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ORIGINAL ARTICLE

Corynebacterium kroppenstedtii subsp. *demodicis* is the endobacterium of *Demodex folliculorum*

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Abstract

Background *Demodex* spp. mites are the most complex member of the human skin microbiome. Mostly they are commensals, although their pathophysiological role in inflammatory dermatoses is recognized. Demodex mites cannot be cultivated *in vitro*, so only little is known about their life cycle, biology and physiology. Different bacterial species have been suggested to be the endobacterium of Demodex mites, including *Bacillus oleronius*, *B. simplex*, *B. cereus* and *B. pumilus*.

Objectives Our aim was to find the true endobacterium of human Demodex mites.

Methods The distinct genetic and phenotypic differences and similarities between the type strain and native isolates are described by DNA sequencing, PCR, MALDI-TOF, DNA-DNA hybridization, fatty and mycolic acid analyses, and antibiotic resistance testing.

Results We report the true endobacterium of *Demodex folliculorum*, independent of the sampling source of mites or life stage: *Corynebacterium kroppenstedtii* subsp. *demodicis*.

Conclusions We anticipate our finding to be a starting point for more in-depth understanding of the tripartite microbe–host interaction between *Demodex* mites, its bacterial endosymbiont and the human host.

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Conflict of interest

The authors declare no conflicts of interest.

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Introduction

The term 'endosymbiosis' was formerly just meant as a form of living together, coined by Russian botanist Konstantin Mereschkowski in 1905. It can be roughly divided into beneficial, neutral and harmful interactions, depending on the effect of the symbiont towards the host.[1] However, the classification is not always straightforward, as for example *Demodex* spp. mites can infest the human skin as a commensal, but can also cause skin diseases, like rosacea-like demodicosis, pityriasis folliculorum or granulomatous demodicosis.[2]

Endobacterial symbionts, which are most comprehensively described in the class of insects, also occur in the class of arachnida – both being in the phylum of arthropods. Endobacteria-bearing arthropods of humanpathological relevance include head/body lice *Pediculus humanus capitis/corporis* (insect; endosymbiont: *Riesia pediculicola*), 'bed bugs' *Cimex lectularius* (insect; endosymbiont: *Wolbachia* spp.), 'kissing bugs'/Chagas disease vector *Triatoma infestans* (insect; endosymbiont: *Corynebacterium* sp.), common ticks *Ixodes ricinus* (arachnida; endosymbiont: *Midichloria mitochondrii*) and face mites *Demodex* spp. (arachnida; presumed endosymbiont: *Bacillus* spp.).[1,3]

Several bacterial species have been proposed to be the endosymbiont of *Demodex* spp. mites. Already in 1961, Spickett proposed *Demodex* spp. as a vector of leprosy after identifying acid-fast bacteria in the digestive tract of the mites.[4] In 2007, Lacey *et al.*[5] isolated a strain of *Bacillus oleronius*; however, they did not state any method of decontaminating the outer surface of the isolated mites. As literature states, bacteria adhere firmly to the mite exoskeleton, so external decontamination appears to be crucial.[6]

Additionally, other *Bacillus* spp. strains have been described to be *Demodex*-associated. Tatu *et al.*[7] isolated *Bacillus simplex*, later in the same year also *Bacillus cereus*.[8] Subsequently, they proposed *Bacillus pumilus* a year later.[9] *Bacillus* spp. strains occur ubiquitously in the environment, however, and results of the above reports have unfortunately not been reproduced.

In this study, we attempt to resolve the void of knowledge concerning the endobacterial symbiont of *Demodex folliculorum* in a reproduceable fashion with special attention to avoiding external contamination. Further knowlegde about the endobacterium could lead to an extended understanding of the biology of these mites, the elucidation of the mechanism of action of antibiotic agents used in the treatment of associated diseases like rosacea, and possibly reveal additional strategies to disturb the microbe–host interaction.

Materials and methods

Chemicals

All chemicals and buffers were purchased from Sigma Aldrich (Steinheim, Germany) except where noted otherwise.

Bacterial reference strain

The bacterial reference strain *Corynebacterium kroppenstedtii* DSM 44385 was obtained from the DSMZ (Leibniz Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Mites

Living and dead mites in human sebum and skin scrapings were obtained anonymously from the diagnostics facility of the department of dermatology at the Ludwig-Maximilian-University in Munich, Germany. The use of anonymized remaining patient sebum samples after diagnostic analysis was approved by the local ethics committee (project 17-450 UE). The samples were kept in a humid environment and used on the same day of sampling. For analyses, *D. folliculorum* mites from six distinct, anonymized sebum samples were used.

Brightfield and fluorescence microscopy

Brightfield and fluorescence microscopy was performed using an inverted Axio Observer 7 microscope (Carl Zeiss AG, Oberkochen, Germany) with 200–400× total magnification and the ZEN Imaging Software (Zeiss). The mites were observed using either visible (VIS) or UV light with a blue (DAPI; Filterset 02 or 49; Zeiss) or green (GFP) filter set (Filterset 38; Zeiss) without any staining due to their autofluorescence. For resazurin staining, they were observed using UV light with a red filter set (PI; Filterset 43 HE; Zeiss).

Bacterial isolation strategies

Rupture of adult mites The diagnostically collected sebum was spread on microscope slides, and single adult mites with dark-stained opisthosomata were picked and transferred to clean sites on the same slide. Mites were rolled around to get rid of coarse debris and attached sebum. Apparently, clean mites were submersed in 3% H_2O_2-solution for 5 min and transferred to different agar plates (lysogeny broth, MacConkey, mannitol salt, nutrient, tryptic soy, blood, chocolate, brain heart infusion and reinforced clostridial medium). The different conditions were used to increase the probability of culturing fastidious microorganisms. The mites were positioned in a way they could move around freely on the agar surface (ventral side down), thereby stripping off remaining attached bacteria (Fig. 2a). After 48 h incubation at 30°C with high humidity (80–100%), the mites were transferred to a new agar plate and aseptically ruptured to release their internal contents (Fig. 2b). The plates were incubated for up to 7 days at 37°C with high humidity (80–100%) and examined daily for bacterial growth.

Dissolution from nymph exoskeleton during ecdysis The diagnostically collected sebum was spread on microscope slides, and single moulting mites (nymph to adult transition; Fig. 2c, internally localized adult marked by brace) with visible 'bacterial spheres' (Fig. 2c, arrows, Fig. 2f) were picked and transferred to clean sites on the same slide. Mites were mechanically cleaned and treated with 3% H_2O_2-solution for 5 min. After transferral to different agar plates, the mites were aseptically ruptured on the agar surface to release the 'bacterial spheres' onto the agar surface. The plates were incubated for up to 7 days at 37°C with high humidity (80–100%) and examined daily for bacterial growth (Fig. 2d).

Characterization of the obtained bacterial isolates

16S sequencing Bacterial isolates were initially identified by 16S sequencing after the partial amplification of the 16S rRNA gene by colony PCR with the HotStarTaq Master Mix Kit (Qiagen, Qiagen GmbH, Hilden, Germany) using the primerpairs 27f/1492r (initial analyses) with standard conditions (for primer sequences see Appendix S1, Supporting Information). Obtained sequences were compared to 16S reference genomes using nucleotide BLAST.[10,11]

Biomass production for further analyses After sequencing and identification of the species, the isolates were grown on brain heart infusion agar with 1% Tween-80 to increase biomass production.

Additionally, the novel '*C. kroppenstedtii* selective medium' (CKSM) published by Wong *et al.*[12] was used as liquid and solid growth medium to further improve results. Also, a simplified version of CKSM was used for the cultivation of our isolates, ommitting esculin and ferric citrate, optionally Tween-80 can be substituted by Tween-20.

rpoB gene sequencing for identification of Corynebacterium species For accurate identification of the heterogeneous group of corynebacteria, *rpoB* sequencing was performed according to Khamis *et al.*[13] Briefly, a section of the *rpoB* gene was amplified by colony PCR with forward primer rpoB-F and reverse primer

rpoB-R using the conditions described. Obtained sequences were compared to reference genomes using BLAST.[10,11]

nanI amplification To further ascertain species identity, a nested colony PCR targeting *C. kroppenstedtii*-specific sialidase gene, *nanI*, using the primers CKOut-F, CKOut-R, CKIn-F and CKIn-R was performed (GenBank accession no. ACR18588)[14] as described by Wong *et al.*[15] The target gene *nanI* is specific for *C. kroppenstedtii* with no *in silico* or *in vitro* cross-reactivity against other *Corynebacterium* spp. The amplified 141-bp section, corresponding to the first lectin domain, was visualized by electrophoresis on 1% agarose gel in TAE buffer with ethidium bromide unter UV illumination.

MALDI identification Additional non-DNA dependent identification was performed using a MALDI biotyper® (Bruker Daltonik GmbH, Bremen, Germany) automated mass spectrometry system.

ERIC-/REP-PCR To analyse the genome architecture and the genomic relationship between our isolates and the reference genome (*C. kroppenstedtii* strain DSM 44385), PCR of repetitive regions of the genome was performed. Enterobacterial Repetitive Intergenic Consensus (ERIC-) PCR and repetitive-element (REP-) PCR were performed by colony PCR with the primer pairs ERIC1R/ERIC2 and REP1R-1/REP2-1 at different T_m in increments of 3°C from 40–55°C.[16] Resulting ERIC- and REP-fingerprints were visualized by agarose gel electrophoresis. Furthermore, six different bacterial isolates were compared by ERIC-/REP-PCR to each other to verify the robustness of both methods.

DNA–DNA hybridization To further compare the isolates to the reference organism *C. kroppenstedtii* DSM 44385, DNA–DNA hybridization was performed. This technique is deemed necessary when describing new species with more than 97% 16S rRNA sequence similarity.[17,18]

Cells were disrupted by using a Constant Systems TS 0.75 KW (IUL Instruments GmbH, Königswinter, Germany). DNA in the crude lysate was purified by chromatography on hydroxylapatite as described by Cashion *et al.*[19]

DNA–DNA hybridization was carried out as described by De Ley *et al.*[20] under consideration of the modifications described by Huss *et al.*[21] 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 Analytical Instruments, Walnut Creek, CA, USA).

GC content Briefly, cells were disrupted as described by Cashion *et al.*[19]. The DNA was hydrolyzed, and the nucleotides were dephosphorylized. The resulting desoxynucleosides were analysed by HPLC. Chromatograms were analysed using the OpenLAB 2 software (Agilent, Santa Clara, CA, USA). The GC content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to Mesbah *et al.*[22] For more details, see Appendix S1 (Supporting Information).

Mycolic acids Analysis of mycolic acids was carried out by the DSMZ as described in the literature.[23]

Fatty acids Fatty acid methyl esters were obtained according to Miller[24] and Kuykendall[25] and separated using Sherlock Microbial Identification System (MIDI, Microbial ID, Newark, DE, USA). For more details, see Appendix S1 (Supporting Information).

Phenotypic characterization The isolate was analysed using the API® identification system (bioMérieux Deutschland GmbH, Nürtingen, Germany) by the DSMZ with 'API CORYNE' test strips.

Antibiotic resistance Analysis of mycolic acids was carried out by the DSMZ according to national standards using the agar diffusion method.[26]

Deposit in the DSMZ open collection The bacterial isolate was deposited in the DSMZ open collection in compliance with the Nagoya protocol under the name *C. kroppenstedtii* subsp. *demodicis*, DSM-No. 109755.

Results

Brightfield and fluorescence microscopy

Microscopical examination of native mite preparations (sebum spread on specimen slides) revealed 'pigment granules' in all life stages (egg, larva, nymph, adult and pregnant adult) of *Demodex* spp., as described by Stromberg and Nutting[27] (Fig. 1, lower row). The granules showed moderate fluorescence using the GFP filter set (green) and no fluorescence using the DAPI filter set (blue). Staining with resazurin revealed increased metabolic activity in those 'pigment granules' using the PI filter set (red), as the dye gets transformed to fluorescent resorufin in a reducing environment. Isolated 'pigment granules' (after mechanical disruption of the mite) showed the same fluorescent behaviour (Fig. 1, upper row).

Bacterial isolation strategies

Bacterial growth from adult mites' opithosomal contents and nymph exosceletons occurred on RCM and BHI agar plates, yet very faintly and slowly (Fig. 2d). Growth of bacterial colonies was visible after 2–3 days and suspended after approximately 10 days. Also in liquid media, the growth stopped after that time. However, enough biomass could be acquired to perform 16S sequencing by colony PCR. All colonies had identical microscopic morphology and appearance, further analyses showed the same species in all mites that yielded bacterial growth. No additional bacterial species were detected in the mites prepared in this fashion.

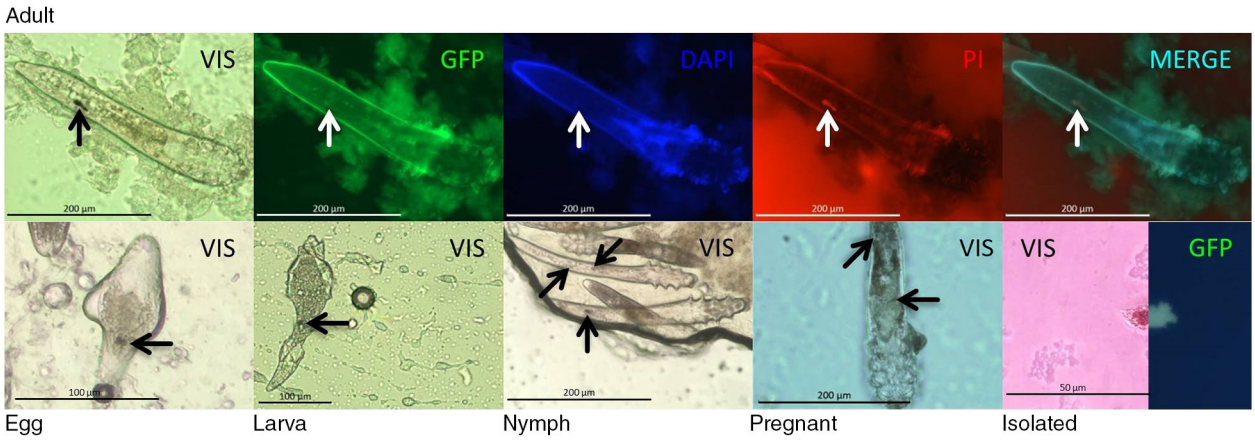

Figure 1 Upper row: brightfield (VIS) and fluorescent (GFP, DAPI, PI and MERGE) pictures of an adult mite harbouring a 'bacterial inoculum' (arrows). The mite was stained with resazurin, a dye that turns fluorescent once it is reduced, thereby showing the metabolic activity of the bacteria. Natively, the bacteria also fluoresce in the GFP channel. Lower row: brightfield pictures of an egg, a larva, two nymphs and a pregnant mite – all harbouring the 'bacterial inoculum'/'pigment granule', thereby suggesting a vertical transmission. The last picture is divided into a brightfield and fluorescent picture of a microdissected bacterial inoculum from an adult mite, showing the distinctive brownish (VIS) and green-fluorescent (GFP) colour, respectively.

Characterization of the obtained bacterial isolates

16S sequencing The 16S sequence had 99.88% sequence identity with the 16S partial gene sequence of *C. kroppenstedtii* strain DSM 44385. Sequences and alignment can be found in the Appendix S1 (Supporting Information).

Biomass production for further analyses Using the simplified CKSM, all isolates grew consistently, yet slowly in liquid and solid media. Incubation with 5% CO_2 for at least 24 h seems to be necessary to induce growth/resuscitation. Visible bacterial colonies could be macroscopically detected on day 3.

rpoB gene sequencing for identification of Corynebacterium species Partial *rpoB* sequences had 98.8% and 97.58% sequence identity with the *rpoB* gene sequence of *C. kroppenstedtii* strain DSM 44385, indicating the same species. Sequences and alignment can be found in the Appendix S1 (Supporting Information).

nanI amplification Colony PCR of the isolated endobacterium did not result in any amplified DNA, while using *C. kroppenstedtii* strain DSM 44385 as a positive control yielded a band at approximately 140 bp. This result indicates distinctive differences at the genetic level between the two bacterial probes.

MALDI identification Automatically software-evaluated mass spectra yielded *C. kroppenstedtii* in every individual bacterial isolate with a reliably high score value according to system standards. Scores can be found in the Appendix S1 (Supporting Information).

ERIC-/REP-PCR To analyse the genome architecture of the isolate, amplification of repetitive sequences with two different primer pairs was performed. The resulting gel electrophoresis fingerprints were significantly different from the reference strain; however, different isolates were almost identical to each other (Fig. 3).

DNA–DNA hybridization Performing DNA–DNA hybridization of our isolate against the reference genome of *C kroppenstedtii* DSM 44385 yielded a 78.0% DNA–DNA similarity. Therefore, the isolated bacteria belong to the species *C. kroppenstedtii* DSM 44385 when the recommendations of a threshold value of 70% DNA–DNA similarity for the definition of bacterial species by the *ad hoc* committee[28] are considered.

GC content The GC content of the isolate was determined to be 57.3 mol% G+C. The GC content of *C. kroppenstedtii* DSM 44385 is 62.0 mol% G+C, as determined by the DSMZ.

Mycolic acids The isolated strain contained mycolic acids, in complete contrast to *C. kroppenstedtii* DSM 44385. The analysis yielded C34:0 (34%), C36:0 (10%), C38:0 (24%), C40:0 (15%) and C42:0 (16%). This result also indicates distinctive differences at the genetic level between the two bacterial probes.

Fatty acids The analysis yielded C16:0 (29%), C18:1ω9c (26%), C18:0 (18%) and tuberculostearic acid (10-R-methyl-

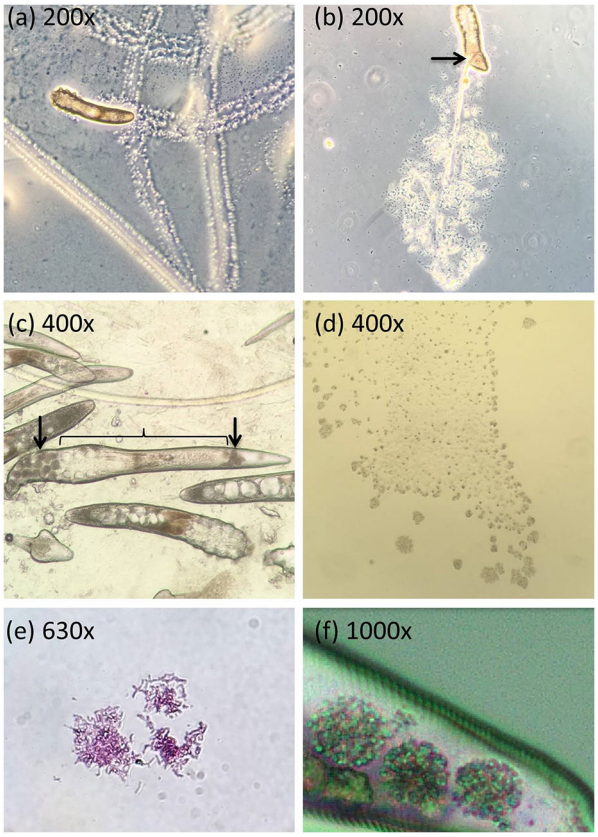

Figure 2 Isolation of endobacteria: a freshly isolated mite crawls on an agar surface, after removing debris and submersion in 3% H_2O_2-solution to decrease the chance of isolation of externally attached bacteria (a). Mechanical disruption of the chitin exoskeleton on an agar surface in order to cultivate the internal bacteria (b). Microscopic view of a moulting nymph with cranial and caudal 'bacterial spheres' (arrows), the adult animal (braces) can be seen inside the old nymph exoskeleton (c). Bacterial colonies 3 days after disruption of the isolated and decontaminated mites (d). Gram stain of the isolated bacteria (e). Digital zoom of the caudal 'bacterial spheres' during ecdysis (f).

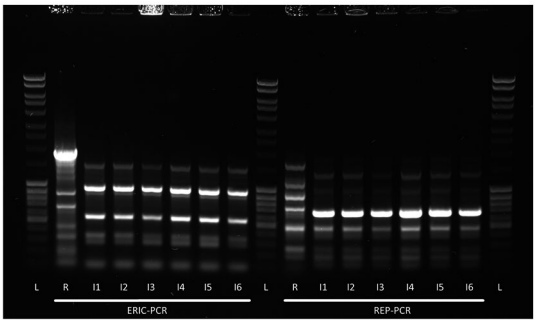

Figure 3 Agarose gel electrophoresis of the resulting 'finger print' after performing Enterobacterial Repetitive Intergenic Consensus- (left) and repetitive-element-PCR (right), amplifying the repetitive regions of the bacterial genome. L = ladder, R = reference strain (*Corynebacterium kroppenstedtii* DSM 44385), I1–6 = bacterial isolates from different mites. Isolates resulting in a very identical fingerprint, however very different from the genetically closest reference strain *C. kroppenstedtii* DSM 44385.

octadecanoic) (12%), a unique cell component within the Actinomycetales.[29] Human sebum unique C16:1ω10c (sapienic acid) and 18:2ω10,13c (sebaleic acid) were not detected to a significant amount. Identically, cultured *C. kroppenstedtii* DSM 44385 had a similar fatty acid composition, although it additionally contained arachidonic acid (C20:4ω6,9,12,15c) (2%). Chromatograms can be found in the Appendix S1 (Supporting Information).

Phenotypic characterization Acid is produced from glucose after 3 days, acid is not produced from ribose, xylose, mannitol, maltose, lactose, sucrose and glycogen. Alkaline phosphatase, urease and pyrazinamidase are produced. *N*-acetylglucosaminidase, β-galactosidase, α-glucosidase, β-glucuronidase and pyrrolydonyl arylamidase are not detected. Aesculin is not hydrolysed. Gelatin is not hydrolysed. Nitrate is not reduced to nitrite. Catalase is not produced. For comparison with *C. kroppenstedtii* DSM 44385 refer to Table 1.[30,31]

Antibiotic resistance Resistance testing showed susceptibility against most of the 35 tested chemotherapeutics with the exception of aztreonam, lincomycin, colistin, nitrofurantoin, clindamycin and fosfomycin, where no growth inhibition occurred. The tested substances and extent of inhibition can be found in the Appendix S1 (Supporting Information).

Discussion

Bacterial endosymbiont

One of the most prominent features when observing Demodex mites through a microscope is the central dark staining in the cranial opisthosoma. This staining is 'strongly associated with viability of mites and diminished as mites became less viable', as Lacey *et al.* reported.[32] This dark staining forms three or more finger-like spurs and fluoresces faintly blue.[33] This structure resembles the acarine gut in the order of trombidiformes. Upon closer microscopic view, the blue fluorescence comes from moving, approximately 1 μm measuring particles, we identified as endobacteria.

Earlier literature stated 'pigment granules' in the caudal opisthosoma, occuring in every life stage of the mites,[27] however, presumably not refering to the mite gut. In the same publication, it was noted that these 'pigment granules' get transmitted vertically during mite pregnancy – a result we were able to confirm. However, it was hypothesized those structures were nitrogenous waste disposals and serve as a crystal nucleus to accumulate

Table 1 Phenotypic/metabolic comparison of the isolated bacteria vs. *Corynebacterium kroppenstedtii* DSM 44385 (modified after[30,31])

Feature	*C. kroppenstedtii* DSM 44385	*C. kroppenstedtii* subsp. *demodicis*
Lipophilism	+	+
Acid is produced from		
Glucose	+	+
Maltose	(+)	–
Ribose	–	–
Sucrose	+	–
Xylose	–	–
Mannitol	–	–
Lactose	–	–
Glycogen	–	–
Alkaline phosphatase	–	+
Reduction of nitrate	–	–
Hydrolysis of urea	–	+
Hydrolysis of aesculin	+	–
Tuberculostearic acid	+	+
Mycolic acid	–	+

guanine derivatives. In our work, we could show these 'pigment granules' were metabolically very active and upon isolation yielded the newly described bacterial subspecies.

Hence, we propose the model of *C. kroppenstedtii* subsp. *demodicis* being the vertically transmitted endosymbiont of *D. folliculorum* mites, showing a phenotypic heterogeneity with one form aiding actively in digestion of the lipid-rich diet of the mites and a 'stock form' as a reserve supply and for vertical transmission. As *C. kroppenstedtii* belongs to the group of lipophilic Corynebacteria, it is highly suited to aid the digestion of lipids by providing numerous hydrolytic exoenzymes, additionally it is also known as a mastitis-causing pathogen.[15]

Another pro-symbiont reasoning is the fact that Demodex mites have a very small arthropod genome (authors' observations) and even their mitochondrial genome is fairly reduced,[34] so the mites probably rely on an additional set of genes to dwell in their ecological niche.

Characterization of the obtained bacterial isolates

Extensive analyses of the isolated endobacterium pleaded for *C. kroppenstedtii* at first, however, a confirmatory DNA–DNA hybridisation only showed a 78% identity – so the threshold for describing a new species was not met, yet it was different enough to encourage us to further investigate the new strain's features. The fatty acid composition was similar, as was the GC content. The most striking differences were, firstly, the lack of *nanI*, a *C. kroppenstedtii*-specific sialidase gene, which may act as a pathogenic factor in infections. Secondly, the DNA fingerprint of repetitive genomic regions showed completely different patterns, suggesting an altered genomic architecture. Thirdly, in crass contrast to *C. kroppenstedtii* DSM 44385, our isolate contains mycolic acids in its cell membrane, a fact that explains in synopsis with its lipophilism (a term used to describe the need to consume exogenous fatty acids, since the strain is missing a fatty acid synthase capable of initiating fatty acid synthesis (FAS-I) and only being able to elongate acquired fatty acids (via FAS-II)) it's slow growth. In summary, the isolated endobacterium is very distinct from the reference strain, yet is classified as a subspecies or biovar by convention.

Conclusion and future clinical relevance

The bacterial subspecies described explains multiple features of Demodex mites. It can be speculated that it lives in mutualistic symbiosis with Demodex mites, as it is transmitted vertically during pregnancy and also as its metabolic capacity is optimized to live in and from a lipid-rich environment. Additionally, the fluorescence of the bacteria was described earlier in literature,[32,35–37] although presumably with no knowledge about its bacterial origin. It was stated that mites with loss of fluorescence are dying; therefore, viable bacteria seem to be an obligatory criterion for their host's viability. If this is true, the clinical consequence is that demodicosis could also be ameliorated by administration of most lipophilic antibiotics and due to their susceptibility also the mechanism of action of low-dose doxycycline can be explained (in addition to its anti-inflammatory effect). Epidermal growth factor receptor (EGFR-) inhibitor-treated patients with acneiform skin-eruptions also show an increased Demodex density,[38] so skin improvement upon treatment with lipophilic antibiotics might also be explained by the effect on their endobacteria.[39]

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Characterization results and description of the new subspecies *C. kroppenstedtii* subsp. *demodicis*.