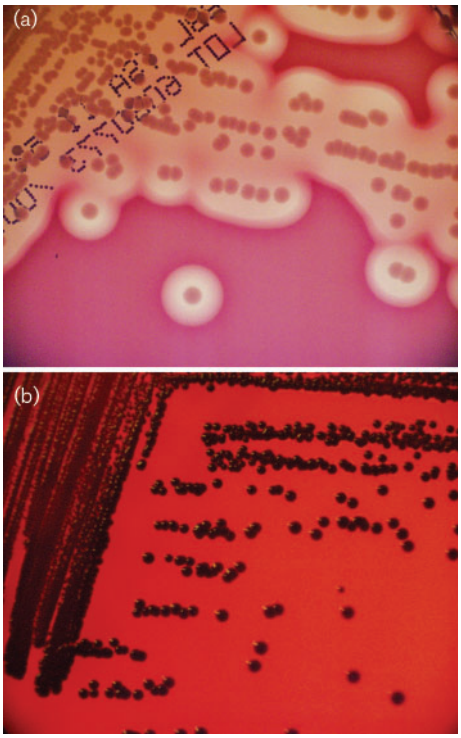

Fig. 2. Strain MDA0585^T with marked haemolysis (a) and *C. violaceum* ATCC 12472^T with deep violet pigmentation (b) on sheep blood agar after 24 h culture. The change of the red cell colour in (a) was due to transmission of the background fluorescent light through the haemolysed zone as compared with no light transmission in (b).

The cellular fatty acids of strain MDA0585^T contained mainly $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{16:1}\omega6c$ (summed feature 3) and $C_{18:1}\omega7c$ and $C_{18:1}\omega6c$ (summed feature 8), with a low similarity (index of <0.441 on a scale of 1) to *C. violaceum* (see Supplementary Table S1, in IJSEM Online). However, the highest similarity indices indicated moderate similarity to *Photobacterium angustum* ATCC 25915^T (0.663), *Photobacterium damselae* ATCC 33539^T (0.543) and *Photobacterium leiognathi* ATCC 25521^T (0.512). The similarity index of strain MDA0585^T with *C. subtsugae* could not be assessed, but the latter strain also contained 41.9 % of $C_{16:1}\omega7c$ (Martin *et al.*, 2007), similar to strain MDA0585^T.

The growth, biochemical reactions and results of antibiotic susceptibility tests are shown in Table 1. Both strain MDA0585^T and *C. violaceum* ATCC 12472^T grew readily on a few common agar plates, with some differences in colony morphology, pigmentation and haemolysis. Strain MDA0585^T also grew well on trypticase soy agar but poorly on MacConkey agar and hardly at all on Hektoen enteric agar (after extended incubation), differing from the growth of *C. violaceum* ATCC 12472^T on these plates. Both organisms were regular Gram-negative rods and some cells of strain MDA0585^T showed intracellular vacuoles that intensified with the ageing of the culture. For biochemical tests, the Vitek GNI+ card, which tested 30 biochemical reactions, yielded bionumbers 6022000060 for *C. violaceum* ATCC 12472^T and 7022000060 for strain MDA0585^T (both with 99 % confidence for *C. violaceum*), with the sole difference being that strain MDA0585^T gave a positive

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Table 1. Growth, biochemical reactions and results of antibiotic susceptibility tests for strain MDA0585^T and *C. violaceum* ATCC 12472^T

+, Positive; −, Negative; MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant. Both strains showed positive results in the following biochemical tests: nitrate reduction to nitrite, arginine dihydrolase, gelatin hydrolysis and utilization of glucose, gluconate, caprate, malate and *N*-acetylglucosamine. Both strains gave negative results for urea hydrolysis, aesculin hydrolysis, phenyl-galactopyranosidase, H_2S production (by lead acetate strip and triple-sugar iron agar) and utilization of adipate, arabinose, maltose and phenylacetate. Both strains give an alkaline/acid reaction in triple-sugar iron slants/butts.

Characteristic	MDA0585^T	*C. violaceum* ATCC 12472^T
Growth on agar (37 °C, 5 % CO_2, 24 h):	All non-pigmented	All purple
Sheep blood	2 mm, β-haemolysis	2 mm, no haemolysis
Chocolate	2 mm	2 mm
Buffered charcoal yeast extract	2 mm, flat, dull	2 mm, raised, shiny
Trypticase soy	1.8 mm	1 mm
MacConkey	0.2 mm, pinpoint	1 mm
Hektoen enteric	No growth	0.2 mm, pinpoint
Indole production (tryptophanase)	−	+
Glucose fermentation	+ (weak)	+
Utilization of:		
Mannose	−	+
Mannitol	+	−
Citrate	+	−
Oxidase	+	−
Catalase	+ (weak)	+
Antimicrobial susceptibility (MIC, μg ml^{-1})		
Amikacin	16, S	3, S
Cefepime	>32, R	2, S
Ceftriaxone	>32, R	>32, R
Ciprofloxacin	0.003, S	0.006, S
Imipenem	6.0, S	1.0, S
Penicillin	>32, R	>32, R
Piperacillin/tazobactam	>256, R	3, S
Ticarcillin/clavulanate	>256, R	32, I
Trimethoprim/sulfamethoxazole	0.094, S	0.094, S

result for DP3 (inhibition of glucose fermentation by hydroxydiphenylether) whereas *C. violaceum* ATCC 12472^T gave a negative result. The API 20NE test, which examined 21 reactions, yielded a bionumber of 7152550 for *C. violaceum* ATCC 12472^T (99.9 % confidence) at both 24 and 48 h of incubation. The bionumbers for strain MDA0585^T were 1154555 at 24 h (98 % confidence for *Pseudomonas aeruginosa*) and 5154555 at 48 h when glucose fermentation became positive (99.6 % confidence for *C. violaceum*). Therefore, strain MDA0585^T would be identified as *C. violaceum* by these tests; however, different API 20NE codes can be used to separate them. In addition, the oxidase test was positive for strain MDA0585^T but negative for *C. violaceum*. The catalase test was weakly positive for strain MDA0585^T but positive for *C. violaceum* ATCC 12472^T. The results of antimicrobial susceptibility tests also differed. Strain MDA0585^T was more resistant overall, with higher minimum inhibitory concentrations for most drugs. Specifically, strain MDA0585^T was resistant to cefepime, piperacillin/tazobactam and ticarcillin/clavulanate, whereas *C. violaceum* ATCC 12472^T was susceptible or intermediate to these antibiotics. Both organisms were resistant to penicillin and ceftriaxone, but susceptible to ciprofloxacin, imipenem, amikacin and trimethoprim/sulfamethoxazole. The resistance profile prompted a review of the history of the patient from whom strain MDA0585^T was isolated. The patient suffered from oesophageal cancer and had not received prior antibiotics. Thus, there was no antibiotic selection effect.

Direct biochemical comparisons between strain MDA0585^T and the type strain of *C. subtsugae* were not made, owing to the recent description of *C. subtsugae*. However, by analysis of the reported data for the type strain of *C. subtsugae* (Martin *et al.*, 2007), these strains differed in the utilization of citrate and mannose. *C. subtsugae* also grows well on MacConkey agar, which is similar to *C. violaceum* ATCC 12472^T but different from strain MDA0585^T (Table 1). Antimicrobial susceptibility data for *C. subtsugae* are not available for comparison.

Taken together, the genotypic and phenotypic features of strain MDA0585^T suggest that the new strain represents a

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novel species of the genus *Chromobacterium*, that can be distinguished from both the well characterized species *C. violaceum* as well as the recently reported species, *C. subtsugae*. The name *Chromobacterium haemolyticum* sp. nov. is proposed for the novel strain.

Before the recognition of *C. haemolyticum* sp. nov., many cases of *C. violaceum* infections had been reported. For instance, Lee *et al.* (1999) and Kim *et al.* (2005) reported infections caused by three non-pigmented but β-haemolytic strains of *C. violaceum*. In retrospect, these features, along with their nearly identical API 20NE codes, make these strains more likely to be *C. haemolyticum* sp. nov. instead of *C. violaceum*. The clinical significance of *C. subtsugae* is unknown, but this recently described species is barely distinguishable from *C. violaceum* based on morphology and commercial identification kits (such as the Biolog assay).

Description of *Chromobacterium haemolyticum* sp. nov.

Chromobacterium haemolyticum (hae.mo.ly′ti.cum. Gr. n. *haima haimatos* blood; Gr. adj. *lutikos* loosening, dissolving; N.L. adj. *lyticus -a -um* dissolving; N.L. neut. adj. *haemolyticum* dissolving blood).

Gram-negative bacillus that grows readily on several types of agar plates (sheep blood, chocolate, trypticase soy and buffered charcoal yeast extract). The colonies are grey, round and elevated and may reach 2 mm after 24 h incubation. On sheep blood agar, colonies are surrounded by a clear haemolytic zone (5 mm); a remarkable feature. The colonies are non-pigmented, contrasting with the violet colonies of other known chromobacteria. Poor growth on MacConkey agar and no growth on Hektoen enteric agar. In liquid culture, a potent haemolysin is secreted into the culture medium in the mid- to late-exponential growth phase, causing the haemolysis of human erythrocytes. In biochemical tests, reduces nitrate, ferments glucose, hydrolyses arginine and gelatin and utilizes several sugars aerobically, such as glucose, mannitol, *N*-acetylglucosamine, gluconate, caprate, malate and citrate. It is also positive for oxidase activity and weakly positive for catalase activity. Does not produce indole. Generally more resistant to antibiotics than *C. violaceum*. The cellular fatty acids mainly contain $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{16:1}\omega6c$ (summed feature 3) and $C_{18:1}\omega7c$ and $C_{18:1}\omega6c$ (summed feature 8).

The type strain, MDA0585^T (=CCUG 53230^T=JCM 14163^T=DSM 19808^T), was isolated in June 2001 from a sputum culture from a patient.

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