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Characteristics of *Bordetella hinzii* Strains Isolated from a Cystic Fibrosis Patient over a 3-Year Period

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Over a 3-year period, an adult cystic fibrosis patient underwent eight episodes of pulmonary exacerbation of his disease. At least one of two different strains of *Bordetella hinzii* could be isolated from sputum samples in every instance. The differentiation of *B. hinzii* from related taxa and its role as an etiologic agent of infections are discussed. The two isolates of *B. hinzii* reported are the third and fourth human-derived strains described in the literature.

At present, the genus *Bordetella* comprises the species *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. avium*, as well as the recently defined species *B. holmesii* (17) and *B. hinzii* (14). *B. pertussis* and *B. parapertussis* are the causative agents of whooping cough in humans, whereas *B. bronchiseptica* is primarily a respiratory pathogen found in animals. *B. bronchiseptica* may also cause pneumonia and bacteremia in humans. *B. avium* causes coryza in poultry but has never been described as causing infections in humans; recently, a *B. avium*-like organism was isolated in mixed culture from a patient with chronic otitis media (6). Most strains of *B. holmesii* have been isolated from young adults with septicemia (17). *B. hinzii* was proposed as the species of some strains isolated from poultry with respiratory disease, but the etiologic role of *B. hinzii* had been unclear. Only two human-derived isolates of *B. hinzii* have been reported so far: one strain was recovered from a sputum sample collected in 1957 (no clinical data were available) (14), and one isolate came from cultures of blood from a human immunodeficiency virus-infected patient (3). We report here multiple isolations of *B. hinzii* from sputum samples of a cystic fibrosis (CF) patient during a 3-year period. This report adds further evidence that *B. hinzii* can cause disease in humans.

Case report. During his youth, a 51-year-old artist had seen numerous physicians until the final clinical diagnosis, i.e., a mild form of CF, was established by a positive sweat test at the age of 25. At that time he presented exclusively with gastrointestinal symptoms. His exocrine pancreas insufficiency was treated with oral pancreatic enzyme substitution, and he later also developed diabetes mellitus. In 1990, the patient presented for the first time with significant pulmonary symptoms. Lung function tests revealed a moderate to severe obstructive ventilatory defect with mild hyperinflation. X rays as well as computed tomography scans showed tubular bronchiectases in both lower lobes. Molecular genetic investigations revealed that none of the most frequent mutations (DF508, 3905insT, R553X, 1717-1G→A, G542X) found in Swiss CF patients could be detected in our patient. Repeated tests for human immunodeficiency virus antibodies remained negative (the patient's risk factor was homosexuality). From March 1992 on, the patient was seen in the outpatient clinics of the Zürich University Hospital, and from March 1993 onward he was seen in the Kantonsspital Winterthur on every occasion of pulmonary exacerbation. The patient presented with increased cough, occasional low-grade fever, exertional dyspnea, and mild deterioration in spirometry results. A sputum sample taken in March 1992 grew *Staphylococcus aureus* and a nonfermenting gram-negative rod which was primarily diagnosed as *B. avium* (see below) but which was later shown to be *B. hinzii* (Table 1). The patient was treated with a combination of oral amoxicillin-clavulanic acid (625 mg four times daily) and ciprofloxacin (750 mg twice daily) for 3 weeks (same regimen for the other exacerbations) and improved gradually. One year later, the patient reported with similar symptoms, and *B. hinzii* was again isolated from a sputum sample (Table 1). However, antimicrobial susceptibility testing revealed two strains of *B. hinzii* with different resistance patterns; as shown below, strain DMMZ 1277 was resistant to more antibiotics than strain DMMZ 1281. Over the next 2 years the patient had six further episodes of pulmonary exacerbation of his underlying disease, and in every instance *B. hinzii* was isolated in significant amounts. *S. aureus* was isolated from six of eight sputum samples over the total 3-year period (Table 1). Three of eight times the multiresistant strain *B. hinzii* DMMZ 1277 was grown in parallel with the more susceptible strain *B. hinzii* DMMZ 1281 (Table 1). Since 1990 no further radiologic changes in the patient's lung appeared and the patient's pulmonary function was preserved, but the mild exertional dyspnea remained unchanged.

Microbiological investigations. Sputum samples were cultured aerobically at 37°C with 5% CO_2 on Columbia agar with 5% sheep blood, the same medium with colistin and nalidixic acid, Columbia chocolate agar with bacitracin, and MacConkey agar without CO_2 (all media were from Becton Dickinson, Cockeysville, Md.). The cultures showed round, convex, glistening, greyish colonies about 1 to 2 mm in diameter after 24 h of incubation on sheep blood agar. The cells were asporogenous, gram-negative rods which were motile (hanging drop method). Catalase and oxidase activities were detected, and the triple sugar iron reaction was slant/butt alkaline/alkaline. The commercial API 20 NFT (NE) system (bioMérieux, Marcy l'Etoile, France) gave the numerical code 0000067 (i.e., caprate assimilation was negative), which was compatible with the identification of the isolates as *B. avium* (percent identifica-

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TABLE 1. Culture results for the sputum samples from our patient

Date of collection (mo/yr)	Culture results[a]
3/1992	10^4 *B. hinzii* (resistant)[b], 10^5 *S. aureus*
3/1993	10^5 *B. hinzii* (resistant), 10^5 *B. hinzii* (susceptible)[b]
6/1993	10^4 *B. hinzii* (susceptible), 10^4 *S. aureus*
7/1993	10^5 *B. hinzii* (resistant)
9/1993	10^4 *B. hinzii* (susceptible), 10^4 *S. aureus*, 10^4 *Streptococcus pneumoniae*
10/1993	10^4 *B. hinzii* (resistant), 10^5 *S. aureus*
10/1994	10^5 *B. hinzii* (resistant), 10^5 *B. hinzii* (susceptible), 10^5 *S. aureus*
2/1995	10^4 *B. hinzii* (resistant), 10^4 *B. hinzii* (susceptible), 10^5 *S. aureus*

[a] The numbers represent the number of CFU of bacteria per milliliter. Data for the normal oral flora are not reported.

[b] One strain of *B. hinzii* was more resistant (DMMZ 1277) than the other strain (DMMZ 1281); for details, see the text.

tion, 95.6%; *T* value, 1.00). Antimicrobial susceptibility testing by the disk diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (11) revealed that strain DMMZ 1281 was susceptible to amikacin, amoxicillin-clavulanic acid, cefamandole, ceftazidime, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, moxalactam, netilmicin, piperacillin-tazobactam, ticarcillin-clavulanic acid, and trimethoprim-sulfamethoxazole but was resistant to ampicillin, aztreonam, ceftriaxone, cefotaxime, cefuroxime, and tobramycin, whereas strain DMMZ 1277 was additionally resistant to cefamandole, cephalothin, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole. In the beginning of 1995, we became aware of the newly described species *B. hinzii* (14). Further laboratory investigations revealed alkali production from malonate (the medium used was 0.5% Bacto beef extract [Difco, Detroit, Mich.], 1% Trypticase peptone, 0.5% NaCl, 0.0006% bromothymol blue, and 0.5% malonate) by our isolates. This feature separated our two strains from *B. avium* in which this reaction is negative (14); therefore, the two strains were preliminarily identified as *B. hinzii*. For confirmation, the two isolates DMMZ 1277 (LMG 15873) and DMMZ 1281 (LMG 15872) and reference strains of all presently defined *Bordetella* and *Alcaligenes* species were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins. The strains were grown, whole-cell protein extracts were prepared, and SDS-PAGE was performed as outlined in detail before (13, 14). Duplicate protein extracts were prepared to check the reproducibility of the growth conditions and the preparation of the extracts. Densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed by using the GelCompar software package, version 3.1 (Applied Maths, Kortrijk, Belgium). The level of correlation between duplicate protein patterns was very high ($r \geq 0.94$). Figure 1 shows the dendrogram obtained after numerical analysis of the protein patterns of all strains examined. All *B. hinzii* strains and strains DMMZ 1277 (LMG 15873) and DMMZ 1281 (LMG 15872) formed a separate cluster distinct from all other taxa included. In particular, both isolates from

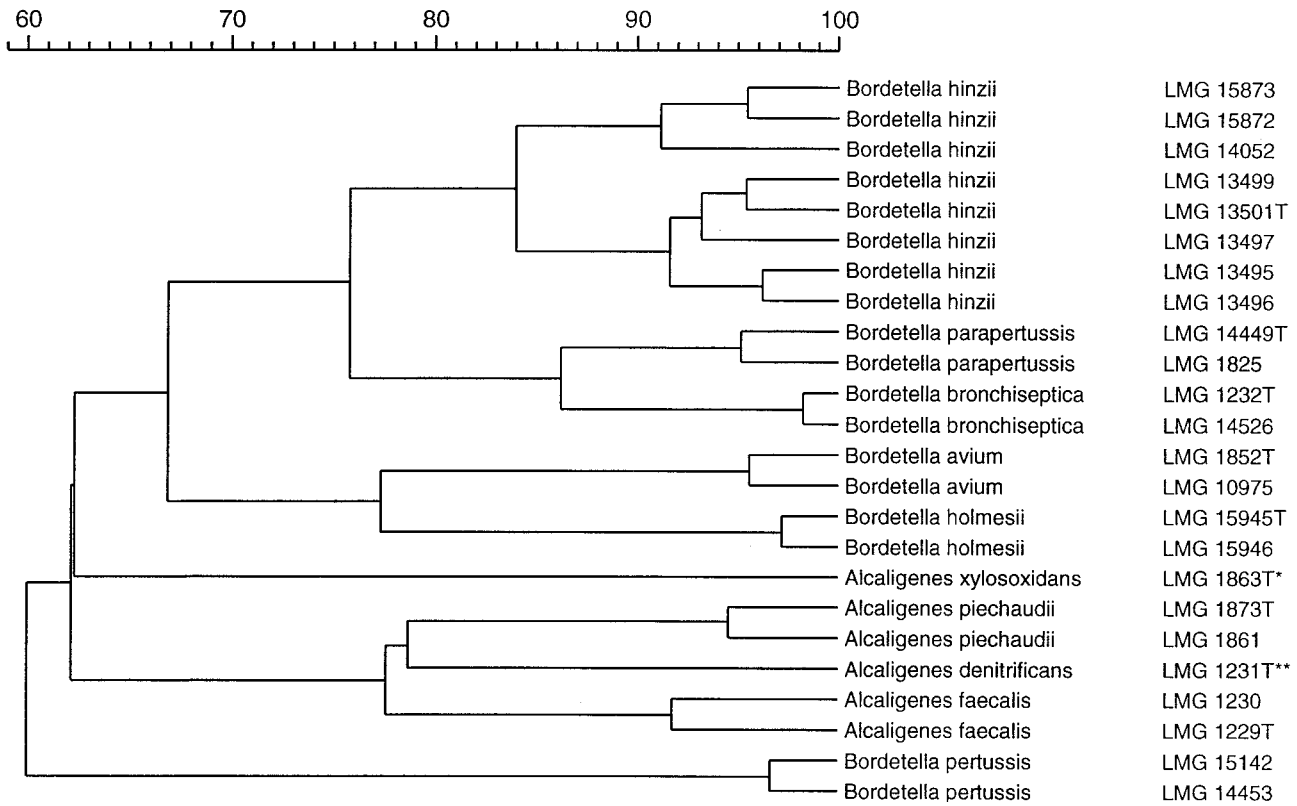

FIG. 1. Dendrogram derived from the unweighted pair group average linkage of correlation coefficients (*r*; expressed for convenience as a percentage) between the protein patterns of all strains studied. *, *A. xylosoxidans* subsp. *xylosoxidans*; **, *A. xylosoxidans* subsp. *denitrificans*. All strain numbers are LMG (Culture Collection of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium) numbers; T refers to type strains. Strain DMMZ 1277 is LMG 15873, and strain DMMZ 1281 is LMG 15872.

our patient were shown to be closely related but not identical. The closest neighbor of our two strains was strain LMG 14052, which is another isolate from a human (14).

For determination of the cellular fatty acid (CFA) patterns, cells were grown for 24 h at 37°C in parallel on (i) Trypticase soy agar (TSA) incubated in air, (ii) TSA supplemented with 5% sheep blood incubated in air, and (iii) TSA supplemented with 5% sheep blood incubated in a 5% CO_2-enriched atmosphere. Analysis of CFA patterns was performed with the MIDI system (Microbial ID, Newark, Del.) as outlined previously (15). The results were almost identical for both isolates (DMMZ 1277 and DMMZ 1281) incubated under different conditions and showed the presence of $C_{16:1\omega7c}$ (17 to 24%), $C_{16:0}$ (38 to 40%), and $C_{17:0\ cyclo}$ (16 to 22%) as the predominant CFAs.

Discussion. The clinical picture as well as the laboratory data were clearly consistent with episodes of infectious pulmonary exacerbations in a CF patient. The etiological role of *B. hinzii* may be questioned in six of the eight episodes, because *S. aureus* was grown in parallel in the sputum samples; however, in two instances *B. hinzii* was the only bacterium cultured (apart from the normal oral flora), suggesting a disease association. In addition, the isolation of two different strains of the same species from a CF patient is not unusual, because this is frequently observed for *Pseudomonas aeruginosa* in CF patients (7). We did not obtain tracheal aspirates or bronchial lavages from the patient, but sputum cultures reflect the flora of the lower airways in adult CF patients (7).

It has been documented extensively for many bacterial groups (4), including the genus *Bordetella* (13), that a high degree of similarity in whole-cell protein content is a reflection of a high degree of DNA homology and, as a consequence, species identity. We therefore conclude that the numerical analysis of the whole-cell protein patterns of strains DMMZ 1277 and DMMZ 1281 unambiguously reveals that both isolates belong to the species *B. hinzii.*

Nonfermenting gram-negative rods other than *P. aeruginosa* or *Burkholderia cepacia* are infrequently isolated from CF patients, and these rods usually do not persist for longer periods of time (7). In particular, members of the family *Alcaligenaceae* (5) are rarely encountered in respiratory specimens of CF patients (1, 9). To our knowledge, this report is the first one demonstrating multiple isolations of a *Bordetella* species over an extended period of time from a CF patient, while the role of *B. hinzii* as a respiratory tract pathogen in poultry is not yet established (12). However, it is obvious that the underlying disease in our patient was the predisposition for at least two episodes of infections caused by *B. hinzii*. We had no clues how the acquisition of *B. hinzii* occurred in our patient, since the patient lived in the metropolitan area of Zürich, had traveled only to urban areas throughout Europe, South Africa, and Central America, and denied any close contacts with animals over the years.

The commercial API 20 NFT (NE) system is only capable of differentiating the *B. avium-B. hinzii* complex (as "*B. avium*") from related taxa. However, observation of alkali production from glycine, malonamide, malonate, or valerate (all positive for *B. hinzii* but negative for *B. avium*) may be necessary for final differentiation, as outlined by Vandamme et al. (14). We can support the CFA data for *B. hinzii* given by Cookson et al. (3), although incubation on a sheep blood-containing agar and incubation in a 5% CO_2 atmosphere does not seem to be necessary. Our data from CFA analyses are in contrast to other published data for *B. hinzii* (14) in which, most significantly, $C_{16:1\omega7c}$ could not be detected, but this was one of the major CFAs observed in our strains. This discrepancy may be explained by the fact that in the study of Cookson et al. (3) and in our investigations, cells were harvested after 24 h of growth, whereas Vandamme et al. (14) grew their cells for 48 h; it is known that cells harvested after an extended period of time contain more cyclopropane CFAs (e.g., $C_{17:0\ cyclo}$) but fewer monoenoic acids (e.g., $C_{16:1\omega7c}$), which serve as precursors for the cyclopropane CFAs (16).

Despite appropriate antimicrobial therapy, *B. hinzii* was not eradicated from our patient, a phenomenon which is well known for other nonfermenting gram-negative rods causing infections in the respiratory tracts of CF patients (7). In a recent report, *B. bronchiseptica* was shown to persist over a 2.5-year period in a patient with bronchopneumonia with no underlying condition (8).

Only two reports regarding the antimicrobial susceptibility patterns of *B. hinzii* can be found in the literature (2, 12). These reports demonstrated that *B. hinzii* isolates are uniformly resistant to ampicillin and that most isolates are susceptible to trimethoprim-sulfamethoxazole. Our two isolates were susceptible to all aminoglycosides tested (except tobramycin) as well as to β-lactam antibiotics combined with β-lactamase inhibitors. Most significantly, both strains were resistant to several cephalosporins including cefuroxime, ceftriaxone, and cefotaxime; only strain DMMZ 1281 remained susceptible to cephalothin, cefamandole, and ceftazidime. Resistance to cefuroxime has also been reported for *B. avium* and *B. bronchiseptica* isolates (10).

We acknowledge that this case report cannot entirely clarify the role of *B. hinzii* in human infections but, nevertheless, adds further evidence that *B. hinzii* may cause disease in selected patients.

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