REVIEW ARTICLE

Pathogenicity of the highly leukotoxic JP2 clone of Aggregatibacter actinomycetemcomitans and its geographic dissemination and role in aggressive periodontitis

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For decades, Aggregatibacter actinomycetemcomitans has been associated with aggressive forms of periodontitis in adolescents. In the middle of the 1990s, a specific JP2 clone of A. actinomycetemcomitans, belonging to the cluster of serotype b strains of A. actinomycetemcomitans and having a number of other characteristics, was found to be strongly associated with aggressive forms of periodontitis, particularly in North Africa. Although several longitudinal studies still point to the bacterial species, A. actinomycetemcomitans as a risk factor of aggressive periodontitis, it is now also widely accepted that the highly leukotoxic JP2 clone of A. actinomycetemcomitans is implicated in rapidly progressing forms of aggressive periodontitis. The JP2 clone strains are highly prevalent in human populations living in Northern and Western parts of Africa. These strains are also prevalent in geographically widespread populations that have originated from the Northwest Africa. Only sporadic signs of a dissemination of the JP2 clone strains to non-African populations have been found despite Africans living geographically widespread for hundreds of years. It remains an unanswered question if a particular host tropism exists as a possible explanation for the frequent colonization of the Northwest African population with the JP2 clone. Two exotoxins of A. actinomycetemcomitans are known, leukotoxin (LtxA) and cytolethal distending toxin (Cdt). LtxA is able to kill human immune cells, and Cdt can block cell cycle progression in eukaryotic cells and thus induce cell cycle arrest. Whereas the leukotoxin production is enhanced in JP2 clone strains thus increasing the virulence potential of A. actinomycetemcomitans, it has not been possible so far to demonstrate such a role for Cdt. Lines of evidence have led to the understanding of the highly leukotoxic JP2 clone of A. actinomycetemcomitans as an aetiologic factor of aggressive periodontitis. Patients, who are colonized with the JP2 clone, are likely to share this clone with several family members because the clone is transmitted through close contacts. This is a challenge to the clinicians. The patients need intense monitoring of their periodontal status as the risk for developing severely progressing periodontal lesions are relatively high. Furthermore, timely periodontal treatment, in some cases including periodontal surgery supplemented by the use of antibiotics, is warranted. Preferably, periodontal attachment loss should be prevented by early detection of the JP2 clone of A. actinomycetemcomitans by microbial diagnostic testing and/ or by preventive means.

Keywords: Virulence factors; spreading; geographical dissemination; leukotoxin; cytolethal distending toxin; host response

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For decades, particular attention has been given to the oral bacterium, Aggregatibacter actinomycetemcomitans as a species implicated in the aetiology of periodontitis among the young (1–5). In addition to an exotoxin of A. actinomycetemcomitans, called cytolethal distending toxin (Cdt) (6), one of the major and intensely studied virulence factors of A. actinomycetemcomitans is the leukotoxin which is able to affect, impair the function, and kill important cells of the human immune system (6–12). Although much research has already focused on the structure, function, secretion, and the role of the leukotoxin (6, 12–22), researchers still pay attention to this important area of research.

Population genetic analysis, as a tool to study the population structure of A. actinomycetemcomitans, has
demonstrated a mainly clonal population structure with evolutionary lineages corresponding to serotypes (23–26). The highly leukotoxic clone, which is termed the JP2 clone and which belongs to the group of serotype b strains, was described in the middle of the 1990s (27). That was shortly after the presence of a 530-basepair (bp) deletion was demonstrated in the promoter region of the leukotoxin gene operon in A. actinomycetemcomitans strains with an enhanced leukotoxic activity (28). The JP2 clone, with an estimated origin more than 2000 years ago (29), is highly conserved based on analyses of a collection of JP2 clone strains that were collected through more than 20 years from individuals of diverse origin and living geographically widespread (29). Despite the demonstration of minor evolutionary changes within the genome of JP2 clone strains, these strains constitute a unique clonal type, the characteristics of which among other findings include the 530-bp deletion in the leukotoxin operon most likely implicated in the enhanced leukotoxic activity (28–31).

The JP2 clone of A. actinomycetemcomitans is endemically present in Northwest Africa and particularly associated with Northwest African populations (27, 29, 30, 32–34). Furthermore, it appears to play a prominent role in the initiation of periodontal attachment loss among Northwest African adolescents compared with other non-JP2 genotypes of the species (33–35). As demonstrated in prospective cohort studies in Morocco and Ghana, the presence of the JP2 clone in dental plaque confers a markedly increased risk for the development of aggressive periodontitis (33, 35). This suggests that the JP2 clone is an important aetiological agent in aggressive periodontitis among the young.

The aim of this report is to review the latest findings concerning the characteristics, pathogenic mechanisms, geographic dissemination, and the role of the JP2 clone of A. actinomycetemcomitans in aggressive periodontitis among adolescents.

Differences in the occurrence of periodontitis in adolescent populations living worldwide

Prevalence estimates of periodontal attachment loss in adolescent populations from geographically widespread parts of the world are difficult to compare, and part of these differences may be due to differences in study designs and diagnostic criteria (36–41). However, prevalence differences cannot be ascribed entirely to variations in study design and diagnostic criteria. Periodontitis among the young occurs relatively frequently in some countries, for example, Uganda, Morocco, Ghana, Sudan, Israel, and certain groups in Brazil and the United States (32, 35, 42–47), whereas it is a rare disease with a prevalence of less than 1% in many other parts of the world, for example, Northern Europe (37–39, 41, 48, 49).

Studies on the epidemiology of periodontitis carried out in the United States and in other countries with an ethnically mixed population for decades, for example, the United Kingdom, have often subdivided the study population into subgroups according to ethnicity. The overall findings in such studies have often been that black populations are more likely to have periodontitis than white populations (37, 38, 50, 51). It is also generally accepted that the total periodontal disease burden in Africa is high. For example, periodontal attachment loss was found in 107 (21.4%) individuals out of 500 participants (mean age 13.2 years) in a recent study undertaken in the Ghanaian adolescent population (34), and 16.3% out of 1200 Sudanese students (mean age 15.9 years) had at least one tooth with 4 mm attachment loss or more (47). Hence, these findings are consistent with conclusions in other previous epidemiological studies carried out in African countries, for example, Uganda and Nigeria (52, 53). However, human populations show many differences with regard to several parameters: ethnicity (genetic profile), knowledge regarding oral and general health, dietary habits, oral hygiene practices, other behavioral traits, socioeconomic status, access to dental health care services, and the microbial composition of the dental plaque (6, 39, 41, 54, 55). All of these factors could result in different levels and patterns of disease both within and outside of the African continent.

In recent years, after the reporting of a high prevalence of the highly leukotoxic JP2 clone strains of A. actinomycetemcomitans in Northwest African countries, it has become more and more clear that this part of the African continent has a relatively high prevalence of severe forms of periodontitis among the young (31–35). However, the complete picture of the geographic dissemination of the JP2 clone and the manifestation of the JP2 clone-associated periodontitis is not fully elucidated. In a very recent epidemiological study carried out in the Eastern part of Africa, JP2 clone-positive Sudanese adolescents were not found (47). In addition, some A. actinomycetemcomitans strains from periodontitis patients living in Somalia, Tanzania, and Kenya, subtype according to the non-JP2 or the JP2 leukotoxin promoter genotypes, did not belong to the group of JP2 clone strains of A. actinomycetemcomitans (30). Thus, there are patients with aggressive periodontitis around the world, and also in Africa, where the JP2 clone is not necessarily implicated as an aetiological factor of the disease. Thus, the JP2 clone of A. actinomycetemcomitans is not the only factor involved in and cannot explain all cases of aggressive periodontitis in the adolescent population. More research on the pathogenesis and aetiology of periodontal disease among the young is still needed.

Surprisingly, a more extensive spreading of the JP2 clone around the world than initially assumed seems to have occurred (56, 57). Although the JP2 clone-associated
periodontitis is only one piece of the puzzle in the understanding of the aetiology and the pathogenesis of periodontitis among the young, the JP2 clone-associated periodontitis is a clinical manifestation of periodontal disease that needs intensive attention by the researchers and clinicians. Due to the highly progressive nature and thus the aggressive type of periodontitis linked to the presence of the JP2 clone of *A. actinomycetemcomitans*, there are many reasons to increase our understanding of the epidemiology and treatment of this disease.

**The association between the presence of the JP2 clone of *A. actinomycetemcomitans* and periodontal disease**

Pioneering studies from the middle of the 1990s indicated that highly leukotoxic JP2 clone strains of *A. actinomycetemcomitans* were closely associated with aggressive periodontitis (27, 30, 43, 58–60). Moreover, some early studies on the geographic dissemination of the JP2 clone of *A. actinomycetemcomitans* indicated that colonization with this clone appeared to be particularly linked to aggressive periodontitis in individuals of North African descent (27, 30). An updated list of studies that have aimed at elucidating various aspects concerning characteristics, function, occurrence, geographic dissemination, and the role of the JP2 clone in periodontitis is presented in Table 1 (original version of Table 1, see Ref. 31). The majority of studies that have included JP2 clone strains are either experimental studies, studies based on patient groups with various periodontal disease profiles, or cross-sectional studies. Some studies are based on previously collected *A. actinomycetemcomitans* isolates kept as a part of microbial culture collections at various microbiological laboratories. A weakness in such studies can be the selection of the bacterial isolates, the representativeness, and that there may be a lack of detailed information on the periodontal condition of the patients from whom the dental plaque samples were originally collected. Few longitudinal studies on the association between the presence of the JP2 clone of *A. actinomycetemcomitans* and periodontal disease are available, and some of these do not have a clear follow-up time or do include both periodontally healthy and diseased subjects at baseline (43, 81). That makes it impossible to determine the temporal relationship between the colonization with the JP2 clone and the development of periodontitis. In addition, the number of individuals included in those longitudinal studies was limited, thus resulting in risk estimates with wide confidence intervals (43, 81). To address the question concerning the temporal relationship between the colonization with the JP2 clone of *A. actinomycetemcomitans* and the development of periodontal attachment loss in adolescents, more recent longitudinal studies with improved study design have been published (33, 35).

The first longitudinal study on JP2 clone-associated aggressive periodontitis, conducted in an African country, was cross-sectional and based on a group of 301 Moroccan adolescents in Rabat, Morocco (32). That study clearly demonstrated a strong association between the presence of the JP2 clone of *A. actinomycetemcomitans* and periodontal attachment loss in Moroccan adolescents (32). In further support of the role of the JP2 clone in periodontal disease, diseased individuals, who were positive for the JP2 clone, had more extensive periodontal attachment loss than those without detectable levels of the JP2 clone (82). Furthermore, a 2-year follow-up examination of this Moroccan population showed that the prevalence of the JP2 clone of *A. actinomycetemcomitans* was strongly associated with the progression of periodontal attachment loss (81). To elucidate the causal role of the JP2 clone of *A. actinomycetemcomitans* in disease, information on the temporal relation between the colonization and the disease is important. The first study, carried out in Morocco, provided no possibility for studying the temporal relation between the presence of the JP2 clone and the initiation of disease because only four periodontally healthy carriers were present at baseline (32). However, a good infrastructure and some general information obtained on periodontal diseases among adolescents in Morocco during the first Moroccan population-based cohort study provided an excellent platform for designing another longitudinal study in Morocco that could focus on the temporal relation between colonization with *A. actinomycetemcomitans* and the initiation of disease. Thus, taking the already obtained experiences, results, and knowledge on aggressive periodontitis in Moroccan adolescents into account, another prospective cohort study was designed (33). In that second, prospective cohort study, a screening at baseline of 700 Moroccan school children (mean age 12.5 years) revealed that only 18 (2.6%) were periodontally diseased (33). Thus, a total of 682 individuals provided the basis for the second longitudinal study performed in the Moroccan population of which 428 (62.8%) returned for the 2-year follow-up. The results showed a strong association between the presence of the JP2 clone of *A. actinomycetemcomitans* and the development of aggressive periodontitis among the Moroccan adolescents (RR = 18.0; 95% CI [7.8, 41.2]). Exclusion of the individuals, who had received antibiotics less than 3 months before examination or had received any type of periodontal treatment since baseline, did not change the estimates (33). Furthermore, that second prospective Moroccan cohort study provided some evidence, although less pronounced, that the non-JP2 genotypes of *A. actinomycetemcomitans* are also associated with aggressive periodontitis in adolescents (RR = 3.0; 95% CI [1.3, 7.12]) (33). This association had not been clear in the first Moroccan cohort study where no power calculation was carried out before the initiation of the study (32). Non-JP2
Table 1. Geographic origin of donors of the JP2 clone of *A. actinomycetemcomitans* reported on in various types of research reports

| References                  | Number of subjects in the study | Country of residence | Number of subjects positive for JP2 clone strains | Geographical origin of subjects positive for the JP2 clone |
|-----------------------------|---------------------------------|----------------------|-----------------------------------------------|----------------------------------------------------------|
| Poulsen et al. (23)         | > 60                            | Northern Europe      | 0                                             |                                                          |
| Brogan et al. (28)          | 17<sup>a</sup>                  | Not reported         | 3                                             | Not reported                                             |
| Haubek et al. (24)          | 88                              | Finland              | 0                                             |                                                          |
| Haubek et al. (27)          | 17                              | Denmark and Sweden   | 11                                            | Moroccan (3), Algerian (2), the Cape Verde islands (6)    |
| Zambon et al. (59)          | 256<sup>b</sup>                 | USA                  | 61                                            | Not reported                                             |
| Haubek et al. (30)          | 326                             | Geographically widespread on five continents | 38                                            | Moroccan (7), Algerian (2), Ghanaian (1), from the Cape Verde Islands (6), Brazilian (4), Israeli (1), African–American (17) |
| Haubek et al. (29)          | 82                              | Geographically widespread on five continents | 66                                            | African–American (28), Algerian (2), Ghanaian (1), from the Cape Verde Islands (6), Brazilian (4), Israeli (3), Turkish (1), from the Mediterranean area (2), Portuguese (1), African–American (16), unknown (2) |
| Tinoco et al. (61)          | 36                              | Brazil               | 5                                             | Brazilian (5)                                             |
| Saarela et al. (62)         | 163                             | Finland and USA      | 3                                             | African–American (2), American (1)                        |
| Bueno et al. (43)           | 58                              | USA                  | 8                                             | African–American (8)                                      |
| Macheleidt et al. (63)      | 238                             | Germany              | 1                                             | Ghanaian (1)                                              |
| Mombelli et al. (64)        | 185                             | China                | 0                                             |                                                          |
| He et al. (65)              | 43                              | Japan                | 0                                             |                                                          |
| Contreras et al. (66)       | 94                              | USA                  | 12                                            | African–American (2), Hispanic (1), Jamaican (9), Asian (0), Caucasian (0) |
| Haraszthy et al. (60)       | 146                             | USA                  | 41                                            | African–American (33), Caucasian (1), Hispanic (7), Asian–American (0) |
| Tan et al. (67)             | 92                              | China                | 0                                             |                                                          |
| Haubek et al. (32)          | 217                             | Morocco              | 19                                            | Moroccan (19)                                             |
| Müller et al. (68)          | 97                              | Germany              | 0                                             |                                                          |
| Saddi-Ortega et al. (69)    | 35<sup>c</sup>                 | Brazil               | 6                                             | Brazilian (6)                                             |
| Kaplan et al. (25)          | 33<sup>a</sup>                  | USA                  | 8                                             | African or African–American (8)                          |
| Cortelli et al. (70)        | 136                             | Brazil               | 11                                            | Brazilian (11)                                            |
| Cortelli et al. (71)        | 203                             | Brazil               | 13                                            | Brazilian (13)                                            |
| Leung et al. (72)           | 56                              | China                | 0                                             |                                                          |
| Orru et al. (56)            | 81                              | Italia (Sardinia)    | 6                                             | Not reported                                              |
| Junior et al. (73)          | 40                              | Brazil               | 2                                             | Not reported                                              |
| Haubek et al. (29)          | 82                              | Geographically widespread on five continents | 66                                            | Moroccan (28), Algerian (2), Ghanaian (1), from the Cape Verde Islands (6), Brazilian (4), Israeli (3), Turkish (1), from the Mediterranean area (2), Portuguese (1), African–American (16), unknown (2) |
| Fine et al. (74)            | 1075                            | USA                  | 7                                             | African–American (6), Hispanic (1)                        |
| Van der Reijden et al. (75) | 107                             | Indonesia            | 0                                             |                                                          |
| Haubek et al. (33)          | 700                             | Morocco              | 95                                            | Moroccan (95)                                             |
| Viera et al. (76)           | 86<sup>d</sup>                 | Brazil               | 0                                             |                                                          |
| Sakellari et al. (77)       | 228                             | Greece               | 0                                             |                                                          |
| Åberg et al. (34)           | 500                             | Ghana                | 44                                            | Ghanaian (44)                                             |

<sup>a</sup> Not reported for country of residence;<br><sup>b</sup> Not reported for number of subjects positive for JP2 clone strains;<br><sup>c</sup> Geographically widespread on five continents;<br><sup>d</sup> Geographically widespread on five continents;<br><sup>e</sup> Geographically widespread on five continents;
genotypes of *A. actinomycetemcomitans* also being associated with the development of periodontal attachment loss are in concordance with results from other longitudinal studies performed in Indonesia (JAVA-project) and in the United States (74, 83, 84). The discrepancy in the conclusions concerning the role of the non-JP2 genotypes of *A. actinomycetemcomitans* between the first and the second Moroccan cohort study can be explained by a number of factors, for example, the disease status at baseline and the sample size of the study populations. Thus, the second longitudinal study, performed in the Moroccan population, showed evidence of *A. actinomycetemcomitans* as a risk factor for the initiation of periodontal attachment loss, but is also the first evidence of a strong association particularly between the presence of the JP2 clone of *A. actinomycetemcomitans* and the initiation of attachment loss, suggesting the JP2 clone to be an etiological factor of aggressive periodontitis (33).

Continuously, it is an unanswered question if the JP2 clone-associated periodontitis is linked to a specific genetic constitution and disposition of the North African populations, for example, Berbers and Arabs. On the other hand, several epidemiological studies reporting on the disease prevalence of aggressive periodontitis among the young in the United States have found a particularly high prevalence of periodontal disease among black Americans, but not reported on a particularly high prevalence in populations originating from the Mediterranean parts of Africa. However, determination of ethnic origin may be difficult or even impossible due to lack of information on the genetic origin of many groups of humans. In particular, it may be a challenging task in a continent like the United States, which has a long history of ethnically mixed inhabitants (50). In the study on periodontal epidemiology, carried out in 2010 in Sudan by Elamin and coworkers (47), the study population was selected in a multistage, stratified sampling design, and ethnicity was categorized into Afro-Arab tribes and non-African tribes. In that study, a significantly higher prevalence of aggressive periodontitis was found among African tribes than Afro-Arab tribes (6% versus 2.3%, \( p = 0.01 \)) (47). Hence, this study also lends support of a particularly high disease prevalence of periodontitis among young black Africans.

In an attempt to further elucidate the topic on periodontal disease among black Africans and the colonization with the JP2 clone of *A. actinomycetemcomitans*, we decided more recently to study aggressive periodontitis in a sub-Saharan country. In 2008, when a study in Ghana was designed and initiated, we found no West African study on periodontal epidemiology that included microbiological analyses of *A. actinomycetemcomitans*, which addressed the JP2 clone issue. Thus, no direct evidence for the presence of the JP2 clone of *A. actinomycetemcomitans* in the sub-Saharan region of West Africa was available at that point of time. However, case reports had indicated that the JP2 clone of *A. actinomycetemcomitans* may be present and implicated in the disease aetiology in that part of the African continent (63, 85). In addition, some reports on the periodontal conditions of West African adolescents had been published, but those reports had focused only on clinical aspects (52, 86). Many studies have reported black Africans as particularly vulnerable to the development of rapidly progressing and severe aggressive periodontitis, but the studies did not include microbiological findings. Therefore, we hypothesized that the JP2 clone of *A. actinomycetemcomitans* could be implicated in the high prevalence and severe disease manifestation in black Africans (34).

To generate more direct information on the conditions in the sub-Saharan area of Africa, another prospective cohort study was planned and carried out in Ghana more recently. Five hundred Ghanaian school children (mean age 13.2 years) were included in that prospective cohort study (34). No exact information was available.

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### Table 1 (Continued)

| References                        | Number of subjects in the study | Country of residence | Number of subjects positive for JP2 clone strains | Geographical origin of subjects positive for the JP2 clone |
|-----------------------------------|---------------------------------|----------------------|--------------------------------------------------|-----------------------------------------------------------|
| Bandhaya et al. (78)              | 453                             | Thailand             | 0                                                | –                                                         |
| Martinez-Martinez et al. (79)     | 75\(^\alpha\)                   | Mexico               | 0                                                | –                                                         |
| Wahasugui et al. (80)             | 113                             | Brazil               | 64                                               | Not reported                                              |
| Höglund Åberg et al. (35)         | 397                             | Ghana                | 38                                               | Ghanaian (38)                                            |

\(^{\alpha}\)The study is based on *A. actinomycetemcomitans* (Aa) isolates and does not report on subjects.

\(^{\alpha}\)165 fresh Aa isolates from patients and 91 Aa strains previously collected from patients and non-human primates.

\(^{\alpha}\)21 Aa isolates of human origin and 14 Aa isolates from captive marmosets.

\(^{\alpha}\)Aa isolates were detected from Brazilian Indians from the Umutina reservation, Mato Grosso, Brazil.

\(^{\alpha}\)Aa isolates were obtained from patients with Down syndrome with or without periodontitis.
concerning periodontitis epidemiology in Ghana when the Ghanaian study was designed. However, all previous periodontal studies on subjects of African descent had indicated that the disease level in black Africans was presumably high, and that suggested that a slightly smaller sample size than in the Moroccan study would be necessary in a study carried out in Ghana (33, 34).

At baseline, the Ghanaian cohort study showed that there was a relatively high carrier rate of both JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* in the Ghanaian adolescent population, and the overall carrier rate of *A. actinomycetemcomitans* was 54.4%. Furthermore, the presence of this bacterium was associated with the occurrence of periodontal attachment loss (34). The highly leukotoxic JP2 clone was detected in 9%, and that was comparable or slightly lower than the level found previously in Morocco (32, 33). Thus, the JP2 clone is also frequently found in the Ghanaian population. Furthermore, this study reported on some demographic, social level, and oral hygiene factors. In individuals attending public schools compared to those attending private schools, more individuals were identified as carriers of *A. actinomycetemcomitans* (63% versus 35%, respectively) and periodontal attachment loss was more frequently found (28.3% versus 5.8%, respectively) (34). In addition, logistic regression analysis showed a significant association between the presence of *A. actinomycetemcomitans* and no use of a tooth brush versus if a tooth brush was used (p = 0.003). At baseline, the Ghanaian participants (mean age 13.2 years) were young. Therefore, baseline data according to the presence of non-JP2 and JP2 genotypes of *A. actinomycetemcomitans* and periodontal status could not distinguish between the two possible patterns of disease progression that might manifest at an older age (33, 35).

Two years after baseline, follow-up data on the Ghanaian adolescent population were collected (35). Although many aspects, such as genetics, life style, living condition, oral hygiene habit, food intake, and social condition were very different from the living circumstances in Morocco, the overall results of the Ghanaian follow-up study were in agreement with the results reported on the Moroccan population (33, 35). Periodontally healthy adolescents in Ghana who carried the JP2 genotypes of *A. actinomycetemcomitans* at baseline had a significantly increased risk (RR = 7.3; 95% CI [4.5, 11.9]) of developing attachment loss greater than or equal to 3 mm at one or more periodontal sites over a 2-year period compared with a reference group without detectable *A. actinomycetemcomitans*. A less pronounced risk was found in those individuals who carried the non-JP2 genotypes of *A. actinomycetemcomitans* (RR = 3.6; CI 95% [2.2, 6.0]) (35). Thus, the results from the prospective cohort study performed in Ghana fully support the previous findings in Morocco (32–35, 81, 82).

**Acquisition, stability, and transmission of the JP2 clone of *A. actinomycetemcomitans***

It is well-known that a substantial proportion of human populations worldwide are colonized with *A. actinomycetemcomitans* (31, 41, 87). By use of DNA-based methods, it is not unusual to report that up to 60–70% of a group of individuals are positive for *A. actinomycetemcomitans*. Hence, *A. actinomycetemcomitans* is a bacterial species frequently found in dental plaque.

*A. actinomycetemcomitans* can be detected in the oral cavity at an early age (31). It is also generally accepted that *A. actinomycetemcomitans* is vertically transmitted from parent to child (29, 88–97). Transmission of *A. actinomycetemcomitans* between spouses has also been reported (28, 98–100). In addition, not family-related horizontal transmission may occur, although it is more difficult to show direct evidence for it (101). Family studies are the most frequent study design for the demonstration of transmission of microorganisms through close contacts (29, 94).

Limited information is available concerning the stability of *A. actinomycetemcomitans*. However, *A. actinomycetemcomitans* appears to be a rather stable colonizer with persistence for 1–6 years (determined by findings of isolates of the same serotype twice) (102). Furthermore, identical genotypes of *A. actinomycetemcomitans* (examined by AP-PCR and/or ribotyping) were repeatedly detected in each of 52 subjects 0.5–11.5 years apart (103). Therefore, it was concluded that spontaneous or treatment-induced change of *A. actinomycetemcomitans* strains appears to be extremely rare, and that colonization with the same strain(s) seems to be remarkably persistent (103). In contrast, other researchers have concluded that *A. actinomycetemcomitans*, examined at the species level, only appears to colonize transiently with random concordance between results of the first and the second sampling (104–106). However, due to the extensive genetic diversity of *A. actinomycetemcomitans* that is well-known today (107), it is necessary to study the persistence of bacterial isolates at the DNA level (clonal level), to be sure that it is indeed an identical bacterial strain found at the follow-up.

Information on the acquisition, stability, and transmission of the JP2 clone of *A. actinomycetemcomitans* is scarce. It is known that the JP2 clone strains are present at an early age. JP2 strains were detected in dizygote twins at 7 years of age (95), and it has been reported that the original donor of the JP2 strain was an 8-year-old child with prepubertal periodontitis (108). Since the clone is most likely acquired by vertical transmission from parent to child, transmission of JP2 clone strains probably occurs even before the ages mentioned above.
It has been hypothesized that an age predilection for the infection with JP2 clone strains [leukotoxic strains according to Tsai and Taichman (5)] may exist, and that the JP2 clone is more frequently found in children and adolescents than in adults (60, 71). Haraszthy and coworkers found a higher prevalence of highly leukotoxic strains in children equal to or under the age of 14 years than in subjects older than 14 years (81% versus 26%, \( p < 0.001 \)) (60). In addition, longitudinal data on Moroccan adolescents reports that few individuals are colonized with the JP2 clone de novo after puberty (109). Thus, it is likely that the JP2 clone preferentially infects younger individuals. However, it cannot be completely excluded that a disappearance of the clone in older individuals is merely due to a decrease in the numerical level of the JP2 clone with increasing age. A decreased number of bacteria would reduce the likelihood of capturing the highly leukotoxic JP2 clone strains at the sampling occasions, although JP2 clone strains are still present in the oral cavity. An age predilection for JP2 clone infection of only the young appears, however, to be in contrast with more recent results obtained in Morocco (110). In the Moroccan study by Ennibi and coworkers, it was investigated if the JP2 clone was particularly linked to the localized forms versus the generalized forms of aggressive periodontitis (110). Among Moroccan patients seeking periodontal treatment at the dental school in Rabat, Morocco, it was found that localized as well as generalized aggressive periodontitis patients were positive for the JP2 clone of \textit{A. actinomycetemcomitans} (83\% versus 69\%, \( p = 0.17 \)) (110). Since patients with generalized periodontitis are generally older than patients with localized periodontitis, it appears that adults could be colonized with the JP2 clone as well as children and adolescents, but presumably it may be a less frequent event or that the clone comprises a less dominating proportion of the oral microbiota. Cortelli and coworkers also studied periodontitis patients, positive for JP2 and non-JP2 genotypes of \textit{A. actinomycetemcomitans}, in three different age groups (14–28, 29–39, or 40–76 years old) (71). The JP2 clone strains were more prevalent among the younger patients, whereas the non-JP2 genotypes were more prevalent among the adult periodontitis patients (71). Furthermore, a report on a Swedish family, surprisingly without known ancestors from Africa (ancestry testing was performed), recently showed that some of the family members were positive for the JP2 clone of \textit{A. actinomycetemcomitans}. Two adult family members, a 33-year-old daughter and her 62-year-old mother, were colonized with the JP2 clone of \textit{A. actinomycetemcomitans}. Thus, the JP2 clone may be more prevalent among children and adolescents, but apparently can also be detected in adults (111).

The stability of JP2 and non-JP2 genotypes of \textit{A. actinomycetemcomitans} has also been studied in the Moroccan population (109). Nearly half of the JP2 clone carriers were persistently infected during the 2-year follow-up period, which indicates a level of the stability of the colonization with the JP2 clone similar to that previously reported for the non-JP2 genotypes of \textit{A. actinomycetemcomitans} (103, 109, 112). However, the relative risk for the development of aggressive periodontitis was highest for individuals with stable JP2 clone colonization (33, 109). Although the method used (polymerase chain reaction) in the first and the second Moroccan cohort studies was not quantitative, these results add to the evidence for a causal role of the JP2 clone in aggressive periodontitis (109).

Studying the stability of JP2 and non-JP2 genotypes of \textit{A. actinomycetemcomitans} also revealed that a simultaneous occurrence of these genotypes appears to be an unstable situation (109). The majority of the co-infected Moroccan adolescents at baseline lost one of the clonal types during the 2-year observation period. This outcome might suggest a competitive exclusion between the different \textit{A. actinomycetemcomitans} genotypes. However, the outcome of the competition is seemingly a stochastic process, as either of the genotypes of \textit{A. actinomycetemcomitans} appeared to take over with similar frequencies in the age group studied. Thus, the fitness between the JP2 and non-JP2 clonal types of \textit{A. actinomycetemcomitans} in periodontal pockets seems to be similar (109).

Transmission of JP2 clone strains has been demonstrated in several studies (29, 95, 111). As for other genotypes of \textit{A. actinomycetemcomitans}, the JP2 clone is transmitted through close contacts. Thus, the JP2 clone does not seem to present another colonization pattern than other genotypes of the species. Transmission of the JP2 clone of \textit{A. actinomycetemcomitans} within families has been reported (29, 95), but the most exact evidence of familial transmission of the JP2 clone has been proven at the single nucleotide level (single mutation) in a study on the microevolution of the JP2 clone of \textit{A. actinomycetemcomitans} (29). Thus, the colonization of family members with JP2 clone strains with unique point mutations provides the most exact and strongest evidence that intrafamilial transmission of the JP2 clone of \textit{A. actinomycetemcomitans} occurs (29).

**Geographical dissemination of the JP2 clone of \textit{A. actinomycetemcomitans}**

Knowledge on human migration routes out of Africa and worldwide spreading of the JP2 clone of \textit{A. actinomycetemcomitans} is of major interest to obtain further insight into the epidemiology of the JP2 clone-associated type of periodontitis. Although mapping of the geographic occurrence of the JP2 clone of \textit{A. actinomycetemcomitans}
in periodontitis patients has revealed that its colonization is largely restricted to individuals of African descent, it is still unknown how widespread in Africa the JP2 clone of *A. actinomycetemcomitans* really is (Fig. 1). Characteristic mutations allow JP2 clone isolates from the Mediterranean area to be distinguished from isolates from West Africa, including the Cape Verde islands. These results suggest that the JP2 clone strains initially emerged as a distinct genotype in the Mediterranean part of Africa and subsequently spread to West Africa, and from there they were transferred to the American continents during the transatlantic slave trade (29). What is the situation concerning potential spreading of the JP2 clone strains to the Eastern parts of Africa? The JP2 genotype of *A. actinomycetemcomitans* was not found in a recent study on adolescents, 14–19 years old, living in Sudan (47) and in a previous report, including isolates obtained from subjects originating from Kenya, Tanzania, and Somalia (30). Thus, the JP2 genotype of *A. actinomycetemcomitans* is apparently more prevalent in the Northern and Western parts of Africa than in countries in the Eastern part of Africa. No report, which has studied the epidemiology of the JP2 clone of *A. actinomycetemcomitans* in South African populations, has been published.

In the middle of the 1990s, studies on the population structure of *A. actinomycetemcomitans* led to the conclusion that the highly leukotoxic JP2 clone of *A. actinomycetemcomitans* apparently is absent in Northern Europe (24). This finding created further interest in the actual dissemination of the JP2 clone considered in a worldwide perspective. Early reports from the 1980s mentioned that strain JP2 was isolated from an 8-year-old African–American child with prepubertal periodontitis (108). That strain was subsequently found to have the characteristic 530-bp deletion in the promoter region of the *ltx* operon and the strain belongs to the JP2 clone. Information on the dissemination of the JP2 clone in a global perspective was not available at that point of time.

Generous sharing of *A. actinomycetemcomitans* isolates collected from individuals living in geographically widespread areas and of different ethnic origin provided an opportunity for studying the geographic dissemination of the JP2 clone of *A. actinomycetemcomitans* (30). That study, in which 38 out of 326 *A. actinomycetemcomitans* strains collected turned out to be JP2 clone strains, indicated a link to the African continent. Numerous individuals from African countries (Algeria, Morocco, Ghana, and the Cape Verde islands), among whom some were recent immigrants to European countries, carried the JP2 clone strains of *A. actinomycetemcomitans* (30). In addition, a substantial number of strains collected from black Americans were positive for the JP2 clone of *A. actinomycetemcomitans*. Another interesting finding was that individuals from countries previously colonized by the Portuguese, for example, the Cape Verde islands and Brazil, were positive for the JP2 clone (30). Altogether, these findings led to the hypothesis that the JP2 clone of *A. actinomycetemcomitans* has emerged in the African continent more than 2000 years ago, and since then it has disseminated worldwide through the migration of African populations (29, 30).

A population genetic study attempted to elucidate the genetic diversification of the JP2 clone at the DNA sequence level by multilocus sequence typing. The goal was to obtain information on the natural history, origin, and global dissemination of the JP2 clone of *A. actinomycetemcomitans*. Although genetically highly conserved, the study revealed that JP2 clone strains have a number of point mutations, particularly in the pseudogenes, *hbpA* and *tbpA* (29). Among a total of 66 JP2 clone strains, 11 sequence types with minor

**Fig. 1.** (A) Illustration of the geographic area of Africa proposed to be the site of emergence (+) of the JP2 clone of *A. actinomycetemcomitans*. After the characteristic mutational event, a 530-bp deletion in the promoter region of the leukotoxin operon, the JP2 clone has disseminated to many parts of the world. (B) Dots illustrate the countries from where JP2 clone-positive patients have been identified (as indicated in Table 1).
differences were identified. Such detailed studies on specific point mutations revealed characteristic mutations that allowed isolates from individuals from the Mediterranean area and from West Africa, including the Cape Verde islands, to be distinguished (29). The patterns of mutations indicate, as previously mentioned that the JP2 clone initially emerged in the Mediterranean part of Africa and subsequently spread to West Africa, from where it was transferred to the American continent during the transatlantic slave trade. However, the entire picture of the dissemination of the JP2 clone, even within the African continent, is still not completely known.

Sporadic signs of the spreading of the JP2 clone to Caucasians have been reported in the literature (60, 66, 111, 113). The proportion of individuals that are positive for the JP2 clone is probably underestimated, because it has only recently become clear that it is important to know the exact subtype of A. actinomycetemcomitans that the patients actually carry. Previously, microbiological diagnostic testing was solely focused on if the patients were positive or negative for A. actinomycetemcomitans at the species level, and subtyping according to the leukotoxin promoter type was probably not done. Subtyping according to the leukotoxin gene promoter type is a more recent phenomenon. Recently, analyses of a collection of A. actinomycetemcomitans strains from a microbiological laboratory in Umea in Sweden surprisingly showed that JP2 clone-positive individuals were occasionally found among patients previously found to be positive for A. actinomycetemcomitans at the species level. Among 2529 A. actinomycetemcomitans strains obtained from subgingival plaque samples, collected from 1084 patients during a 12-year period, the proportion of JP2 clone-positive patients was 2.6% [unpublished data, abstract (58)]. Thus, JP2 clone strains seem to be more widespread than initially believed, and they also seem to be spread among individuals of non-African origin, particularly in the Mediterranean part of the Middle East. The mechanisms behind the dissemination of the JP2 clone are not fully understood. Biologically as well as socially-determined factors (a host tropism for particular populations and/or a tendency towards a social separation of ethnically diverse population groups) might be involved.

Methods for the detection of the JP2 clone of A. actinomycetemcomitans

Methods for microbiological diagnostics have been available for years with the purpose to get information on the composition of the oral microbiota in patients, and to guide the clinicians in their choice of antimicrobial agent in the periodontal treatment of patients (114). However, clinicians have questioned, if microbiological diagnostic testing provides any additional information that would lead to different treatment approaches than if the clinicians did not have any information regarding the content of the dental plaque collected from patients before the initiation of the periodontal therapy. In the light of the possibility for the presence of the JP2 clone of A. actinomycetemcomitans, which is strongly associated with disease progression (33, 35, 81), it might be valuable and relevant information for clinicians treating periodontitis patients to know about the JP2 clone infection profile. For this purpose, it has been an aim of various studies to develop techniques that can be used to obtain such information.

PCR has been known for years and has been used to detect A. actinomycetemcomitans at the species level. In addition, a PCR specifically able to detect non-JP2 and JP2 genotypes of A. actinomycetemcomitans was developed and was described in the early 2000s by Poulsen and coworkers (115). More recently, Seki and coworkers developed a new method, loop-mediated isothermal amplification method (LAMP), for the identification of JP2 clone-positive plaque samples (116). One of the reported benefits of this method is the independency of special equipment and thereby that it can be used in settings with no access to various types of PCR equipment. In addition, the LAMP method is highly specific and sensitive. The LAMP test was found to have a specificity equivalent to and a sensitivity exceeding those described for PCR methods. Concerning the sensitivity, the detection limits for LAMP assay and PCR were 10 and 100 genome copies, respectively (116). Thus, the LAMP reaction is easy to set up, is not time-consuming to perform, and does not require special equipment. This provides several advantages in clinical settings and in population-based studies with limited access to laboratory technology (116). LAMP has been used by Elamin and coworkers in a clinical study on Sudanese adolescents (47). However, no patients in that study were positive for JP2 clone strains, neither by PCR nor by the LAMP technique (47). The LAMP technique was also used by Martinez-Martinez and coworkers to examine plaque samples from Mexican periodontitis patients with Down syndrome, but none of the patients included in that study was positive for the JP2 clone of A. actinomycetemcomitans (80). Two studies have reported on real-time PCR developed for the purpose of being able to quantitatively discriminate the highly leukotoxic JP2 clone strains from the non-JP2 genotypes of A. actinomycetemcomitans in the diagnosis of aggressive periodontitis (56, 117). So far these methods have not been used in clinical studies. Therefore, the real-time PCR method has not yet contributed with results or conclusions on quantitative aspects of the presence of JP2 and non-JP2 genotypes of A. actinomycetemcomitans and the initiation and development of periodontal attachment loss among the young.
Obviously, a number of techniques for the detection of the JP2 clone of *A. actinomycetemcomitans* are available, and can be used to generate information on the status of the JP2 clone infection in parallel to other types of periodontal intervention carried out concomitantly in these periodontitis patients.

### Exotoxins of *A. actinomycetemcomitans*

In the light of the overall topic of this review being particularly virulent clonal types of *A. actinomycetemcomitans* and their association with periodontal disease, it cannot be a surprise that attention is also given to updating research on the exotoxins of *A. actinomycetemcomitans*. Among many different virulence properties of *A. actinomycetemcomitans* (for reviews see Refs. 6, 8, 21, 118–125), the two exotoxins, being leukotoxin (LtxA) and cytotoxic distending toxin (Cdt), have been intensely studied over the years. Leukotoxin has been known for the longest period of time, and it is of specific interest for the virulence of the JP2 clone due to the high expression of leukotoxin from this genotype (31). In the following, the two exotoxins of *A. actinomycetemcomitans* and the most recent results within this field of research are addressed.

### Leukotoxin

**Structure and function of leukotoxin**

The ability of *A. actinomycetemcomitans* extracts to cause death of leukocytes was initially reported more than 30 years ago (126, 127). A protein, named leukotoxin (LtxA), was identified as the responsible molecule for the leukotoxic effect that was restricted to human polymorphonuclear leukocytes (PMNs) and monocytes (126–128). Later, it was shown that LtxA can also affect lymphocytes, erythrocytes, and endothelial cells from humans and cells of animal origin. However, higher concentrations of the toxin are needed than the concentrations which lyse PMNs and monocytes (129–133).

The *A. actinomycetemcomitans* LtxA operon consists of four coding genes designated *ltxC*, *ltxA*, *ltxB*, and *ltxD*, and an upstream promoter (134). The gene, *ltxA*, encodes for the structure of the toxin, *ltxC* encodes for components required for posttranslational acylation of the toxin, and *ltxB* and *ltxD* are involved in the activation and transport of the toxin to the bacterial outer membrane. There is great variation in the leukotoxin expression *in vitro*, although all *A. actinomycetemcomitans* strains harbour a complete leukotoxin operon (6). Zambon and coworkers showed that *A. actinomycetemcomitans* isolated from periodontally diseased subjects showed significantly enhanced leukotoxicity compared to isolates from periodontally healthy subjects (135). Interestingly, certain clones of the bacterium with enhanced leukotoxin expression have been shown to have a modified promoter in the LtxA operon (28, 65). The most well-known phenomenon is the previously mentioned highly leukotoxic JP2 genotype of *A. actinomycetemcomitans*, characterised by the 530-bp deletion in the promoter of the LtxA operon (28, 31).

Leukotoxin is a large pore-forming protein that consists of 1055 amino acids encoded by *ltxA* in the toxin operon (134, 136). The molecule can be divided into four regions based upon analysis of the amino acid sequence: the N-terminal region, the central region, the repeat region, and the C-terminal region (137). These four regions in the molecule structure are shared among many of the bacterial proteins in the RTX family (137, 138). The N-terminal region of LtxA, residues 1–408, exhibits alternating hydrophobic and hydrophilic clusters, and the pore-forming region have been suggested to be mediated by the hydrophobic clusters located between residues 175–400 (9, 18, 138). A cholesterol-binding site (CARC336), necessary for an efficient binding to the target cell membrane, has recently been demonstrated at residues 333–339 (139). The central region of the RTX proteins at residues 409–729 contains large hydrophilic domains, and two acylation sites of LtxA are located at lysine562 and lysine687 (140). The fatty acids at these positions have been shown to be necessary for the activity of the toxin and are suggested to contribute to the anchorage at the target cell membrane (9, 140). The repeat region of the RTX proteins consists of tandem repeats of a cassette with nine amino acids located between residues 730–900, and 14 such repeats have been identified in this region of LtxA (9, 18). The target cell receptor lymphocyte function-associated antigen-1 (LFA-1) binds to the repeat region, and this interaction has been shown to be responsible for the host cell specificity of LtxA (19, 141). In addition, the glycine-rich repeats in this region have strong capacity to bind Ca²⁺, and the presence of these cations mediates increased binding of the toxin to LtxA-sensitive LFA-1 expressing cells (142). Finally, residues 901–1055 at the C-terminal end of the RTX proteins have been shown to be necessary for the export of the toxin to the bacterial outer membrane by interactions with secretory proteins (138). This region of LtxA contains 20 extra basic amino acid residues in comparison with other RTX proteins and this is the reason for its high isoelectric point (9.7) (134).

The expressed LtxA is transported to the bacterial outer membrane by a type I secretion system (6, 143). Whether the expressed and exported LtxA remains associated with the bacterial outer membrane or is secreted into the environment is a topic of controversy, and the mechanisms that keep the toxin associated with the membrane are still not fully understood. However, the serum-mediated release of the toxin (144, 145), as well as its highly systemic immunogenic response (146), indicate...
a substantial release of the toxin from bacteria growing in an oral biofilm in vivo.

LtxA exhibits a unique specificity against cells of haematopoetic origin from humans and some other primates (19). This restricted host cell specificity suggests that the species-specific effect of LtxA is mediated through a unique receptor on the target cells (LFA-1), and a distinct region in the toxin that recognizes and interacts with the receptor (147, 148). The principal feature of this species recognition region of LtxA is that it contains a series of 14 tandemly repeated amino acid sequences in the repeat region of the toxin (18, 142). The domain of LtxA that recognizes the target cell receptor has been shown to be residues 688–941, examined by epitope mapping with monoclonal antibodies (141).

The LFA-1 molecule, identified as the LtxA target cell receptor, is a heterodimer consisting of the $\alpha_4$ (CD11a) and $\beta_2$ (CD18) subunits. The residues 1–128 on human CD11a have been shown to be important for the human specificity of LtxA-induced cell lysis (148). In addition, the extracellular region of human CD18 (residues 500–600) has been shown to be critical for conferring susceptibility to LtxA-induced cell lysis (147, 149). The most important host-related ligand of LFA-1 is the intercellular adhesion molecule 1 (ICAM-1), but this molecule binds to another region of the receptor than the residues identified for the LtxA binding (147, 148, 150).

It has been suggested that the role of LFA-1 in LtxA-mediated cell lysis is to help the protein to have a correct orientation on the target cell membrane, which might explain the enhanced sensitivity to LtxA in the LFA-1-expressing cells (9). Furthermore, the two fatty acids strengthen the anchorage of the toxin when inserted in the target cell membrane and the hydrophobic domain forms small pores in the membrane. It has been suggested that low concentrations of the toxin might induce apoptosis through loss of membrane integrity caused by the small pores, and that higher concentrations of the toxin allow oligomerization of LtxA-LFA-1 complexes on the target cell membrane, thus mediating a rapid and complete membrane collapse (9). In addition, LtxA has been shown to require lipid rafts for target cytolysis and a specific cholesterol-binding site has recently been identified on the LtxA molecule, which also indicates the importance of a high mobility on the target cell membrane (139, 151). The binding of LtxA to LFA-1 has been shown to result in internalization to the lysosomal compartment of the target cell (152).

The actual production of the toxin varies among A. actinomycetemcomitans strains. One of several factors that affect the toxin production is the presence of the 530-bp deletion in the promoter region of the leukotoxin operon (28, 66). In the JP2 clone strains with the 530-bp deletion, the leukotoxin production is reported to be 10 to 20-fold higher than in the non-JP2 genotypes of A. actinomycetemcomitans (28, 153). This is supported by recent transcriptomic analyses which show that the expression of LtxA is enhanced in serotype b strains, including the JP2 genotype (154). In addition, the expression of leukotoxin is reported to be regulated by other genetic and environmental factors (15, 155–158).

Host response to leukotoxin

Leukotoxin interacts with different host cells in a variety of ways that activate cellular and molecular mechanisms, some of which are associated with the pathogenesis of periodontitis. Taken together, the many virulence mechanisms of LtxA described below indicate an important role of this toxin in A. actinomycetemcomitans-induced periodontal breakdown, specifically for the JP2 genotype-associated periodontitis. However, it is still not fully known if other highly leukotoxic clones of A. actinomycetemcomitans besides the JP2 clone might have a significant association with disease progression (28, 65).

Polymorphonuclear leukocytes

LtxA and leukotoxins bacteria have been shown to efficiently cause death of human PMNs, and consequently LtxA is assumed to protect A. actinomycetemcomitans against phagocytic killing (6). Furthermore, analyses of PMNs exposed to leukotoxin have shown an extracellular release of proteolytic enzymes from both primary and secondary granules (11). Moreover, the interaction between LtxA and PMNs mediates activation and release of matrix metalloproteinase 8 (159). Taken together, these findings indicate that beyond causing death of the PMNs, LtxA also induces activation and release of proteolytic enzymes from these cells, which might contribute to the disease progression. Impaired PMN function is closely associated with periodontitis, and functional PMNs seem to be of importance when A. actinomycetemcomitans is present in the subgingival biofilm (160–163).

Lymphocytes

Lymphocytes were initially described as LtxA-resistant cells (126, 127). The first observation of LtxA-susceptible cells of lymphocytic origin was made by Simpson and coworkers (164) who showed that several lymphoid cell lines were killed in the presence of LtxA. In addition, LtxA was shown to suppress the function of peripheral blood lymphocytes (165). A few years later, Mangan and coworkers showed that T-cells isolated from human peripheral blood were affected by LtxA (129). This LtxA-induced T-cell death was a slow process compared to that which lysed human cells of myeloid origin and the cell death was shown to be induced through apoptosis (129). It has also been shown that the human natural killer (NK) cells are affected in a similar way by LtxA as the T-cells are, while the effects of LtxA on human B-cells or plasma cells have not been specifically addressed (165).
A recent report also showed a substantial LtxA effect on lymphocytes of rat origin (133).

Human lymphocytes show a great heterogeneity in regard to LtxA sensitivity and a subgroup of these cells has been shown to be lysed at approximately the same concentrations as human PMNs (130). Cells of lymphoid origin are rare in the infected periodontal pocket, but reside in high numbers in the surrounding tissues and in the lymph glands (166). It was shown more than 30 years ago that the onset of periodontitis involves a switch from a T-cell lesion to one involving large numbers of B-cells and plasma cells (166). A shift occurs in the balance between the so-called Th1 and Th2 subsets of T-cells with Th2 cells being associated with chronic periodontitis (167). More recently, T-regulatory (Treg) and Th17 cells have been detected in periodontal tissues indicating that these cells are also important in the host response and pathogenesis of periodontal disease (168). The strong acquired systemic humoral immune response induced by LtxA indicates direct contact between this molecule and cells of lymphoid origin (146, 169). The ability of LtxA to induce apoptosis in lymphocytes might contribute to a locally impaired acquired immune response in periodontal infections. The ability of LtxA to also affect lymphocytes indicates a possible role of this molecule in Th1/Th2/Th17 differentiation, a process that seems to be of great importance in the pathogenesis of inflammatory diseases, such as periodontitis (168).

Monocytes/macrophages

Previously, it was shown that human monocytes are sensitive targets for LtxA, and it has been described that the sensitivity of these subsets of leukocytes is at a similar level as for human PMNs (126). Characterisation of the LtxA-induced monocyte killing has been described in three different phases: (1) cessation of the membrane undulating folding and an accumulation of granulae in the perinuclear area; (2) abnormal membrane movement and strings of cytoplasm projecting from the cell; and (3) explosive release of cytoplasmic material from the cell (128). However, it should be taken into consideration that this study was made with ‘crude LtxA extract’ that contained a large number of other bacterial components. Rabie and coworkers showed that purified LtxA caused a rapid death of human monocytes in mixtures of the toxin with peripheral blood mononuclear leukocytes (MNLs) (165). More recently, analyses of different subsets of leukocytes separated from peripheral blood of a single donor showed that monocytes have an enhanced sensitivity to LtxA compared with PMNs and lymphocytes (130). The LtxA-induced monocyte lysis was shown to involve activation of caspase-1, which indicates involvement of pro-inflammatory intracellular signalling (Fig. 2). Caspase-1 is a cytosolic cysteine proteinase that specifically induces activation and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (170). Both of these pro-inflammatory cytokines are expressed as biologically inactive precursors and have to be cleaved by caspase-1 for activation and secretion. Caspase-1 is activated by incorporation in a cytosolic multimer complex named the inflammasome (171). The intracellular signalling pathways involved in LtxA-induced inflammasome activation in human monocytes/macrophages have not yet been determined. A partial characterisation of this process indicates involvement of K⁺ efflux and ATP release that might activate purinergic receptors, such as the P2X₇ (172).

The observation that LtxA induces activation of caspase-1 in human inflammatory defence cells indicates a new role for this virulence factor as a mediator of the pro-inflammatory host response. Human macrophages (adherent blood monocytes), exposed to LtxA, activate a rapid and abundant secretion of bioactive IL-1β (173). Moreover, exposure of human macrophages to components of Gram-negative oral pathogens causes an increased accumulation of cytosolic pro-IL-1β and inflammasome molecules that is not activated and released (reformulate) (174, 175). LtxA or leukotoxic A. actinomycetemcomitans induces cleavage and secretion of these accumulated pro-inflammatory molecules, a property that is abolished in A. actinomycetemcomitans mutants without LtxA expression (174). The IL-1β secretion is activated already at a concentration of one bacterium/macrophage in interactions with bacteria from the highly leukotoxic JP2 genotype of A. actinomycetemcomitans and with a similar activation by non-JP2 genotype strains, however, at a 10-fold higher concentration (174). Taken together, these data show that LtxA is the major component of A. actinomycetemcomitans for the induction of the activation and release of IL-1β from human macrophages, and that this effect is further enhanced by priming of the macrophages with other bacterial components.

Macrophages are rare cells in a healthy periodontium, but they are often found in high numbers in tissues from periodontal lesions (166). These cells are recruited to the infected site from the peripheral blood monocytes that are attracted by ICAM-1-expressing endothelial cells. The monocytes pass through the vessel wall and migrate towards a gradient of compounds in the connective tissue that are released from the oral biofilm and the activated host cells (176). During diapedesis, the monocytes differentiate into macrophages and the inflammatory machinery is up-regulated during this process and further during the migration towards the infected site. This process involves an accumulation of pro-inflammatory precursor molecules, such as IL-1β and IL-18 in the migrating macrophages (170). A secondary stimulus is needed to induce activation and release of the accumulated precursors of IL-1β and IL-18 in the primed
macrophages (172, 174). In case of an infection containing *A. actinomycetemcomitans*, the gradient of bioactive components in the connective tissue will contain LtxA, and the migrating macrophages will sooner or later meet concentrations of LtxA that activate secretion of these pro-inflammatory cytokines into the surrounding tissues. If this process is activated in the tooth-supporting tissues in the vicinity of the infection, it might cause imbalance in the host inflammatory response and promote pathogenic cellular mechanisms (Fig. 3, modified from Ref. 177).

The highly systemic immunogenic host response against LtxA of *A. actinomycetemcomitans*-infected subjects indicates that direct contact between the antigen-presenting macrophages and LtxA occurs (146, 169). The enhanced LtxA sensitivity of human macrophages indicates that these antigen-presenting cells might be affected during a primary infection with leukotoxic *A. actinomycetemcomitans*, which might cause a delayed acquired immune response.

**Erythrocytes**

The ability of some strains of *A. actinomycetemcomitans* to cause β-haemolysis on blood agar plates has been known for many years (27, 30, 178). It was later reported that the haemolysis of red blood cells of human and animal origin that is caused by *A. actinomycetemcomitans* involves interaction with LtxA (22, 131). Different strains of the bacterium with varying expression levels of LtxA show specific patterns when cultured on blood agar plates containing fresh horse blood. Red blood cells lack the expression of the LtxA receptor, LFA-1, which has been shown to be a prerequisite for the LtxA-induced leukocyte lysis (141). The cellular and molecular mechanisms for this haemolytic effect of LtxA are therefore not fully understood. It has recently been shown that LtxA-induced haemolysis involves interaction with the P2X7R (P2X purinoceptor 7, an ATP-gated ion channel) on the surface of the red blood cells (179). In addition, the extracellular release of ATP is mediated through a pore formed by the toxin (180). Whether this haemolytic property of LtxA is a virulence mechanism is still not known.

**Endothelial cells**

LtxA has recently been shown to induce a substantial pro-inflammatory effect on human brain endothelial cells by up-regulation of ICAM-1 and VCAM-1 (132). Furthermore, a higher concentration of LtxA was found to decrease proliferation and induce apoptosis in microvascular endothelial cells. The involvement in the pathogenesis of periodontitis by these mechanisms is not known, but the endothelial cells may play a role as a link between periodontitis and cardiovascular diseases.

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Fig. 2. *A. actinomycetemcomitans* with its leukotoxin is a useful tool to study important virulence mechanisms for the progression of periodontitis. This bacterium has a strong association with aggressive forms of periodontitis, and the leukotoxin may represent a major virulence factor. We have identified several cellular activation pathways that are induced upon exposure to leukotoxin (Ref. 12). Briefly, leukotoxin binds to the LFA-receptor (1) and activates an extracellular release of ATP (2), which acts as a ligand for the P2X₇-receptor, and results in an efflux of potassium (3). These events activate the formation of an inflammasome multimer (4) that activates the cysteine proteinase caspase-1, resulting in activation (5), and a massive secretion of IL-1β (6). This pro-inflammatory cytokine is a key molecule that regulates the balance between catabolic and anabolic processes in tissue homeostasis, and therefore is of specific interest for tissue degenerative diseases, such as periodontitis (modified from Ref. 177).
Cytolethal distending toxin

The cytolethal distending toxin (Cdt) is a highly conserved exotoxin produced by a number of Gram-negative bacteria (181). It affects mammalian cells by inhibiting cell division and by causing apoptosis (182). The active holo-toxin is a heterotrimeric complex of CdtA, CdtB, and CdtC. CdtA and CdtC are necessary for the secretion of the toxin, while CdtB is responsible for the biological activity (183). CdtB has a sequence homology with mammalian DNaseI, indicating a critical role for nuclease activity in host parasite interactions (184). Apart from blocking cell cycle progression, Cdt also induces expression of the receptor activator of NF-kappaB ligand (RANKL) in human periodontal fibroblasts and lymphocytes (185, 186). RANKL is a key cytokine for bone resorption and could therefore be associated with the pathogenic mechanisms of periodontitis (187). In addition, Cdt affects the oral epithelium ex vivo and therefore might contribute to impair the barrier function of epithelial cell layers against invading microorganisms (188, 189). Initially, the Cdt was discovered in *A. actinomycetemcomitans* by Sugai and coworkers (190). However, already in the 1980s and the beginning of the 1990s, several studies reported on the inhibition of cell proliferation, cell cycle-specific growth inhibitory effect, and immunosuppression induced by *A. actinomycetemcomitans* (191–194).

Of interest in relation to periodontitis, it is known that Cdt resides in the variable region of the pangenome, and consequently it is not present in all *A. actinomycetemcomitans* strains (107). However, the genes are present in the majority of the isolated *A. actinomycetemcomitans*, including the JP2 clone strains, but the proportion of isolates that lack all or some of the genes varies among the populations studied (25, 72, 195–201).

The systemic immunoreactivity to Cdt has also been studied and assumed to be a marker for the presence of Cdt-expressing *A. actinomycetemcomitans* (202, 203). Interestingly, while all carriers of *A. actinomycetemcomitans* exhibit neutralizing antibodies to LtxA (146), the systemic immunoreactivity to Cdt is not always able to neutralize the toxin (202, 203). Thus, Cdt can sometimes act as a virulence factor that is not neutralized by an acquired humoral immune response.

Despite substantial evidence supporting the fact that Cdt can function as a virulence factor of pathogens producing the toxin (181), the importance of Cdt in the pathogenesis of periodontal disease seems to be limited. This topic was addressed in a recent longitudinal study based on 500 Ghanaian adolescents (201). In that study,
the progression of attachment loss was mainly associated with the presence of *A. actinomycetemcomitans*, and progression of the periodontal attachment loss was only weakly associated with the *cdt*-genotype of the bacterium (201).

**Treatment of JP2 clone-associated aggressive periodontitis**

As mentioned above, *A. actinomycetemcomitans* has numerous important virulence properties (6, 8, 21), and the bacterium is considered as a periodontal pathogen. For decades, clinicians and researchers have been aware of the potential role of *A. actinomycetemcomitans* in periodontal disease and particularly its association with disease in adolescents. This focus has been intensified in light of the reporting of particularly virulent clones of *A. actinomycetemcomitans* and their strong association with disease. Although reports on the treatment of JP2 clone-associated periodontitis are limited, this topic will be touched on below as a part of the translational research line addressed in the present review.

Very limited information is available concerning therapeutic aspects of the JP2 clone-associated periodontitis. One report has addressed the topic directly and has focused on the response to periodontal treatment in subjects infected with either JP2 or non-JP2 genotypes of *A. actinomycetemcomitans* (204). The use of antibiotics as a supplement to conventional periodontal therapy has also been studied by Cortelli and coworkers (204). A total of 25 JP2 clone genotype-positive subjects and 25 non-JP2 genotype-positive subjects were included, and participants received scaling and root planning, systemic antibiotic therapy, and periodontal surgery during the first 4 months of the study. The administration of systemic antibiotics (prescription of 21 tablets of metronidazole at 250 mg plus amoxicillin at 500 mg, one tablet every 8 hours for 7 days) was conducted in combination with mechanical debridement in the second month of the study period. In that study, probing pockets depths, clinical attachment level and gingival and plaque indices were monitored at baseline and at a 1-year follow-up, along with PCR-based detection for the presence of JP2 and non-JP2 genotypes of *A. actinomycetemcomitans*. The overall conclusion of the study was that patients infected with JP2 clone strains showed less response to the periodontal therapies than did subjects infected with the non-JP2 genotypes of *A. actinomycetemcomitans*. The overall conclusion of the study was that patients infected with JP2 clone strains showed less response to the periodontal therapies than did subjects infected with the non-JP2 genotypes of *A. actinomycetemcomitans* (204). Surprisingly, more than half of the subjects remained positive for the JP2 clone strains, even after the intensive treatment employed in the study. This made the authors conclude that establishment of a more effective therapy that can eliminate highly leukotoxic clones of *A. actinomycetemcomitans* is required (204). Concerning the use of antibiotics in the treatment of JP2 clone-associated aggressive periodontitis, it is worth mentioning that the potential problems related to antibiotic resistance may vary in different countries. Sanz and coworkers found a variation in the composition of the subgingival microbiota of two periodontitis populations of different geographical origin. Thirty-one adult periodontitis patients from Spain and 30 comparable patients from The Netherlands were included in the study (205). *A. actinomycetemcomitans* was significantly more prevalent (23.3% vs. 3.2%) in the Dutch group, while *Porphyromonas gingivalis* was significantly more prevalent (64.5% vs. 36.7%) in the Spanish group. Thus, the subgingival microbiota from the Spanish group was characterised by a high prevalence of *P. gingivalis* and low prevalence of *A. actinomycetemcomitans*, while the microbiota from the Dutch group was characterised by a high prevalence of both *A. actinomycetemcomitans* and *Peptostreptococcus micros* (205). In addition, the antimicrobial susceptibility profiles of five periodontal bacterial species, being *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Micromonas micros*, isolated from periodontitis patients in Spain and in The Netherlands were analysed (206). Eight different antibiotics were tested on all the bacterial isolates, and it was concluded that differences exist in the susceptibility profiles of the periodontal pathogens isolated from periodontitis patients in Spain and in The Netherlands. This indicates that antibiotic susceptibility testing is necessary to determine efficacy of antimicrobial agents (206). Furthermore, the findings from the study indicate that it may not be possible to develop uniform protocols for the usage of antibiotics in the treatment of periodontitis (206). More recently, a study concluded that *A. actinomycetemcomitans* JP2 homotypic biofilms were more susceptible *in vitro* to doxycycline than amoxicillin plus metronidazole (207), which is the cocktail of antibiotics usually recommended in aggressive periodontitis patients (208–214). The scenario concerning resistance to various antibiotics has not been addressed in relation to the treatment of the JP2 clone-associated periodontitis, for example, in studies carried out in Northwest African countries. Thus, in the Northwest African countries, treatment outcomes of JP2 clone-associated periodontitis, including the use of antibiotics are unknown at present.

**Conclusions and future perspectives**

Accumulating knowledge at the genome level, including rather detailed information on specific virulence factors, and information from transcriptomic and proteomic analyses of *A. actinomycetemcomitans* strains are available today (215–218). These provide an excellent platform for more studies on the details of the pathogenic mechanisms of *A. actinomycetemcomitans* in the future.

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One of the virulent clones that have been intensely studied is the highly leukotoxic JP2 clone of *A. actinomycetemcomitans*. Strong evidence exists for the role of the JP2 clone of *A. actinomycetemcomitans* as an aetiological factor in the pathogenesis of aggressive periodontitis in certain populations. However, it is still not clear if the link to the Northwest African populations is related to a particular host tropism or to a ‘slow’ geographic dissemination of the JP2 clone. A ‘slow’ dissemination of the JP2 clone could occur due to a number of factors that affect the ability of the disease to manifest itself under various conditions in human beings. These factors may include the general health status, living circumstances, social behaviour, level of knowledge concerning possibilities for the prevention of periodontitis, and access to dental care. In addition, humans sometimes migrate from one country to another or from one continent to another, but they still tend to cluster with other humans with a similar ethnic origin, and that could diminish the likelihood of the spreading of the JP2 clone. Although the dissemination of the JP2 clone is more widespread than initially anticipated, the spreading of the clone does not seem to occur easily, as seen in the light of the estimated time of its emergence in the Mediterranean part of Africa more than 2000 years ago (29).

While the leukotoxicity of JP2 clone strains of *A. actinomycetemcomitans* has been studied and reported on, it is still not clear if other highly leukotoxic clonal types of *A. actinomycetemcomitans* exist. One report from Japan has reported on an *A. actinomycetemcomitans* isolate with an insertion in the leukotoxin operon and with an increased leukotoxicity (65). In addition, Åberg and coworkers have contributed with further information on the leukotoxic activity of *A. actinomycetemcomitans* strains (219). This study supports an important role of the leukotoxin in the pathogenesis of aggressive periodontitis. Concerning the Cdt, it has been shown that presence of *A. actinomycetemcomitans* with or without Cdt activity has similar importance for the disease progression, indicating a less important role of this exotoxin (201).

Whereas several techniques have been developed that aim to detect *A. actinomycetemcomitans*, including specifically the JP2 clone strains (56, 115–117), limited information concerning strategies for the treatment of the JP2 clone-associated periodontitis has been reported (204).

A future preventive strategy for aggressive periodontitis might be modulation or blocking of virulence potential. Recently, we have found that an extract from the tropical plant, *Psidium guajava*, efficiently neutralizes the *A. actinomycetemcomitans* leukotoxin (220). This knowledge might contribute to the development of new preventive strategies in the future, strategies that may also be available for individuals in the developing countries.

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