Partial 16S rRNA primary structure of five Actinomyces species: phylogenetic implications and development of an Actinomyces israelii-specific oligonucleotide probe

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The intra- and intergeneric relationships of the genus Actinomyces were determined by comparing long 16S rRNA sequences, generated by reverse transcriptase. All species formed a phylogenetically coherent cluster in which Actinomyces bovis, A. viscosus, A. naeslundii, A. odontolyticus and A. israelii constituted genetically well defined species. A. israelii DSM 43322 (serotype 2) was not closely related to three other strains of this species (serotype 1) and, as judged from phylogenetic distances, could be accommodated within A. naeslundii, or represent a new species. In contrast to previous findings, members of the genus Actinomyces appear to be related to Bifidobacterium bifidum. Sequence information was used to develop an oligonucleotide probe for the A. israelii serotype 1 strains, which did not react with the serotype 2 strain or with rRNA from strains of eight Actinomyces species.

Introduction

In a first attempt to determine the phylogenetic position of the genus Actinomyces, A. bovis and A. viscosus were found to constitute a separate subline of descent within the broad actinomycetes cluster (Stackebrandt & Woese, 1981). In contrast to the traditional taxonomic treatment of Actinomyces, which grouped this genus with Bifidobacterium, Arachnia, Bacterionema and Rothia in the family Actinomyctecaeae (Slack, 1974), Actinomyces and Bifidobacterium were separated to the extent that membership of the same family was excluded. The two Actinomyces species were moderately related to a cluster embracing aerobic organisms which, except for oerskovios, Promicromonospora citrea and Agromyces ramosus were mainly characterized by 'coryneform' morphology, e.g. arthrobacteria, micrococci, brevibacteria, cellulumonas, microbacteria and aureobacteria. In fact, apart from the presence of lysine as a diagnostic amino acid of the peptidoglycan, no common significant chemotaxonomic features in support of the genetic relationship were found.

New techniques allow generation of sequences for large or complete rRNA molecules (Lane et al., 1985) and much improved phylogenetic trees (Olsen et al., 1986). By comparing phylogenetic trees with dendrograms derived from rRNA oligonucleotide catalogues, the limitations of the latter approach became apparent (Woese, 1987). The failure to handle satisfactorily differences in the evolutionary clock, and the smaller sample number and higher sample error of the rRNA catalogues as compared to complete sequences, may cause organisms to branch off at positions which do not necessarily reflect their actual phylogenetic position when catalogs are treated by simple cluster analysis (Woese et al., 1985, 1987; Sneath, 1989). In order to reconfirm the phylogenetic position of Actinomyces, strains of several species were investigated by advanced sequencing methods.

Methods

Organisms. The following strains were obtained from the German Collection of Microorganisms and Tissue Cultures, Braunschweig, FRG: Actinomyces bovis (DSM 43014), A. denticolens (DSM 20671), A. israelii serotype 1 (DSM 43011, DSM 43320, and DSM 43323), A. israelii serotype 2 (DSM 43322), A. naeslundii (DSM 43013), A. odontolyticus (DSM 43331, DSM 43322 and DSM 433760), A. pyogenes (DSM 20594 and DSM 20630), and A. viscosus (DSM 43027, DSM 43329 and DSM 435798). A. kowellei NCTC 11636 was purchased from the National Collection of Type Cultures, Colindale Avenue, London, UK, and A. hordenvalvleri ATCC 35275 and A. meyeri ATCC 35568 were ordered from the American Type Culture Collection, Rockville, Maryland, USA.

Medium and growth conditions. All strains were cultivated in one-litre screw-top flasks at 37 °C in broth with the following composition per litre: glucose, 10 g; tryptone, 10 g; yeast extract, 10 g; 50 ml 8% (w/v)
Na₂CO₃ solution; 75 ml mineral-salt solution [0.6% (NH₄)₂SO₄, 1.2% NaCl, 0.25% MgSO₄, 7H₂O, 0.16% CaCl₂, 2H₂O, 0.6% KH₂PO₄]; 2 ml 0.1% (w/v) resazurin solution; pH adjusted to 7.2. After sterilization, 50 ml 2% (w/v) cysteine/HCl solution was added when the medium had cooled to 60°C.

Isolation of crude RNA. Cells were harvested in the exponential phase of growth and washed twice with sterile NaCl solution (0.8%). Cells (1 g wet weight) were resuspended in 20 ml 1 x SSC (0.15 M-NaCl, 0.01 M-trisodium citrate, pH 8.0) and mixed with 45 g glass beads (0.1-0.11 mm diameter; Braun). Cells were disrupted in a cell homogenizer (Braun) for 60 s, with a cooling step in ice after 30 s. One volume of ice-cold, SSC-saturated phenol was added and the suspension shaken vigorously. After separation of glass beads by centrifugation, the aqueous phase was removed and treated repeatedly with phenol until no interphase material remained. Nucleic acids were precipitated with ethanol (10 h at -20°C); the precipitate was collected by centrifugation, dried in a Speedvac concentrator (Savant) and treated as follows for removal of DNA. Ice-cold sodium acetate (10 ml, 3 M, pH 6.0) was added to the precipitate and homogenized for 10 s using an Ultra Turrax (Jahnke and Kunkel, IKA-Werke, Staufen). The homogenate was centrifuged and the DNA-containing supernatant discarded. Homogenization and centrifugation steps were repeated three times. The purified crude RNA was dissolved in 1-3 ml H₂O, precipitated (10 h at 4°C) and dissolved in 1 ml H₂O. The amount and purity of the RNA were determined spectrophotometrically and the concentration adjusted to 3.5 µg µl⁻¹ with H₂O and frozen at -20°C.

Sequence analysis of 16S rRNA. Sequence analysis followed the protocol of Lane et al. (1985) with modifications as specified below. Primer oligonucleotides were made using a DNA synthesizer (Applied Biosystems, model 381) and the oligomers were purified according to the recommendations of the manufacturer. Primer sequences and positions are listed in Table 1. The hybridization reaction for binding of DNA primers to complementary rRNA stretches consisted of 2 µl RNA (3-5 µg µl⁻¹), 1 µl hybridization buffer (500 mM-KCl, 250 mM-Tris/HCl, pH 8.5), 1 µl primer DNA (100 ng µl⁻¹) and 1 µl H₂O. For denaturation of rRNA the assay was treated for 2 min at 90°C and slowly cooled to 30°C over a period of 30 min.

<table>
<thead>
<tr>
<th>Position of binding*</th>
<th>Sequence of primer</th>
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<tbody>
<tr>
<td>105-124</td>
<td>5CACGTGTTACTCACCACCGTCC 3'</td>
</tr>
<tr>
<td>343-357</td>
<td>5TCCTGCTGCCCTCCGTA 3'</td>
</tr>
<tr>
<td>359-376</td>
<td>5GWATTACCCGGTGGCTG 3'</td>
</tr>
<tr>
<td>691-704</td>
<td>5TCTGCGGATTCACCACG 3'</td>
</tr>
<tr>
<td>786-803</td>
<td>5CATCTSSGGTATCTAATAC 3'</td>
</tr>
<tr>
<td>907-926</td>
<td>5CCGGCTAATCTTTTGATTGTTT 3'</td>
</tr>
<tr>
<td>1056-1073</td>
<td>5ACAGACTGCAGACGRCGCA 3'</td>
</tr>
<tr>
<td>1100-1115</td>
<td>5AGGGTTGGTGCTGGTG 3'</td>
</tr>
<tr>
<td>1224-1242</td>
<td>5CATTTGATCGATCAGTGAA 3'</td>
</tr>
<tr>
<td>1262-1401</td>
<td>5CGCTGTGTTGCAAGGGC 3'</td>
</tr>
<tr>
<td>1492-1510</td>
<td>5GGTATCCCTGTTGACGT 3'</td>
</tr>
</tbody>
</table>

* Nucleotide positions according to the E. coli numbering system (Brosius et al., 1978).

Table 1. Sequences of primers used and positions to which they bind in the 16S rRNA primary structure

Five microclones of each of the primer assays were incubated in an ice bath with 5 µl reverse transcriptase buffer (250 mM-Tris/HCl pH 8.3, 250 mM-KCl, 50 mM-dithiothreitol and 50 mM-MgCl₂), 5 µl Tris/HCl, pH 8.3, containing 7 U reverse transcriptase (AMV) (25000 U ml⁻¹; Boehringer Mannheim) and 1 µl (10 µCi) deoxyadenosine 5'-α-[³²P]deoxythriphosphosphate ([³²P]dATP). A sample (3 µl) of each assay was mixed with 2 µl of each of the four deoxyNTP/α-deoxyNTP solutions with the following composition: A-mix = 55 µl 0.005 mM-dATP, 25 µl each of 10 mM-dCTP, dGTP and dTTP, 10 µl 0.01 M-ddATP; C-mix = 45 µl 1 mM-dCTP, 25 µl each of 10 mM-dGTP and dTTP, 25 µl 0.005 mM-dATP and 10 µl 1 mM-ddCTP; G-mix = 50 µl 1 mM-dGTP, 25 µl each of 10 mM-dCTP and dTTP, 25 µl 0.005 mM-dATP and 20 µl 1 mM-ddGTP; T-mix = 40 µl 1 mM-dTTP, 25 µl each of 10 mM-dCTP and dGTP, 25 µl 0.005 mM-dATP, and 25 µl 1 mM-ddTTP.

After incubation for 30 min at 37°C, 2 µl of a chase-mix reagent was added (10 µl each of 1 mM-dATP, 10 mM-dCTP, 10 mM-dGTP, 10 mM-ddTTP and 150 µl 10 mM-Tris/HCl, pH 8.3) and incubated for an additional 15 min at 43°C. The assays were boiled for 60 s, placed on ice for 10 min, mixed with 32 µl of a solution consisting of 16 µl chase-mix reagent containing dNTPs (1 mM each in 10 mM-Tris/HCl, pH 8.3), 25 µl cetyltrimethyl ammonium (α-c-tsa) adjusted to pH 7.5 with 20% (w/v) KOH, 5 µl CoCl₂, 0.1 µl dithiothreitol (10 mM), 1.5 µl terminal deoxynucleotidyl transferase (25 U µl⁻¹; Boehringer Mannheim) and 88 µl H₂O, and incubated for 90 min at 37°C (Deborde et al., 1986). Sodium acetate (100 µl, 0.1 M) and 350 µl ethanol were added to each assay and kept at -20°C for 10 h. After centrifugation, the precipitate was washed with 1 ml 70% (v/v) ethanol, dried in a Speedvac concentrator and redisolved in 10 µl of a solution consisting of (per ml) 94 µl formamide, 20 µl bromophenol blue, 20 µl Sigma brilliant blue and 20 µl xylene cyanol.

Gel electrophoresis. DNA fragments were separated on a 0.2 mm-0.6 mm wedge denaturing polyacrylamide gel (Chen & Seeburg, 1985) using an LKB Macrophore system at 60 W. Two gels were prepared for each primer assay, one for separation of small fragments (2.5 h), and a second for separation of fragments of about 300 nucleotides (5 h). Urea was removed by washing the gels for 20 min each in 5% (v/v) acetic acid, 5% acetic acid/glycerol solution and H₂O. Gels were dried in a vacuum oven for 10 h at 80°C Autoradiography was for 3 to 7 d.

Analysis of data. Sequences were aligned and homologies determined with the aid of the Microgenie program (Beckman) (Queen & Korn, 1984). Positions whose nucleotide composition could not be determined unambiguously (recorded N) were removed prior to the calculation, leaving a blank position. Homology values were transformed into evolutionary distance values (K₁₈; Hori & Osawa, 1979), and phylogenetic trees were calculated using the Fitch & Margoliash (1967) algorithm contained in a Phylip 2 version for the IBM PC (Felsenstein, 1982).

Oligonucleotide probe synthesis and hybridization conditions. A 20-mer oligonucleotide complementary to a highly variable stretch of the 16S rRNA from three A. israelii strains was synthesized as described above. 5' in vitro labelling with [³²P]dATP and purification of labelled oligonucleotides were described by Krupp & Gross (1983). The dot-blot apparatus (Minifold SRC 96, Schleicher & Schüll) was treated with the RNAase inhibitor diethylpyrocarbonate (Sigma) (0.1%) for several hours and rinsed carefully with H₂O. A Hybond N membrane filter (Amersham) was adjusted and each blot position moistened with 100 µl 0.1 x SSC. Cool RNA (25 ng) was dissolved in 100 µl 0.1 x SSC, denatured by boiling and applied to the filter. After rinsing each blot with 100 µl 0.1 x SSC, the filter was removed, placed on Whatman MM paper and allowed to dry at room temperature for 30 min. UV treatment (254 nm for 10 min) was as recommended by the Amersham.
Results and Discussion

Phylogeny

The sequenced portions of the 16S rRNA primary structures of *A. boryi* DSM 43014, *A. israelii* DSM 43320, *A. naeslundii* DSM 43013, *A. odontolyticus* DSM 43331 and *A. viscosus* DSM 43027 are shown in Fig. 1. Except for a few positions, sequences of the *A. israelii* strains DSM 43011 and DSM 43323 were identical to that of strain DSM 43320 (not shown). All belong to serotype 1. Also included is a shorter sequence of a serotype 2 strain (*A. israelii* DSM 43322). This strain was later included in the analysis when an *A. israelii* DSM 43011-specific DNA probe failed to give a hybridization signal with rRNA from strain DSM 43322 (see below).

Three different data sets were individually aligned, homologies calculated and trees generated because of incompleteness of 16S rRNA sequences obtained from *Actinomyces* spp. and non-*Actinomyces* reference strains. The most complete data set is based on 1151 nucleotides (position 97–511 and 642–1379, according to the *Escherichia coli* numbering system; Brosius et al., 1978). This set was used to produce the tree depicted in Fig. 2. For the inclusion of *A. israelii* DSM 43322, the second set is restricted to 624 positions taken from the 5’ and middle parts of the molecule (97–335, 415–511 and 675–965). The patterns of the two trees derived from the two data sets were identical with respect to the branching of those organisms included in both kinds of analyses. The third set is based on the analysis of a continuous stretch of the 16S rRNA from four species, ranging from position 194 to 1445 (according to the *E. coli* numbering system; Brosius et al., 1978), in which all positions shown to be invariant in the sequences of more than 90 actinomycete strains from 25 genera were omitted (Hahn et al., 1989). This set allowed placement of the genus *Actinomyces* within the phylogenetic tree of the order *Actinomyces* and related taxa (Fig. 3).

Table 2. Homology values among partial 16S rRNA sequences of *Actinomyces* spp. based on the analysis of 1165 nucleotides

<table>
<thead>
<tr>
<th>Strain 1 (100%)</th>
<th>2.</th>
<th>3.</th>
<th>4.</th>
<th>5.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. boryi</em> DSM 43014</td>
<td>0.724</td>
<td>0.560</td>
<td>0.947</td>
<td>0.773</td>
</tr>
<tr>
<td><em>A. naeslundii</em> DSM 43013</td>
<td>0.475</td>
<td>0.625</td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td><em>A. viscosus</em> DSM 43027</td>
<td>0.678</td>
<td>0.108</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. israelii</em> DSM 43320</td>
<td>0.1661</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. odontolyticus</em> DSM 43331</td>
<td>0.876</td>
<td>0.897</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The lower left triangle shows homology values ($\%$); the upper right triangle shows evolutionary distance values ($K_{nav}$), derived from homology values.

The tree in Fig. 2 was constructed by a distance matrix method (Felsenstein, 1982) that optimizes branch length. The branching pattern of *A. naeslundii* and *A. viscosus* illustrates the fact that in the presence of varying evolutionary rates, species with the highest nucleic acid sequence similarity are not necessarily the most closely related ones. A numerical phenetic analysis that assumes a constant evolutionary rate, e.g. unweighted pair group matrix analysis (UPGMA), would in fact cluster these two species as neighbours. The high degree of relatedness between members of *A. viscosus* and *A. naeslundii* has been recognized previously in serological (Collins et al., 1973; Holmberg & Forsum 1973; Gerencser & Slack, 1967) and numerical taxonomic (Holmberg & Nord, 1975; Fillery et al., 1978; Schoefeld & Schaaf, 1981) studies. Schoefeld & Schaaf (1981) confirmed that these were independent, although closely related, species; Coykendall & Munzenmaier (1979) reached the same conclusion in a study of DNA reassociation values.

The other three *Actinomyces* species are also well separated from each other, and this supports the results of numerical phenetic analyses (Melville, 1965; Holmberg & Hallander, 1973; Homberg & Nord, 1975; Slack & Gerencser, 1975; Fillery et al., 1978; Schaaf & Schoefeld, 1981; Schoefeld & Schaaf, 1981). The phylogenetic proximity of the species, together with the phylogenetic coherence of the genus, do not support suggestions to separate *Actinomyces* into two or three genera, as discussed by Schaaf & Schoefeld (1981) and Schaaf & Gatzer (1985).

Two of the three serotype 1 *A. israelii* strains (the type strain DSM 43320 = ATCC 12102 and DSM 43011 = ATCC 10048), which cannot be distinguished by 16S rRNA primary structure, were also
investigated by Schofield & Schaal (1981) and recovered as members of a single cluster, although located in the neighbouring subclusters 1b and 1a, respectively. No comparative phenotypic data are available for *A. israelii* DSM 43322 (ATCC 29322). As derived from the data set 2 (not shown), this serotype 2 strain shows only slightly higher 16S rRNA homology (95-1% to the three serotype 1 *A. israelii* strains than those found for serotype 1 and 2 strains and *A. naeslundii* (94-1% and 93-8%, respectively). At this stage, with no information available on the phylogenetic depth of the individual *Actinomyces* species and without comparative phenotypic characterization based on many *A. israelii* strains, it is not possible to decide whether strain DSM 43322 is a member of *A. israelii, A. naeslundii*, or even representative of a new *Actinomyces* species.

Fig. 3 depicts the position of the genus *Actinomyces* within the phylogenetic confines of Gram-positive eubacteria with a high DNA G+C content. All of the species investigated form a homogeneous cluster exhibiting no close relationship to any of the reference taxa. Based on data set 3 (homology values not shown in detail) the intrageneric homology values range from 81-0 to 89-6, while the mean value for *Actinomyces* spp. and other actinomycetes, bifidobacteria and propionibacteria is only 73-0. The branching pattern implies that *Actinomyces* and *Bifidobacterium* (represented by *B. bifidum*; R. Hensieck & E. Stackebrandt, unpublished) share a common ancestry. This is in contrast to earlier reports on the phylogeny of the genus *Actinomyces* (Stackebrandt & Woese, 1981; Stackebrandt, 1985) where *A. bovis* and *A. viscosus* clustered with aerobic, mainly non-mycelial actinomycetes. However, phylogenetic trees are dynamic, their topography changing when a more balanced selection of members of different genera and outside reference organisms is used. The majority of reference strains used in this study had not been analysed by the previous cataloguing approach and many of the organisms compared to *Actinomyces* spp. by 16S rRNA cataloguing have not been reinvestigated by the reverse transcriptase sequencing method. It is therefore too early to define the phylogenetic neighbour of *Actinomyces* more precisely. However, in contrast to the earlier report on the phylogenetic position, the relatively close relationship between *Actinomyces* and *Bifidobacterium* is made
more convincing by their common metabolic and chemotaxonomic properties, e.g. major fermentation end products, peptidoglycan structure and mol% G + C of their DNA.

**Diagnostic DNA probes**

A 16S rRNA stretch unique for serotype 1 strains of *A. israelii* was found in the highly variable region around position 200. As compared to *A. naeslundii*, the sequences of *A. bovis*, *A. viscosus* and *A. odontolyticus* have a gap of 13 to 15 nucleotides while the three *A. israelii* strains (DSM 43320, DSM 43011 and DSM 43322) lack only three nucleotides. The region 5'UCACUUUUGUG-GUGUUUUGG 3' (Fig. 1) was selected as the target for a 20-mer probe of the sequence 5'CACCAACACCA-CAAAAGTGA 3'. Sequence homologies of the respective rRNA stretch between the three *A. israelii* strains and the other strains ranged from 25% (A. viscosus) to 67% (*A. naeslundii*). The specificity was tested against isolated crude rRNA from 34 strains of various actinomycete genera [i.e. *Propionibacterium*, including *P. (Arachnia) propionicus*, *Bifidobacterium*, *Streptomyces*, *Streptowerictillium*, *Nocardioidei*, *Terra bacter*, *Tsukamurella*, *Nocardiosis*, *Corynebacterium*, *Nocardia*, *Dermatophilus*, *Geodermatophilus* and *Frankia*] and against 15 strains of nine *Actinomyces* species including those for which no sequence data are available, i.e. *A. odontolyticus* (DSM 43329 and DSM 43760), *A. viscosus* (DSM 43329 and DSM 43798), *A. pyogenes* (DSM 20594 and DSM 20630), *A. denticolens* DSM 20671, *A. meyeri* ATCC 35568, *A. howelli* NCTC 11636 and *A. hordeovulnaris* ATCC 35275. A 'universal' probe, directed against a highly conserved region (691–704) served as a positive control for available 16S rRNA. Using the *A. israelii* probe, positive hybridization signals were only obtained with rRNA of the three *A. israelii* strains whose rRNA sequence information was originally used for the development of the probe. Surprisingly, rRNA isolated from *A. israelii* DSM 43322 did not hybridize to this probe. Sequence analysis of the respective region (Table 1) revealed that only 80% homology exists between the target sequences of the two *A. israelii* sequences, and this explains the failure of the probe to bind.

As discussed above, phylogenetic analysis does not necessarily support the identification of strain DSM 43322 as *A. israelii*, and extensive comparative phenotypic characterization is needed to solve this problem. The DNA probe developed to detect certain *A. israelii* strains of serotype 1 should now be evaluated in a broad survey that includes a large number of isolates, to confirm the specificity required for routine use in clinical diagnosis. The high degree of sequence variation in regions around positions 460, 840 and 1020 (Fig. 1) indicates that oligonucleotide probes can potentially be synthesized for all other strains as well.

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**References**


Phylogeny of Actinomyces


