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Microbiological and immunological characteristics of young Moroccan patients with aggressive periodontitis with and without detectable *Aggregatibacter actinomycetemcomitans* JP2 infection

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SUMMARY

Cross-sectional and longitudinal studies identify the JP2 clone of *Aggregatibacter actinomycetemcomitans* as an aetiological agent of aggressive periodontitis (AgP) in adolescents of northwest African descent. To gain information on why a significant part of Moroccan adolescents show clinical signs of periodontal disease in the absence of this pathogen we performed comprehensive mapping of the subgingival microbiota of eight young Moroccans, four of whom were diagnosed with clinical signs of AgP. The analysis was carried out by sequencing and phylogenetic analysis of a total of 2717 cloned polymerase chain reaction amplicons of the phylogenetically informative 16S ribosomal RNA gene. The analyses revealed a total of 173 bacterial taxa of which 39% were previously undetected. The JP2 clone constituted a minor proportion of the complex subgingival microbiota in patients with active disease. Rather than identifying alternative aetiologies to AgP, the recorded infection history of the subjects combined with remarkably high concentrations of antibodies against the *A. actinomycetemcomitans* leukotoxin suggest that disease activity was terminated in some patients with AgP as a result of elimination of the JP2 clone. This study provides information on the microbial context of the JP2 clone activity in a JP2-susceptible population and suggests that such individuals may develop immunity to AgP.

INTRODUCTION

Aggressive periodontitis (AgP) is a rapidly progressive form of marginal periodontal disease, characterized by severe destruction of the osseous support of the dentition at an early age and a distinctive tendency for cases to aggregate in families (Tonetti & Mombelli, 1999). Although relatively rare among adolescents of European ancestry (prevalence 0.1–0.2%) (Saxen, 1980; Kronauer *et al.*, 1986; Brown *et al.*, 1996), the disease occurs at a prevalence of 2.6% among African-Americans (Löe & Brown, 1991; Brown *et al.*, 1996). More recent

investigations showed a strikingly higher incidence (up to 15%) of localized and generalized AgP among Moroccan adolescents (Haubek *et al.*, 2001) and in selected Israeli populations (5.9%) (Levin *et al.*, 2006). As the disease may result in extensive loss of teeth before the age of 20 it represents a significant health problem.

It is generally accepted that bacterial biofilms formed on tooth surfaces in the subgingival compartment and yet undefined genetic susceptibility determinants of the host are decisive factors in the pathogenesis of AgP (Slots, 1976; Michalowicz *et al.*, 1991; Nibali *et al.*, 2008; de Carvalho *et al.*, 2009). However, the complexity of the bacterial biofilm associated with disease has hampered identification of specific aetiological agents. Several candidates were proposed based on association studies, identification of putative virulence factors, and detection of candidate-reactive antibodies in the serum of affected patients (Slots, 1976; Zambon, 1985; Ebersole *et al.*, 1995; Califano *et al.*, 1997). One additional difficulty related to cross-sectional association studies in particular is that loss of periodontal attachment is, by necessity, taken as sign of active disease, although it may reflect previous rather than ongoing activity. Recent longitudinal investigations of initially healthy Moroccan adolescents provided the yet strongest evidence of a specific and infectious aetiological agent, i.e. a particular clone, termed JP2, of *Aggregatibacter* [formerly *Actinobacillus* (Nørskov-Lauritsen & Kilian, 2006)] *actinomycetemcomitans* which, like traditional pathogens, may be isolated from multiple patients in geographically distinct areas (Haubek *et al.*, 2008). This clone is characterized, among other genetic traits (DiRienzo *et al.*, 1994; Haubek *et al.*, 1996; Hayashida *et al.*, 2002), by enhanced leukotoxin production because of a 530-base-pair (bp) deletion in the promoter region of the leukotoxin gene operon (Brogan *et al.*, 1994). Cross-sectional studies show that it is associated with disease in individuals of African descent, e.g. Moroccans, certain Israeli populations, African-Americans, Brazilians and Jamaicans with high disease prevalence (Haubek *et al.*, 1997; Contreras *et al.*, 2000; Haraszthy *et al.*, 2000), but not in populations of European or Asian ancestry (Haubek *et al.*, 1995; Mombelli *et al.*, 1998; He *et al.*, 1999). Direct proof that the JP2 clone by itself is responsible for AgP is difficult to obtain because the leukotoxin affects human leucocytes only, which limits the relevance of animal models. So far, no information is available on other parts of the subgingival microbiota in patients with AgP in these populations.

Evolutionary reconstruction based on analysis of polymorphic sites in selected genes indicate that the JP2 clone emerged in the Mediterranean part of Africa in a population of Arabs and/or Berbers approximately 2000 years ago and subsequently spread to Africans in the western part of the African continent (Haubek *et al.*, 2007). Apparently, the exclusive tropism for members of these populations has been maintained and the occurrence in North and South America and in Europe of the JP2 clone and associated disease is explained by population movements from northwest Africa rather than by unrestricted dissemination of the clone. No specific aetiological agent of AgP in other ethnic populations has been identified, but *A. actinomycetemcomitans* of a multitude of clonal lineages of different serotypes show association with clinical disease although associated with significantly lower relative risk than the JP2 clone (Zambon, 1985; Slots & Ting, 1999; Haraszthy *et al.*, 2000; Kaplan *et al.*, 2002; Cortelli *et al.*, 2005; Fine *et al.*, 2007). Nevertheless, a recent comprehensive, though not exhaustive, mapping of the microbial diversity in subgingival biofilms from 10 patients with AgP in Brazil demonstrated no association between particular species and disease, and relative proportions of *A. actinomycetemcomitans* were so low that their detection required selective amplification by polymerase chain reaction (PCR) (Faveri *et al.*, 2008).

Despite the strong association of the JP2 clone with AgP in both cross-sectional and longitudinal studies (DiRienzo *et al.*, 1994; Haraszthy *et al.*, 2000; Haubek *et al.*, 2001, 2008), it is striking that approximately 50% of Moroccan adolescents who had developed AgP at the end of a 2-year observation period, did not carry the JP2 clone (Haubek *et al.*, 2008). This finding raises several questions crucial to our understanding of the pathogenesis of AgP. (i) Is sustained infection with the JP2 clone of *A. actinomycetemcomitans* necessary for progression of disease activity or can tissue degradation be induced by only transient infection, which in these cases was missed? (ii) Is there an alternative microbial aetiology of AgP in the Moroccan population that may provide information on the aetiology of AgP in ethnic populations not

infected by the JP2 clone? (iii) Or are some cases clinically diagnosed as AgP in JP2-susceptible populations merely the result of temporary degradation of the periodontium that subsequently stopped as a result of immune elimination or inactivation of the JP2 clone?

This report describes studies designed to test these hypotheses. This was attempted by comprehensive mapping of the complex subgingival microbiota of eight young Moroccans, four of whom were diagnosed with clinical signs of AgP. A total of 2834 cloned PCR amplicons of the phylogenetically informative 16S ribosomal RNA (rRNA) gene generated from subgingival plaque samples were sequenced and analysed by taxonomic clustering principles. In addition, the JP2 infection history spanning a period of 4 years was established by PCR, and further analysed by quantitative determination of serum antibodies to the *A. actinomycetemcomitans* leukotoxin. Our findings demonstrate that the JP2 clone of *A. actinomycetemcomitans*, when present in patients with AgP, constitutes a remarkably small proportion of a highly complex subgingival microbiota of which no other species appears to be specific to patients. The findings further support the hypothesis that, in some patients with AgP, an immune reaction may result in elimination of the JP2 clone and termination of disease activity.

METHODS

Subject population

Eight subjects were selected among young Moroccans recruited from the Department of Periodontology of Rabat University (Morocco) in 2007. These included four subjects with AgP according to the definitions proposed by the American Academy of Periodontology (Armitage, 1999, 1996) and four periodontally healthy subjects. The complete clinical examination included intra-oral examination, full-mouth periodontal probing, and recording of medical and dental histories. None of the patients received any professional dental treatment during the course of the study. The protocol for all procedures was approved by the Ethical Committee for the Medical and Pharmaceutical Faculty of Rabat, Morocco. All the subjects signed the committee-approved informed consent form and, when the subject was <18 years old, the consent was signed by one parent. Four of the eight Moroccans were part of a previously reported longitudinal study from which relevant data were extracted (Haubek *et al.*, 2008).

Inclusion criteria

Subjects with AgP were <24 years old, had >20 teeth present, pocket probing depth >4 mm, and clinical loss of attachment >3 mm at a minimum of two teeth localized at the first molars or incisors. Radiographs were taken to assess the alveolar bone levels and to confirm the diagnosis. The periodontally healthy age-matched control subjects had >20 teeth present, no periodontal pockets >4 mm, and no clinical attachment loss >1 mm at more than one site in the mouth.

Exclusion criteria

Subjects with diabetes mellitus type 1, coronary heart disease, pregnancy, smoking, <20 teeth, antibiotic treatment within 6 months before sampling, or daily intake of medication were excluded from the study.

Clinical examination

The clinical examination was performed by one trained examiner (M.R.). All subjects enrolled were given a full-mouth periodontal examination. Bleeding on probing (1/0), pocket probing depth (mm) and clinical attachment level (mm) were measured at six sites per tooth (mesio-buccal, buccal, disto-buccal, disto-oral, oral and mesio-oral) at all teeth, excluding third molars if present. Pocket depth and clinical attachment level measurements were recorded to the nearest millimetre using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL).

Microbiological sampling

After removal of supragingival plaque with a cotton pellet, a pool of subgingival plaque samples collected with sterile Gracey curettes (11/12) was obtained from eight sites: the mesio-buccal aspect of each first molar and the disto-buccal aspect of each first incisor in both jaws. In the patients with AgP, all these sites showed significant loss of attachment (4–8 mm). The samples were directly suspended in 255 μl lysis buffer (20 mM Tris–HCl, pH 8.0, 2 mM ethylene-diamine

tetraacetic acid, and 1.2% Triton X-100), in which they were transported to the laboratory in Denmark.

Blood sampling

Blood samples were taken from all participants in the study. The samples were taken by a medical laboratory technician in a vacuum tube without anticoagulant. Serum was collected aseptically after centrifugation of the coagulated blood and was transported to the laboratory in Denmark.

DNA extraction

Lysozyme (20 mg ml^{-1}) and 625 U ml^{-1} mutanolysin were added to the samples whereupon they were incubated at 37°C for 90 min. DNA from all samples was extracted by using the DNeasy Blood and Tissue kit from Qiagen (Qiagen, Hilden, Germany). The manufacturer's protocol for genomic DNA isolation (with pre-treatment for gram-positive bacteria) and purification of total DNA (spin-column protocol) was used with minor modifications. Incubation time was prolonged to 1 h at 56°C for the proteinase K degradation whereupon an additional 5 μl proteinase K was added and incubation was continued at 37°C over night. Samples were eluted in 200 μl AE buffer, as described in the DNeasy Blood and Tissue kit.

Selective PCR detection of *A. actinomycetemcomitans*

Polymerase chain reactions were performed in thin-walled tubes with PuReTaq™ Ready-To-Go PCR Beads (Amersham Biosciences, Amersham, UK). Ten microlitres of the purified DNA was added to a reaction mixture (final volume, 25 μl) containing 5 pmol of each primer (forward primer Ltx3: GCC GAC ACC AAA GAC AAA GTC T, and reverse primer Ltx4: GCC CAT AAC CAA GCC ACA TAC) (Poulsen *et al.*, 2003). As reference for the JP2 clone and non-JP2 clones respectively the strains HK921 and HK1605 were used. A sample containing water served as negative control. The amplification comprised 5 min of denaturation at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. Final extension was performed for 8 min at 72°C. Amplification products were analysed by electrophoresis on a 1% agarose gel supplemented with ethidium bromide. The presence of the JP2 clone or non-JP2 clones of *A. actinomycetemcomitans* was determined by distinct amplicon bands corresponding to 686 bp for the JP2 clone and 1216 bp for the non-JP2 clone.

PCR amplification of 16S rRNA

An internal fragment corresponding to 1460 bp of the 16S rRNA gene of *Escherichia coli* was amplified by universal bacterial primers 8F (forward primer: 5′-AGA GTT TGA T(C/T)(A/C) TGG CTC AG-3′) and 1492R (reverse primer: 5′-GG(C/T) TAC CTT GTT ACG ACT T-3′) as described Bek-Thomsen *et al.* (2008). The PCR amplification reactions were carried out in a total volume of 25 μl containing 1 μl extracted template DNA (estimated amount, 1–5 ng), 1.2 μM of each primer, molecular-biology-grade water, and Hot Master mix as recommended by the manufacturer (Eppendorf AG, Hamburg, Germany). The amplification comprised 5 min of incubation at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. Final extension was performed for 10 min at 72°C. Amplification products were visualized by electrophoresis on a 1% agarose gel supplemented with ethidium bromide. To eliminate contaminating bacterial genomic DNA, all reagents used for the samples were tested for contamination by PCR amplification of a negative extraction control. For all PCRs, a negative control that included only the PCR reagents was examined in parallel by using procedures identical to those used for the plaque samples. For none of these preparations was a visible band identified after electrophoresis and ethidium bromide staining.

Cloning procedures

The PCR amplification products were ligated directly into the pCR® 4-TOPO® cloning vector (Invitrogen, Carlsbad, CA) and cloned into One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen). Cloning and transformation reactions were performed according to the manufacturer's instructions. The transformed cells were then plated onto 2 × TY agar plates supplemented with 100 μg μl^{-1} ampicillin and incubated overnight at 37°C. Putative positive clones were screened for the presence of an insert of the

correct size by vector-target PCR with universal primers M13Forward/M13Reverse followed by electrophoresis on a 1% agarose gel for visualization.

Sequencing and sequence analysis

Plasmid purification and sequencing were performed at Beijing Genomics Institute (Beijing, China). A total of 2834 cloned 16S rRNA gene sequences from the eight libraries were determined with M13F and M13R primers. A sequence of approximately 800–900 bp was obtained from both ends of the amplicon inserts. Vector trimming and primer removal were performed manually. Alignment of sequences from each of the individuals was performed with CLUSTALX as part of the MEGA 4.0 software (Tamura *et al.*, 2007). Chimeric sequences were identified with the CHIMERA CHECK program of the RDP II database (http://wdcm.nig.ac.jp/RDP/cgis/chimera.cgi?su=SSU) (Cole *et al.*, 2003). A total of 116 sequences identified as chimeric were excluded from further analysis. Validated sequences trimmed to approximately 500 bases spanning the positions 28 to 525 in the *E. coli* 16S rRNA gene were initially used to determine identity and approximate phylogenetic position. The sequences were grouped into clusters of total identity using the FASTGROUPII program, accessible at http://biome.sdsu.edu/fastgroup/fg_tools.htm. The phylogenetic associations of representative sequences of each cluster were then determined with the help of the SEQUENCE MATCH program, available at the RDP II and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases by using the standard nucleotide–nucleotide BLAST program to examine their similarity to designated type strains. All identifications were further checked in the Human Oral Microbiome Database (http://www.homd.org). Sequences that showed total identity to sequences of type strains of recognized species or to sequences of non-cultivated bacteria were identified accordingly after verification by phylogenetic analysis. Sequences without identity to reference strains of recognized taxa were classified to taxonomic position and rank according to phylogenetic analysis. Phylogenetic trees for each major taxonomic unit, which included all relevant available sequences in GenBank, were constructed by the minimum evolution algorithm in MEGA 4.0 (Tamura *et al.*, 2007). Final identification was according to closest neighbour in distinct clusters supported by a bootstrap value of 75 or higher (500 re-samplings) corresponding to a statistical significance level of more than 95%. Assignment to a recognized species required a minimum of 98% identity within the relevant cluster containing a type strain calculated with the maximum composite likelihood method algorithm in MEGA 4.0.

The taxonomic assignment of previously undetected taxa was validated by determination of near complete 16S rRNA gene sequences. These sequences were deposited in GenBank under accession numbers shown in Table S1.

Coverage analysis

The rarefaction curve and richness estimates were calculated using the ESTIMATES program version 8.0 available at http://viceroy.eeb.uconn.edu/estimates. The sample-based Mao Tau method was used to compute the rarefaction curve. The bias-corrected Chao1 estimator of species richness was calculated after 100 randomizations of sampling without replacement.

Quantification of antibodies to leukotoxin of *A. actinomycetemcomitans*

Antibodies to purified leukotoxin in sera collected from the eight subjects from Morocco were quantified by an enzyme-linked immunosorbent assay (ELISA) calibrated for estimation of antibodies in gravimetric units ($\mu g\ ml^{-1}$). Sera from an additional five young Moroccans and three Moroccan patients with AgP, and from three Danish patients with AgP and three healthy controls were used as reference. The protocols for all procedures were approved by the Ethics Committee of the County of Aarhus, Denmark, (and Ethical Committee of Rabat University, Morocco) and all the subjects signed the committee-approved informed consent form. Polystyrene 96-well microplates (Nunc, Roskilde, Denmark; code 269620) were coated overnight with 100 μl purified leukotoxin, 2 $\mu g\ ml^{-1}$, in coating buffer (20 mM Tris–HCl, pH 7.0, 250 mM NaCl, 0.2 mM $CaCl_2$), except for two rows of wells on each plate, which were coated with rabbit antibodies to human immunoglobulin G (IgG) (heavy chain specific) (DAKO, Glostrup, Denmark; code A423), and non-immune rabbit immunoglobulin (DAKO; code X0936), respectively. The leukotoxin used for coating was lipopolysaccharide-free as demonstrated with a probe of labelled polymyxin B

(J. Reinholdt, unpublished data). After washing and blocking of the plates with phosphate-buffered saline containing 0.15% Tween (PBST), test sera were added to leukotoxin-coated wells in serial two-fold dilutions in PBST, range 100–1.024×10^5-fold. Wells coated with antibodies to IgG (or non-immune rabbit immunoglobulin as control) were included to generate a standard curve and received serial two-fold dilutions (range 5×10^3–5.12×10^6 fold) of an international serum immunoglobulin standard with a nominal IgG concentration of 11.0 mg ml^{-1} (DAKO; code X908). After incubation for 2 h, all wells were washed and then received peroxidase-conjugated rabbit antibodies to human IgG (DAKO; code P214) diluted in PBST as suggested for ELISA by the manufacturer. Upon incubation for 2 h, wells were washed and then developed with *O*-phenylenediamine-H_2O_2 chromogenic substrate in citrate–phosphate buffer, pH 5. The staining reaction was stopped after approximately 10 min by the addition of 150 μl 1 M H_2SO_4. Plates were kept at room temperature throughout the assay procedure.

Optical densities (OD) at 492 nm were read with a PC-operated Multiscan RC ELISA reader (Labsystems, Helsinki, Finland). Titration curves for standard and test samples were fitted from a four parameter logistic model using the GENESIS LITE software package (Labsystems). Antibody concentrations for test samples were calculated as the mean of three determinations based on OD readings corresponding to the log-linear part of the standard curve. Generally, the coefficient of variation for the three determinations was below 10%, testifying to the parallelism of titration curves for samples and standard.

As a control, anti-leukotoxin antibodies in one of the sera were quantified also by an alternative protocol involving dissociation of antibodies bound in leukotoxin-coated wells followed by transfer and quantification as IgG in another ELISA relative to an IgG standard. In this assay, serial two-fold dilutions of the test serum were incubated in duplicate rows of leukotoxin-coated wells as described above. After being washed, the wells of one row were incubated with 100 μl 0.1 M glycine–HCl, pH 2.5, for 3 min to dissociate antibodies. The 100 μl of dissociated antibodies in each of these wells was then neutralized by being transferred to new wells supplied in advance with 50 μl 0.5 M Tris, pH 8.0, and 0.45% Tween 20 to prevent binding of protein. The row of wells from which the antibodies had been dissociated was immediately washed and incubated with peroxidase-conjugated rabbit antibodies to human IgG in parallel with the row of duplicate wells in which the antibodies had not been dissociated. By subsequent staining and comparison of OD readings between these rows, the percentage of antibody molecules dissociated by the glycine–HCl treatment could be estimated. The dissociated IgG in two neutralized samples representing sub-saturating concentrations of serum in the original wells was quantified by titration in IgG-specific ELISA along with a serum IgG standard. This ELISA was identical to that used for generation of the standard curve in the previous assay.

Table 1 Clinical characteristics in 2007 of the eight young Moroccans included in the study

				Mean (mm; full mouth)		Number of sites with PPD mm				Mean at sample site (mm)	
Subjects	Age (years)	Sex	BOP (%)	PPD	CAL	≤3	4	5–6	≥7	PPD	CAL
05 AgP	23	F	60	3.9	3.9	104	14	26	24	4.8	4.6
10 AgP	15	M	52	2.4	2.3	145	11	8	4	4.6	4.5
07 AgP	17	F	65	2.9	2.6	117	19	28	4	4.5	4.1
08 AgP	16	M	35	2.1	1.9	158	8	2	0	3.3	3.5
03 C	16	M	37	1.7	1.4	152	0	0	0	1.8	1.5
09 C	16	F	33	1.8	1.6	163	5	0	0	2.1	1.9
06 C	15	F	26	1.8	1.7	168	0	0	0	2.1	1.9
13 C	23	F	52	1.9	1.6	141	3	0	0	1.8	1.6

AgP, aggressive periodontitis; C, healthy control; F, female; M, male; BOP, bleeding on probing; PPD, pocket probing depth; CAL, clinical attachment level.

RESULTS

Clinical data

Demographic characteristics and clinical parameters including pocket probing depth measurements of the eight Moroccan subjects are presented in Table 1. The mean age of the patients with AgP was 17.8 years (range 15–23) and of the healthy control subjects 17.5 years (range 16–23). The mean pocket probing depth and mean clinical attachment level of the eight sampled sites in AgP subjects compared with healthy control subjects differed significantly (unpaired Student's *t*-test, $P < 0.05$; 95% CI, 1.236–3.464 and $P < 0.05$, 95% CI, 1.591–3.309, respectively).

Selective detection of *A. actinomycetemcomitans*

Aggregatibacter actinomycetemcomitans was detected in four of the eight subjects by PCR (Table 2) corresponding to the outcome for this species in the phylogenetic analysis (Table S1). The JP2 clone was found in two of the four AgP subjects whereas the two other subjects diagnosed with AgP in 2007 were not detectably infected with *A. actinomycetemcomitans*. Non-JP2 clones were found in two of the four healthy control subjects. These findings were validated by the size of *ltx* promoter region amplicons corresponding to 686 bp (JP2) and 1216 bp (non-JP2), respectively, as determined by agarose gel electrophoresis.

Table 2 Periodontal status, presence of *Aggregatibacter actinomycetemcomitans*, and concentrations of antibody to leukotoxin in eight subjects included in the study

	Periodontal status			Presence of *A. actinomycetemcomitans*						
				2003		2005		2007		
Subject	2003	2005	2007	Non JP2	JP2	Non JP2	JP2	Non JP2	JP2	Antibody to leukotoxin (μg/ml)
05	ND	ND	AgP	ND	ND	ND	ND	–	+	102.0
10	Healthy	AgP	AgP	–	+	–	+	–	+	58.2
07	ND	ND	AgP	ND	ND	ND	ND	–	–	13.7
08	Healthy	AgP	AgP	–	+	–	+	–	–	6.2
03C	Healthy	AgP	Healthy	+	+	–	–	+	–	15.3
09C	Healthy	AgP	Healthy	+	+	+	–	+	–	20.2
06C	ND	ND	Healthy	ND	ND	ND	ND	–	–	16.9
13C	ND	ND	Healthy	ND	ND	ND	ND	–	–	18.3

ND, no data; AgP, aggressive periodontitis.

Microbiota profile

A total of 2834 cloned 16S rRNA gene sequences were obtained from the gene libraries generated from samples collected from the eight diseased and healthy subjects. Exclusion of truncated sequences and chimeras resulted in a total of 2717 validated sequences. The number of valid sequences per sample ranged from 286 to 398 (median 340). Analysis of a sequence stretch corresponding to nucleotide positions 28 to 525 in the 16S rRNA gene of *E. coli*, which is the phylogenetically most informative part of the gene, was used to determine identity or an approximate phylogenetic affiliation. The analyses revealed a total of 173 bacterial taxa detected in the eight samples, the number per subject ranging between 24 and 71. A complete list of the taxa, arranged according to phyla, in each of the eight individuals is shown in Table S1. Sixty-eight of these 173 taxa (39%) were not previously described and were designated as clones, genomospecies or genomogenus based on their phylogenetic position in the trees and the level at which they were linked to other clusters supported by a significant bootstrap value.

The detected taxa belonged to eight bacterial phyla: TM7, *Proteobacteria, Firmicutes, Synergistetes* according to Jumas-Bilak *et al.* (2009), *Actinobacteria, Spirochaetes, Bacteroidetes* and *Fusobacteria*. The relative proportions of sequences assigned to each of the eight phyla in each of the eight individuals are shown in Fig. 1. No statistically significant difference in phylum proportions was observed between the diseased and healthy subjects.

The as yet uncultured TM7 phylum was represented by 3–21.2% (median 10.9%) of the cloned sequences. The sequences representing this phylum were divided into nine phylogenetic clusters each supported by significant bootstrap values and two unclustered phylotypes represented by a single sequence (Fig. S1). According to a cut-off value of 0.03, each of the nine clusters correspond to phylotypes as defined in most other studies using this technique. Eight of the nine clusters included previously detected phylotypes that are highlighted in the figure. The previously undetected cluster E was

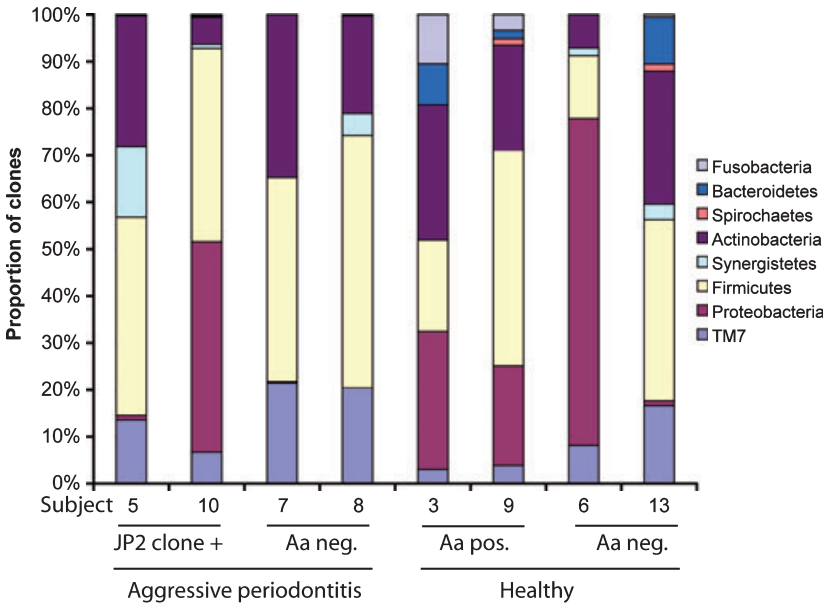

Figure 1 Distribution by phylum of the 2717 sequenced 16S ribosomal DNA clones. Abbreviation: AgP, aggressive periodontitis; Aa, *A. actinomycetemcomitans*; pos., positive; neg., negative.

represented by 31 sequences distributed in 17 sequence variants. Phylotype A was most dominant and accounted for 2.9–20.6% of the sequences in the subjects with aggressive periodontitis and 1.5–5.9% of the sequences cloned from healthy subjects (not significantly different, NS). Particularly high levels of phylotype A members (20.6 and 10.8%) were found in the two patients with AgP who did not carry *A. actinomycetemcomitans* JP2. The position of the three new phylotypes (phylotypes E and two singletons) is shown in Fig. S1.

Members of the phylum *Proteobacteria* were detected primarily in four of the individuals, and in interesting patterns. In agreement with results of the specific detection by PCR the JP2 clone of *A. actinomycetemcomitans* was detected in two of the patients with AgP, constituting 1 and 6.3% of the sequenced clones, respectively. Two healthy subjects (subjects no. 03 and 09), who carried non-JP2 clones of *A. actinomycetemcomitans* (0.6 and 1.5% of sequences) as verified by PCR, were the only ones who carried other fastidious members of the phylum Proteobacteria such as taxa of the family *Neisseriaceae* (6.6% and 8.4%), i.e. *Eikenella corrodens*, *Aggregatibacter aphrophilus* and species of *Neisseria*, *Kingella* and *Cardiobacterium.* Interestingly, the same two individuals carried high proportions (15.3 and 9.8%) of the beta-*Proteobacteria* species *Lautropia mirabilis*, and were also the only ones who were colonized with *Capnocytophaga* spp. (Table S1). The two other healthy individuals and two of the patients with AgP carried bacteria of the family *Enterobacteriaceae*. In two individuals, one diseased and one healthy, *Enterobacter* spp. constituted a surprisingly high proportion of the microbiota (38.1 and 69.4%, respectively).

Bacteria of the phylum *Firmicutes* were detected in all eight individuals and were represented by multiple taxa belonging to four orders. These included *Solobacterium moorei*, which was present in all four patients with AgP and in one of the four healthy subjects, species of *Gemella*, *Abiotrophia*, *Granulicatella*, *Lactobacillus*, *Streptococcus*, *Parvimonas micra* and species of *Peptostreptococcus*, *Mogibacterium*, *Eubacterium*, *Johnsonella*, *Oribacterium*, *Veillonella*, *Selenomonas*, *Dialister* and multiple distinct yet uncultivated lineages of the family *Lachnospiraceae* (Table S1). The genus *Streptococcus* constituted between 2.8 and 23% (median 18.1%) of the sequenced clones generated from the eight individuals. The lowest proportions (2.8 and 5.4%) were seen in the two subjects (one a patient with AgP and one a healthy individual) who carried high proportions of *Enterobacter* spp.

The family *Lachnospiraceae* of the phylum *Firmicutes* was present in higher proportions in subjects

with attachment loss (3.9–14.5%) than in healthy subjects (0.9–3.6%). This difference was statistically significant (unpaired Student's *t*-test, $P = 0.0380$, 95% CI 0.5–12.9). Particularly high proportions (14.5 and 10.3%) were found among sequenced clones generated from the two patients with AgP who did not carry the JP2 clone.

The family *Actinomycetaceae* of the phylum *Actinobacteria* accounted for 2.8–27.8% (median, 15.0%) of the sequenced clones. The proportions of species from the families *Spirochaetaceae, Prevotellaceae, Flavobacteriaceae*, and the order *Fusobacteriales* were generally low, except for one subject, where members of the order *Fusobacteriales* accounted for 10.5% of the clones sequenced (Table S1).

The majority of taxa constituted a minor proportion of the microbiota, when evaluated by the number of sequences representing the taxa. Single taxa that accounted for more than 10% of sequences in one or more individuals included two different phylum TM7 taxa, i.e. phylotype A (in two patients) and phylotype C (in one healthy individual), *Lautropia mirabilis* (in two healthy individuals), *Enterobacter cloacae* and *Enterobacter* uncultured genomospecies 10_2_E12 (in both diseased and healthy subjects), *Streptococcus gordonii* and *Streptococcus sanguinis* (both in healthy individuals), *Streptococcus intermedius* and *Streptococcus anginosus* (both in patients with AgP), *Veillonella parvula* (in one healthy individual) and *Atopobium rimae* (in one patient with AgP).

No single taxon, except for the JP2 clone of *A. actinomycetemcomitans* and taxa detected only in a single individual, was exclusively detected in patients with AgP. However, *Solobacterium moorei*, which has been associated with halitosis (Haraszthy *et al.*, 2007), was detected in all four patients and only in a single healthy individual. In contrast, several taxa were exclusively detected in healthy individuals. Such taxa, detected in more than a single healthy individual, included *Lautropia mirabilis*, *Eikenella corrodens*, species of *Neisseria*, *Campylobacter gracilis*, *Rothia aeria*, *Corynebacterium matruchotii*, species of *Capnocytophaga*, *Fusobacterium* and *Leptotrichia*.

Estimation of Species Level Operative Taxonomic Units richness

The total number of taxa present in the subgingival microbiota of the healthy subjects and patients with AgP, respectively, was estimated from the non-parametric estimator of total richness, Chao1 (Fig. 2A,B). As expected, on the basis of the numerous species represented by a single clone (Table S1), the number of species present in the subgingival samples was not exhaustively revealed by the number of sequences analysed. On the basis of these calculations, the combined bacterial communities in the samples from the healthy subjects contained approximately 143 taxa (95% CI 133–170) and from the diseased subjects contained approximately 147 taxa (95% CI 130–187). Hence, the 127 taxa detected in the healthy subjects (95% CI 119 – 134) represent 88.8% (95% CI 71.6–91.5%) and 117 taxa detected in the diseased subjects (95% CI 107–126) represents 79.6% (95% CI 67–82%) of the estimated number of species present in the subgingival microbiota from healthy subjects and those with

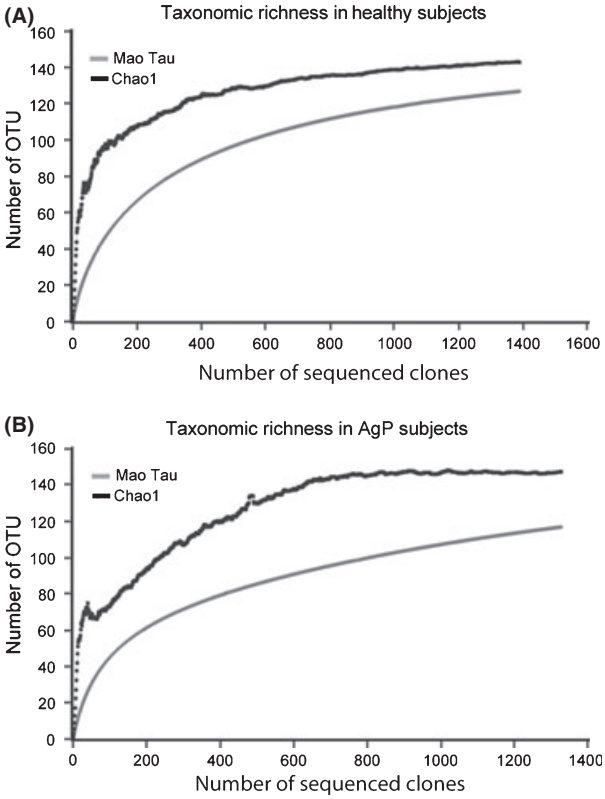

Figure 2 Rarefaction analysis of the bacterial 16S ribosomal RNA gene library of clones obtained from subgingival plaque of the healthy subjects (A) and the patients with aggressive periodontitis (B), respectively. The rarefaction curve, which plots the number of observed operative taxonomic units (Mao Tau) as a function of the number of clones, was computed using the EstimateS program, together with the corrected Chao1 estimator for species richness.

AgP, respectively. Extrapolation of the Mao Tau graph in Fig. 2 indicates that sequencing of an additional approximately 1500 clones would be required to obtain a near complete picture of the remaining taxa, which were all present in portions below 0.3%.

Infection and disease history of the subjects

Longitudinal observations with regard to clinical status and colonization with *A. actinomycetemcomitans* of the JP2 clone or non-JP2 clones, were available for four of the subjects included in the study. These data, summarized in Table 2, show that one of the patients (subject 10) diagnosed with AgP had been infected with the JP2 clone since 2003, when no loss of attachment was detectable, and still carried the JP2 clone in 2007 when substantial attachment loss at multiple sites was detected by the clinical and radiographic examination. Three other subjects studied longitudinally (08, 03C and 09C) had been previously infected with the JP2 clone but the clone was no longer present in 2007. Subject 08 apparently lost the clone after 2005 and showed loss of attachment at first and second molars and at lower incisors in 2007, which represented a limited progression relative to the clinical situation in 2005. In subjects 03C and 09C, the JP2 clone was apparently lost between 2003 and 2005. These subjects had shown no disease progression since 2005 and displayed in 2007 only a single site with clinical attachment loss of 2 or 3 mm. Accordingly, they were considered healthy. These data show that periodontal infection with the JP2 clone of *A. actinomycetemcomitans* may terminate, and that this may lead to amelioration of the clinical condition.

Quantification of antibodies to leukotoxin of *A. actinomycetemcomitans*

To obtain further information about the infection history of the eight subjects we performed a quantitative analysis of serum IgG antibodies to the leukotoxin of *A. actinomycetemcomitans*, which is unique to this species. To validate the approach to use such antibodies as a marker of previous infection we included sera from additional Moroccan and Danish subjects.

Within the log-linear range, the titration curves for individual subject sera in leukotoxin-coated ELISA wells were parallel to the corresponding standard curve of IgG in anti IgG-coated wells (Fig. 3). Accordingly, for each serum, calculated antibody concentrations based on individual OD values for three consecutive dilutions showed limited variation (CV ≤ 10%). Antibody concentrations determined for some of the sera were remarkably high (Table 2). To evaluate the validity of the numerical results, the concentration of IgG antibody to the leukotoxin in one high-titre serum determined at 102 μg ml^{-1} (subject 05) was evaluated also by a protocol involving dissociation of IgG bound in leukotoxin-coated wells followed by its quantification in IgG-specific ELISA. By this method, the concentration of IgG antibodies in this serum was estimated at 68 μg ml^{-1} calculated as the mean of two determinations based on sub-saturating concentrations of serum in leukotoxin-coated wells. Comparison of residual IgG in glycine–HCl-treated wells relative to untreated control wells indicated that 90% of leukotoxin-bound antibodies had been dissociated for transfer and quantification. These observations testified to the validity of the

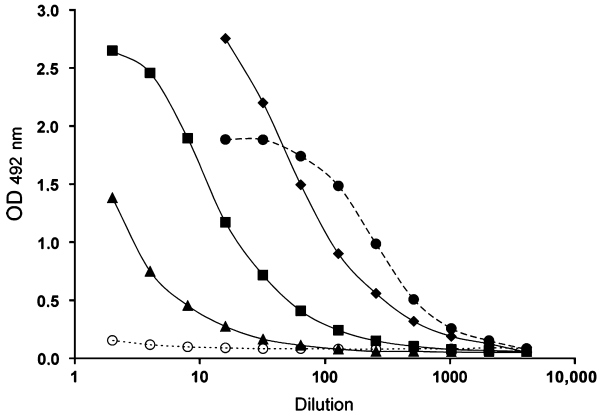

Figure 3 Optical density (OD) data illustrating the performance of the ELISA for quantification in gravimetric units of immunoglobulin G (IgG) antibodies to *Aggregatibacter actinomycetemcomitans* leukotoxin in human sera. Shown are ELISA OD titration curves for sera from subjects 05 (♦), 10 (■) and a Danish healthy control (▲) in leukotoxin-coated wells. Also shown are ELISA OD titration curves for a standard of purified human IgG titrated in wells coated with rabbit antibodies to human IgG (•), and in control wells coated with non-immune rabbit immunoglobulin (○). Note that within the OD range corresponding to the log-linear part of the standard curve, the titration curves for the three sera are parallel to each other as well as to the standard curve. Estimates of antibody concentrations in sera were calculated on the basis of OD readings falling within the log-linear part. The titration curves for the remaining sera (not shown) were parallel to the curves shown.

procedure for estimation of antibody in gravimetric units.

The eight subjects were grouped as follows, irrespective of potential disease activity: (A) individuals with current JP2 infection; (B) individuals with known, previous JP2 infection; (C) individuals with current non-JP2 *A. actinomycetemcomitans* colonization; (D) individuals with no evidence of *A. actinomycetemcomitans* colonization. Antibody data for the eight subjects were supplemented with data from Moroccan and Danish individuals to a total of 22 subjects, distributed according to *A. actinomycetemcomitans* infection history with three, four, five and 10 subjects in groups A–D, respectively. The highest mean antibody concentration (62.1 ± 22.0 μg ml^{-1}; mean ± standard error of mean) was observed in patients with current JP2 infection. The concentration in the subjects with known previous JP2 infection was lower (37.2 ± 24.3 μg ml^{-1}), though this difference was not statistically significant. Significantly lower concentrations were seen both in subjects with current colonization by non-JP2 clones of *A. actinomycetemcomitans* (16.1 ± 1.9 μg ml^{-1}; analysis of variance, $P < 0.05$; 95% CI 3.745–88.093) and in subjects without current presence of *A. actinomycetemcomitans* (7.4 ± 2.6; analysis of variance, $P < 0.01$ 95% CI 16.658–92.689) (Fig. 4).

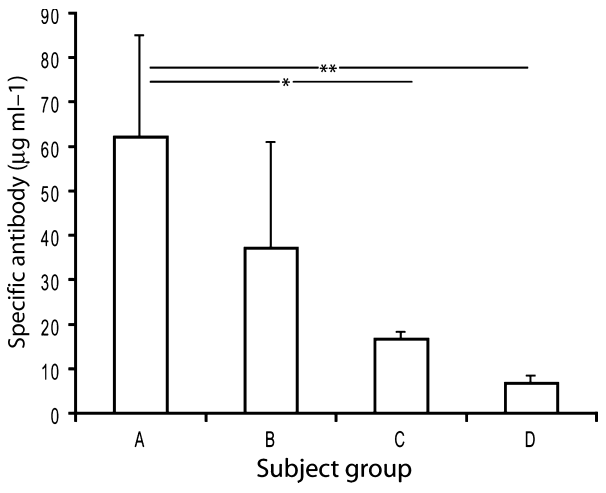

Figure 4 Antibody concentrations in μg ml^{-1} to leukotoxin of *A. actinomycetemcomitans* (Aa) in eight subjects studied supplemented with 14 reference subjects of Moroccan and Danish decent distributed into the following groups: (A) Current Aa JP2 infection, ($N = 3$), (B) Known previous Aa JP2 infection ($N = 4$), (C) Current non-JP2 Aa infection ($N = 5$), and (D) Healthy and currently no detectable Aa. The bars indicate the standard error of the mean.

DISCUSSION

Aggressive periodontitis, which results in severe destruction of the osseous support of the dentition at an early age, has long been recognized as an infectious disease. Identification of particular aetiological agents has been hampered by the complexity of the microbiota associated with disease, by difficulties in diagnosing current disease activity, and by the lack of a relevant animal model. Only recently was a substantial relative risk (18.0; 95% CI 7.8–41.2, $P < 0.0001$) of disease initiation demonstrated to be associated with the JP2 clone of *A. actinomycetemcomitans* in a comprehensive longitudinal study of adolescents in Morocco where the disease occurs with an unusually high prevalence (Haubek *et al.*, 2008). The aim of the present study was to characterize the remaining complex microbiota associated with AgP and health in this population, and to seek explanations to the previous observation that clinical signs of AgP, even in this population, may be found also in the absence of the JP2 clone.

The subgingival microbiota of the eight Moroccan subjects with AgP and healthy controls was mapped by sequencing of clone libraries of phylogenetically informative 16S rRNA genes generated from the samples (Amann *et al.*, 1995). Previous studies using this technique have shown that the bacterial diversity in most environments is severely underestimated by culture-based techniques and have provided considerable insight into the complexity of the human microbiome (Paster *et al.*, 2001, 2002a,b; Munson *et al.*, 2002; Kazor *et al.*, 2003; Faveri *et al.*, 2008). The sensitivity of this method depends on the specificity of primers and their binding efficiency and the number of sequences determined (Hansen *et al.*, 1998; Baker *et al.*, 2003). The primers used in this study had a perfect match with 16S rRNA sequences of 96,811 out of 157,089 sequences representing 34 bacterial phyla in the RDP II database, the majority of which were never associated with humans. Identical or closely similar primers were used in previous studies of the microbiota of the oral cavity and superficial skin (Paster *et al.*, 2001; Bek-Thomsen *et al.*, 2008). Our study detected taxa belonging to eight bacterial phyla in the samples of subgingival plaque, i.e. TM7, *Proteobacteria, Firmicutes, Synergistetes, Actinobacteria, Spirochaetes, Bacteroidetes,* and *Fusobacteria*. Of the 173 taxa detected, 39% repre-

sented phylotypes for which no cultivated representatives have been reported. This is comparable to previous molecular studies of oral microbiotas (Paster *et al.*, 2001, 2002a,b; Kazor *et al.*, 2003).

Interpretation of the relative abundance of taxa identified by this method must be made with care because of the potential biases resulting from differences in DNA release from different bacteria, in the efficiency of amplification and cloning, and in the number of rRNA gene operons per genome. Nevertheless, the number of sequences representing individual taxa indicates that a limited number of taxa dominated the subgingival microbiota of the eight Moroccan individuals (Table S1). The JP2 clone of *A. actinomycetemcomitans*, conceivably the principal pathogen in the patients with AgP, was represented by only 1 and 6.3%, respectively, of the sequenced clones from the two patients who were infected. This low proportion is in agreement with results of previous culture-based studies where *A. actinomycetemcomitans* was present in proportions ranging between 2 and 5% of the total cultivable subgingival microbiota (Moore *et al.*, 1985; van Winkelhoff *et al.*, 2002), and a study of patients with AgP in a Brazilian population using the same method as in our study (Faveri *et al.*, 2008), in which *A. actinomycetemcomitans* was detected only by species-specific PCR amplification.

Availability of critical nutrients and other ecological parameters may explain why certain subgingival bacterial species tend to co-occur in characteristic complexes (Socransky & Haffajee, 2005). In agreement with this hypothesis a multitude of fastidious taxa belonging to the phylum *Proteobacteria*, i.e. *Eikenella corrodens*, non-JP2 clones of *A. actinomycetemcomitans*, *Aggregatibacter aphrophilus*, and species of *Neisseria*, *Kingella* and *Cardiobacterium*, were detected in two of the healthy subjects (subjects no. 03 and 09) and not in any of the remaining six subjects (Table S1). Interestingly, the same two individuals carried high proportions of the beta-*Proteobacteria* species *Lautropia mirabilis*, and were also the only ones that were colonized with *Capnocytophaga* spp. Two other individuals, one diseased and one healthy, carried high proportions of *Enterobacteriaceae* that have entirely different metabolic characteristics and ecological preferences. Enteric rods were previously detected in dental plaque from both healthy subjects and from patients with refractory periodontitis and proportions were higher in developing countries (Slots *et al.*, 1990; Goldberg *et al.*, 1997; Colombo *et al.*, 2002; Botero *et al.*, 2007). The ecological determinants of their dominance, usually regarded as a state of 'superinfection', are not known. Our controls combined with the selective presence in some samples excludes that the finding can be explained by contamination of PCR reagents.

TM7 is one of a number of prominent candidate bacterial phyla detected in studies based on 16S rRNA gene sequencing that lack any cultivated representatives, yet comprising more than 50 phylotypes (Rappe & Giovannoni, 2003). The 16S rRNA gene sequences representing the TM7 phylum were found previously in a variety of habitats, ranging from deep sea ecosystems to the human mouth. Some sequence types within this phylum have been associated with chronic periodontitis in humans (Brinig *et al.*, 2003; Kumar *et al.*, 2003; Ouverney *et al.*, 2003). In our study, phylum TM7 was detected in all subjects and was represented by 3–21.2% (median 10.9%) of the cloned sequences. Three new phylotypes of the TM7 phylum were detected (Fig. S1). Among the previously detected TM7 clustered phylotypes, particularly high levels of phylotype A (20.6 and 10.8%) were found in two subjects with clinical signs of AgP (subjects 7 and 8). The same two individuals also carried significantly higher proportions (14.5 and 10.3%) of previously undetected lineages of the family *Lachnospiraceae* (Table S1). Phylotypes representing the family *Lachnospiraceae* have been previously identified as part of the subgingival microbiota but not associated with periodontal disease (Hutter *et al.*, 2003).

The previous observation by Haubek *et al.* (2008) that AgP disease initiation was detected also in a substantial proportion of Moroccan adolescents, who did not carry the JP2 clone at the outset of the longitudinal study, raised the question whether there is an alternative aetiology of AgP in the Moroccan population. The significant variation between subjects and the potential difference in the pathogenic potentials of clones of the same taxa (Kilian *et al.*, 2006) make it difficult to exclude this hypothesis, but no single taxon, except for the JP2 clone of *A. actinomycetemcomitans* and taxa detected only in a single individual, was exclusively detected in patients with AgP. Only *Solobacterium moorei*, which has been associated with halitosis (Haraszthy *et al.*, 2007), was detected in all four patients and

also in a single healthy individual. Theoretically, the significantly higher proportions of *Lachnospiraceae* in the two patients with AgP without the JP2 clone at the 2007 examination might indicate aetiological involvement. However, the evidence discussed above on *Lachnospiraceae* phylotypes as part of complex microbiotas in combination with clinical findings (Tables 1 and 2, subject 08) rather suggest that disease activity in these patients was about to level off at the 2007 examination because of the absence of the JP2 clone. This interpretation is supported by the evidence of previous but terminated infection with the JP2 clone in subject 08 (Table 2) and in two subjects clinically diagnosed as being healthy in 2007, in spite of previous evidence of AgP and concurrent JP2 clone infection (subjects 03 and 09, Table 2).

Identification of the host or microbial factors that cause cessation or termination of disease activity is, by nature, difficult, if not impossible. In most other infectious diseases, immunity eventually eliminates the pathogen. However, elimination of a member of a complex bacterial biofilm, such as that present in the subgingival compartment of patients with AgP, by mechanisms of immunity is conceivably problematic and, so far, there is no experimental evidence supporting that this may occur. The detected antibody concentrations specific to the leukotoxin in subjects known to be infected with, or known to have been infected with, the JP2 clone were substantial (Table 2 and Fig. 4) and exceeded by far the antibody levels that confer protection against traditional bacterial pathogens (Gergen *et al.*, 1995). The exact level of leukotoxin antibodies in serum that may be associated with elimination of the JP2 clone requires further studies. Data presented in Table 2 suggest that it may exceed 100 μg of specific antibody per ml and that loss of the infection, as expected, results in decreased titres.

Antibodies to *A. actinomycetemcomitans* leukotoxin in the serum of patients with periodontitis have been previously assessed in gravimetric units only by Califano *et al.* (1997) and at levels comparable to those reported here. To obtain leukotoxin free of contaminating lipopolysaccharide, which is an immunodominant antigen of *A. actinomycetemcomitans* (Page *et al.*, 1991; Lu *et al.*, 1993), these authors adopted a complicated assay based on the use of leukotoxin antigen in the form of bands in Western blots (Califano *et al.*, 1997). In the present study this problem was solved by the use of purified leukotoxin free of lipopolysaccharide as demonstrated by a sensitive immunochemical assay. It is conceivable that antibody at the concentrations detected is capable of neutralizing the leukotoxin activity *in vivo* by blocking its interaction with the β_2 integrin ligand on human phagocytic cells (Lally *et al.*, 1997). The JP2 clone, in contrast to most other clones of *A. actinomycetemcomitans*, has lost its ability to acquire iron via a haemoglobin-binding protein (Hayashida *et al.*, 2002). Therefore, eventual elimination from the ecosystem conceivably may result also from loss of iron supply as a consequence of antibody-mediated inhibition of lysis of erythrocytes, which are an additional target of the toxin (Balashova *et al.*, 2006). Notably, data do exist to support the assumption that antibodies to *A. actinomycetemcomitans* leukotoxin, their mechanism of action untold, may offer protection in AgP (Califano *et al.*, 1997). In addition, because the polysaccharide moiety of LPS in *A. actinomycetemcomitans* serotype b, to which the JP2 clone belongs, is an equally strong immunogen generating high concentrations of opsonic and apparently protective antibodies in patients with AgP (Baker & Wilson, 1989; Califano *et al.*, 1996), such antibodies are likely to have been present and, possibly, contributed to the elimination of *A. actinomycetemcomitans* in the subjects.

The results of the study reveal the ecological context of the JP2 clone in young Moroccans with AgP, and demonstrate that the JP2 clone, although conceivably the aetiological agent of the disease in some patients, constitutes a minor proportion of the microbiota. The pattern of co-occurrence and distribution of bacterial species in the subgingival microbiota of both healthy and diseased subjects supports the concept of complexes of species that are supported by particular ecological determinants. Rather than identifying alternative aetiologies to AgP in young Moroccans, the results of the study suggest that disease activity may be terminated in some of the patients with AgP as a result of loss of infection with the JP2 clone of *A. actinomycetemcomitans*. A driving force may be substantial concentrations of antibodies against the leukotoxin and possibly other antigens of this pathogen. The potential role of specific immunity is a matter of controversy because immune reactions to mucosal flora may not only con-

trol but also contribute to pathological inflammatory conditions, notably in the gut but also in periodontal tissues (Taubman *et al.*, 2005; Abraham & Cho, 2009). Presumably, the role of *A. actinomycetemcomitans*-specific antibodies in relation to AgP could be clarified by a controlled longitudinal study in larger groups of Moroccans, involving sequential analysis of microflora and antibodies. However, considering the striking evidence implicating the JP2 clone of *A. actinomycetemcomitans* as being responsible for the high prevalence of AgP in this population, and the fact that *A. actinomycetemcomitans*-associated periodontitis can be treated with standard antibiotics (Pavicic *et al.*, 1994; Herrera *et al.*, 2008), such a study is no longer ethically acceptable. Recent studies have analysed the clinical development (Haubek *et al.*, 2009) and treatment outcomes (Cortelli *et al.*, 2009) among patients with JP2 compared with non-JP2 *A. actinomycetemcomitans*-associated periodontal infection. Persistence of the JP2 as opposed to a non-JP2 clone or no *A. actinomycetemcomitans* was associated with a poorer prognosis in both studies, and periodontitis associated with the JP2 clone appeared to be less successfully treated (Cortelli *et al.*, 2009). Studies are needed to identify protocols that most effectively eliminate or prevent infection with this virulent periodontal pathogen.

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SUPPORTING INFORMATION

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

Figure S1. Phylogenetic tree of bacterial phylotypes in the TM7 phylum representing nine phylogenetic clusters each supported by significant bootstrap values and two unclustered phylotypes represented by a single sequence.

Table S1. Bacteria detected in subgingival plaque from four young Moroccan patients with aggressive periodontitis and four healthy Moroccan controls.

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