# Different growth rates in amoeba of genotypically related environmental and clinical *Legionella pneumophila* strains isolated from a thermal spa

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## SUMMARY

Two cases of legionellosis occurring 3 years apart were acquired in the same French thermal spa and were apparently due to the same strain of *Legionella pneumophila* serogroup 1, as shown by genomic macrorestriction analysis. Minor differences between the two isolates were found by random amplification PCR profiling which showed an additional band with one of the isolates. Analysis of 107 *L. pneumophila* strains isolated from the spa waters by genome macrorestriction failed to identify the infective strain, but a closely related *L. pneumophila* serogroup 3 strain differing from the clinical isolates by only one band was found. To determine if the clinical *L. pneumophila* serogroup 1 isolates was better adapted for intracellular multiplication than related serogroup 3 environmental isolates, the growth kinetics of six isolates were determined in co-culture with *Acanthamoeba lenticulata*. One clinical isolate failed to grow within amoeba, while the other clinical isolate yielded the highest increase in bacterial cell count per amoeba (1200%) and the environmental isolates gave intermediate values. Genetic analysis of *L. pneumophila* isolates by DNA macrorestriction does not therefore appear to reflect their growth kinetics within amoeba, and is not sufficiently discriminatory to identify potentially virulent strains.

## INTRODUCTION

*Legionella pneumophila* [1] belongs to the *Legionellaceae* family, which comprises at least 43 species [2]. *L. pneumophila* serogroup 1 is most frequently associated with legionellosis. Legionellae are inhaled in aerosols created mainly by hot water distribution systems, cooling towers and thermal spa water [3–7]. *L. pneumophila* is a facultative intracellular pathogen that infects human macrophages, monocytes and epithelial cells [8–10], and in the

aquatic environment, it can survive and multiply within amoebae, which act as natural hosts. Uptake by amoeba and survival of *L. pneumophila* is influenced by environmental conditions such as temperature [11]. Bacteria growing within amoeba are changed phenotypically and exhibit an increased resistance to antibiotics and biocides when compared with cells grown in conventional media [12–14].

*In vitro* co-culture models have been developed to study the interaction between amoebae and legionella, and the effects of external conditions (temperature, sunlight, etc.) on the growth of the two organisms

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[5, 15, 16], as well as to identify several legionella genes involved in virulence. The growth kinetics of *L. pneumophila* within amoeba such as *Acanthamoeba* spp. [17, 18] and *Hartmannella* spp. [19] vary according to the bacterial strain and extrinsic factors such as the number of subcultures of the strain [20]. Strains lacking *mip* (macrophage infectivity potentiator gene) [21], *dot* (defect in organelle trafficking gene) [22], *icm* (intracellular multiplication gene) [23], *eml* (early stage macrophage-induced locus) [24], and *pmi* (protozoan and macrophage infectivity loci) [25, 26] grow more slowly than the parent strains in coculture. A study of the general stress response of *L. pneumophila* to a modified cellular environment identified the role of *rpo*S gene [27], which regulates genes that enable its survival within the protozoa [28]. Other genes are also required for intracellular survival during the early stages of infection and include *pil BCD*, *eml*-early macrophage induced locus or intracellular replication (*asd*-aspartate semialdehyde deshydrogenase) [29]. A relationship between growth kinetics and virulence is suspected, as *L. pneumophila* strains with the highest growth rate in co-culture also display enhanced entry into mouse or human cells [17, 18, 30, 31].

In 1994 and 1997, *L. pneumophila* type 1 isolates were recovered from two patients who had developed legionellosis at the same French Alpine thermal spa. In order to determine the precise source of infection, legionella isolates from the spa waters were compared to the two clinical isolates by means of pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD). The growth kinetics of the clinical and environmental legionella isolates within *Acanthamoeba* sp. were compared to determine whether this parameter reflected virulence *in vivo*.

## MATERIALS AND METHODS

### Patient and environmental isolates

The clinical isolates were obtained by bronchoalveolar lavage (BAL) from two patients infected during stays at the same thermal spa. Patient 1 (B.P.) was a 40-year-old man with Still's disease, who was receiving 21 mg of prednisone daily and 40 mg of methotrexate weekly. Against medical advice, he attended the thermal spa in July 1994 for a routine 21-day thermal cure. In August 1994, 5 days after returning home, he developed severe acute pneumonia involving both lungs on the chest X-ray film. He was admitted to an
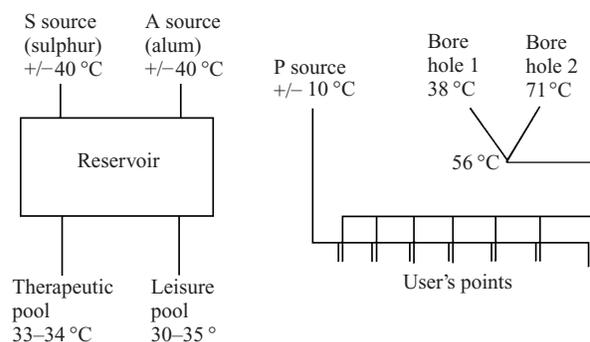


**Fig. 1.** Schematic diagram of the thermal spa water distribution system.

intensive care unit (ICU), where a BAL fluid sample grew *L. pneumophila* serogroup 1 ($> 10^5$ c.f.u./ml). His condition deteriorated rapidly despite appropriate antibiotic therapy and he became neutropenic (500 G/L). He died on the fourth day in ICU. The clinical strain (SC94) was stored at $-80$ °C.

Patient 2 (F. J.) was a 69-year-old man with chronic obstructive bronchopneumonia who attended the spa in August 1997. Fifteen days after his arrival he developed fever, cough and dyspnoea. Crackles were present and the chest X-ray film showed signs of pneumonia. He received an oral *β*-lactam antibiotic but his clinical status worsened and he was admitted to an ICU where he was given ofloxacin and cefotaxime, which was replaced with rovamycin when BAL fluid culture yielded *L. pneumophila* serogroup 1 (10 c.f.u./ml). He was cured after 20 days of antibiotic treatment. The clinical strain (SC97) was stored at $-80$ °C.

To determine the source of infection, 11 water samples were collected throughout the spa's distribution system. The thermal spa receives water from three natural springs (S, sulphur; A, alum and P, cold water) and two bore holes (Bh1 and Bh2). Water from these sources are then mixed as shown schematically in Fig. 1, and distributed throughout the buildings at temperatures optimal for the various uses. The 11 samples yielded 107 legionella strains. The environmental isolates were collected over a 2-year period following the second case of legionellosis at the thermal spa. Eighty-one strains were identified as *L. pneumophila* (27 serogroup 1, 1 serogroup 2, 62 serogroup 3, 3 serogroup 6 and 9 serogroup 13), and 26 as *L. dumoffii* (Table 1). Seven isolates came from source A, 6 from source S, and 3 from source P; 44 isolates came from the thermal water distribution network, 13 from pools and 5 from internal reservoirs (Table 1). All strains were identified by a combination

Table 1. *PFGE types of the* L. pneumophila *isolates from patients and thermal spa water*

| Strain designation | Origin | Serogroup | PFGE type |
|---|---|---|---|
| Clinical strains | | | |
| SC94 | Patient 1 | 1 | U |
| SC97 | Patient 2 | 1 | U |
| Environmental strains | | | |
| AX1–13 | User's point | 3 | A |
| AX15–26 | User's point | 3 | A |
| AX28–29 | User's point | 3 | A |
| AX32 | User's point | 3 | A |
| AX61 | User's point | 13 | A |
| AX14 | Pool PMI | 3 | A |
| AX27 | Pool PMI | 3 | A |
| AX30–31 | Pool PMI | 3 | A |
| AX34 | User's point | 3 | B |
| AX33 | User's point | 3 | C |
| AX35 | User's point | 3 | D |
| AX36–37 | Reservoir | 3 | E |
| AX38 | Source A | 3 | F |
| AX42 | Source A | 3 | F |
| AX40 | Source S | 3 | F |
| AX39 | Pool PMI | 3 | F |
| AX41 | Pool PMI | 3 | F |
| AX43 | Source S | 3 | G |
| AX44 | User's point | 3 | H |
| AX45–46 | Source A | 3 | I |
| AX75–77 | User's point | 6 | J |
| AX72–74 | Source A | 1 | K |
| AX60 | Reservoir | 3 | L |
| AX78 | Reservoir | 2 | M |
| AX55–56 | Source S | 3 | N |
| AX57 | User's point | 3 | O |
| AX58–59 | Pool PMI | 3 | P |
| AX62–68 | User's point | 13 | Q |
| AX69 | User's point | 13 | R |
| AX70 | Source S | 1 | S |
| AX47 | Source S | 3 | T |
| AX80–81 | Source P | 1 | U |
| AX71 | User's point | 1 | U |
| AX48–49 | Pool PMI | 3 | U |
| AX51 | Pool PMI | 3 | U |
| AX52 | User's point | 3 | U |
| AX54 | User's point | 3 | U |
| AX82 | Cold water | 3 | U |
| AX50 | Reservoir | 3 | U |
| AX53 | Reservoir | 3 | U |

of biochemical activity [32] and direct fluorescent assay with adsorbed and unadsorbed sera [33]. The reference *L. pneumophila* strain ATCC 33152 was used as a control.

**Pulsed field gel electrophoresis (PFGE)**

Genomic DNA was prepared as previously described with some modifications [34, 35]. Briefly, legionellae were treated with proteinase K (50 $\mu$g/ml) in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8) for 24 h at 55 °C, and DNA was digested with 20 IU of *Sfi*I restriction enzyme (Boehringer–Mannheim, Meylan, France) for 16 h at 50 °C. Fragments of DNA were separated in 0·8% agarose gel (Fast-Lane, FMC) prepared and run in 0·5 mM Tris-borate-EDTA

buffer (pH 8·3) in a contour-clamped homogeneous field apparatus (CHEF DRII system; Bio–Rad, Ivry sur Seine, France) with a constant voltage of 150 V. Runs were carried out with constant pulse times (25 s) at 10 °C for 11 h and increasing pulse times (35–60 s) at 10 °C for 11 h. The agarose gels were stained with ethidium bromide and photographed under UV light. Band patterns were interpreted with the aid of Taxotron software (Institut Pasteur, Paris, France), based on the unweighted pair grouped with mathematical average (UPGMA) method to construct dendrograms. Isolates with patterns which differed by no more than three restriction fragments were considered to have the same pulsotype, while organisms differing by more than three restriction fragments were considered sufficiently divergent to warrant a separate pulsotype designation [36].

### RAPD technique

DNA was extracted from legionellae by a thermal lysis procedure [37] followed by phenol/chloroform/isoamyl alcohol purification and precipitation in absolute ethanol. Random amplification was performed using previously described random primers [24] (Eurogentec, Seraing, Belgium): AP8 (5′-TT-GCTGGCCTAGTTAAACGTA-3′) and AP9 (5′-ATGCGTAACCGTAACGTGCTGACT-3′). The reaction mixture consisted of 5 $\mu$g of DNA template, 4 mM MgCl$_2$, 0·2 mM of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 2·5 U of *Taq* DNA polymerase (Amplitaq; Perkin–Elmer Cetus, Branchburg, N.J.), and 50 pmol of each primer in PCR buffer (10 mM Tris–HCl pH 8·3, 50 mM KCl, 0·001 % gelatin; Perkin–Elmer Cetus). PCR cycles comprised a 1-min denaturation step at 94 °C, followed by hybridization for 2 min at 30 °C and extension at 72 °C for 1 min (40 cycles) and for 3 min at 72 °C (one final extension cycle). PCR products were run on standard 1·5 % agarose gel (SeaKem GTG; FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and photographed under UV light.

### Co-culture of legionella with amoebae

*Acanthamoeba lenticulata* PD2, an axenized reference strain, was cultured in a 10 % X-ray-inactivated fetal calf serum casein glucose yeast extract medium (SCGYEM) at 30 °C. Amoebae were inoculated into 25 cm² tissue culture flasks (Greiner laboratories, Frickenhausen, Germany) containing 14 ml of SCGYEM liquid medium and incubated for 72 h at 30 °C. SCGYEM medium was then replaced by the same volume of saline solution, as previously described [26]. Bacterial strains were grown on buffered charcoal yeast extract (BCYE) agar supplemented with 0·1 % $\alpha$-ketoglutarate, glycine, vancomycin and colistin (GVPC) for 72 h. Co-culture was conducted at 30 °C in saline solution at pH 7 in tissue culture flasks with a ratio of one bacterium per 100 amoebae. Controls included the saline solution alone, with either legionellae or amoebae. Amoebae and legionellae were both quantified after 1, 2 and 3 days. Amoebae were counted directly in the tissue culture flask, using an inverted microscope, and the whole content of a tissue culture flask was removed. Amoebae were lysed by drawing the suspension 3–5 times through a 27-gauge needle [16]. Legionellae (extra- and intracellular) were counted by dilution plating in triplicate on GVPC agar with incubation at 35 °C in 2·5 % CO$_2$-air for 5–7 days. The ratio of bacterial increase versus the number of amoeba was calculated for each co-culture. Each experiment was repeated three times and standard errors were calculated from the repeated measurements.

## RESULTS

### PFGE patterns of the clinical and environmental isolates

Macrorestriction profiles of the 107 environmental and 2 clinical isolates contained 5–15 fragments ranging in size from 50 to 1000 kb. A total of 21 pulsotypes were identified (Fig. 2), strains within a given pulsotype differing by no more than three bands [38]. Pulsotype A was the most frequent, comprising 32 environmental *L. pneumophila* serogroup 3 isolates. The two clinical isolates, SC94 and SC97, both belonged to pulsotype U, as did 11 environmental isolates (3 serogroup 1 and 8 serogroup 3) originating from pools, reservoirs, the thermal water sources, and the cold water source. No profile identical to that of the clinical isolates was identified among the environmental isolates but the profile of the two clinical isolates differed by only a single band from that of five serogroup 3 environmental isolates, and were thus regarded as highly related to the latter.
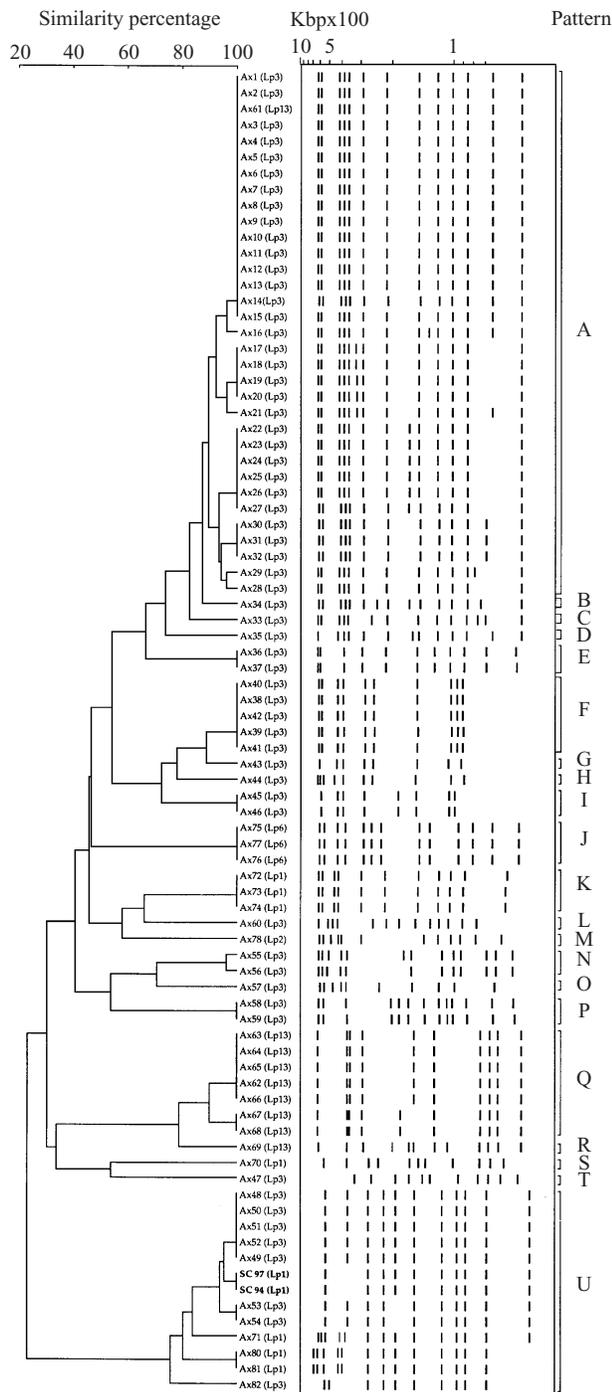
Fig. 2. Dendrogram and schematic representation of the pulsotypes of two clinical and 81 environmental isolates of *Legionella pneumophila*; the error threshold is 4%.



**Fig. 3.** RAPD patterns of clinical and selected environmental isolates from the thermal spa. Lane 1, negative control (no DNA); lanes 2 and 3, clinical isolates (SC94 and SC97); lanes 4, 8, 9 and 10, environmental isolates of serogroup 1 (A×71, A×80, A×81 and A×2); lanes 5, 6 and 7, environmental isolates of serogroup 3 (A×52, A×54 and A×82); lane 11, *L. pneumophila* Philadelphia 1; lane M, size markers.

## RAPD patterns

To confirm the genetic relatedness of the pulsotype U isolates, the two clinical isolates and four representative pulsotype U environmental isolates were tested by RAPD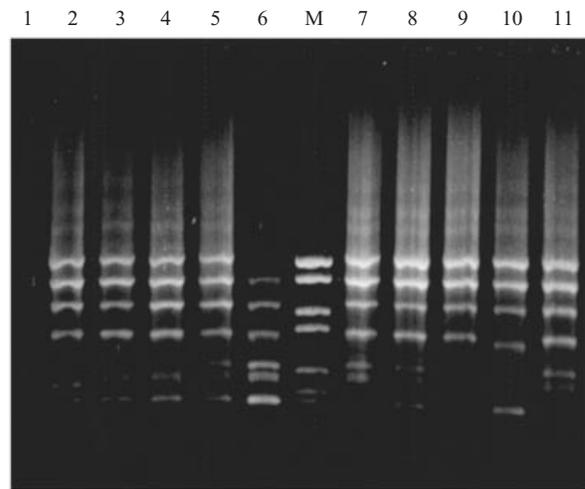. The six isolates differed from one another by a maximum of one band, whereas control isolates (one pulsotype A isolate and the reference strain ATCC 33152) differed from the six strains by 3 or 4 bands (Fig. 2). The RAPD profiles of the two clinical isolates differed from each other by one band, while clinical isolate SC97 had a RAPD profile identical to that of environmental isolate A×71 (Fig. 3, Table 2).

## Intra-amoebic growth rates

The six isolates tested above by RAPD were studied for their growth kinetics in culture with *Acanthamoeba lenticulata*. Values differed considerably among the isolates (Fig. 4, Table 2). Interestingly, clinical isolate SC94 showed a 1200% increase in cell count per amoeba, whereas clinical isolate SC97 failed to replicate. Values for the environmental and control isolates were intermediate between those of the two clinical isolates (Table 2). None of the legionella isolates grew in saline alone. Ranking of the isolates according to their intra-amoebic multiplication rate did not reveal the ascendancy of one of the two serogroups and did not correlate with the RAPD patterns (Table 2). For a selected number of cases, co-culture experiments were also performed in the presence of gentamicin to remove extracellular

Table 2. *Characteristics of clinical strains and closely related environmental strains of* L. pneumophila

| Strain designation | Bacterial increase per amoeba (%) | PFGE type | RAPD type |
|---|---|---|---|
| Clinical strains | | | |
|   *L. pneumophila* serogroup 1 SC94 | 1200·84 | U | A |
|   *L. pneumophila* serogroup 1 SC97 | − 1·10 | U | B |
| Environmental strains | | | |
|   *L. pneumophila* serogroup 3 AX54 | 357·16 | U | C |
|   *L. pneumophila* serogroup 3 AX82 | 103·18 | U | E |
|   *L. pneumophila* serogroup 1 AX71 | 80·23 | U | B |
|   *L. pneumophila* serogroup 3 AX2 | 21·90 | A | D |
|   *L. pneumophila* serogroup 3 AX52 | 17·82 | U | C |
| Reference strain | | | |
|   *L. pneumophila* serogroup 1 reference strain ATCC 33152 | 11·26 | — | E |

legionellae. The absolute number of countable bacteria was always lower but the kinetics of growth were similar to those obtained without antibiotics (not shown).

## DISCUSSION

Despite the broad range of legionella strains recovered from the water system of this thermal spa, in terms of the number of species and pulsotypes, molecular methods (PFGE and RAPD) suggested that a single strain of *L. pneumophila* serogroup 1 (i.e. a clone) caused two cases of legionellosis that occurred 3 years apart. However, none of the environmental isolates had a pulsotype identical to that of the clinical isolates, and the most closely related profiles were those of several serogroup 3 environmental isolates. This suggested that the serogroup 1 clinical strain may have been derived from a serogroup 3 environmental isolate with a related pulsotype. Indeed, Harrisson *et al.* reported that genotypically related strains of *L. pneumophila* could express different serogroup-specific antigens [39, 40]. Other legionella attributes, such as expression of the flagellum, are also modulated by environmental factors [41].

The presence of *L. pneumophila* serogroup 3 along with *L. pneumophila* serogroup 1 and *L. dumoffii* in this thermal spa had been reported in 1988 [6]; unfortunately, these environmental strains were no longer available for this study. More than 10 years later, and despite cleaning programmes and renovation of the water distribution system, the present study shows a very similar species and serogroup distribution. It should be noted that the use of chlorine is not allowed in French thermal spas in order to preserve the characteristics of the mineral water. Long-term persistence (for up to 10 years) of the same *L. pneumophila* serogroup 6 strain in a hospital water distribution system, and its association with sporadic cases of infection, has been reported [42, 43].

The predominance of serogroup 3 in the water distribution system of this thermal spa, and the involvement of a serogroup 1 strain in the only two cases of legionellosis reported, suggest that the serogroup 1 antigen is better adapted to human infection, while the serogroup 3 antigen may be better adapted to the aquatic environment. Acanthamoeba, which is an appropriate model for intracellular multiplication of legionella in mammalian cells [17], was thus used to study the growth of the clinical isolates and closely related environmental isolates of *L. pneumophila*, and its relationship with human virulence. We observed considerable variations in growth rates, even between isolates from the same serogroup (Table 2). Furthermore, the isolate recovered from the patient who died grew rapidly in amoebae, although a differential uptake capacity could play a role, while the other, isolated from the patient who survived, failed to grow at all. Co-culture with macrophages might have given different results. Although patient 1 and 2 had different underlying health problems, our results show that macrorestriction analysis is unable to distinguish between strains with high and low virulence in humans. RAPD, which is an additional discriminatory method to PFGE [42–45], showed that the two clinical strains
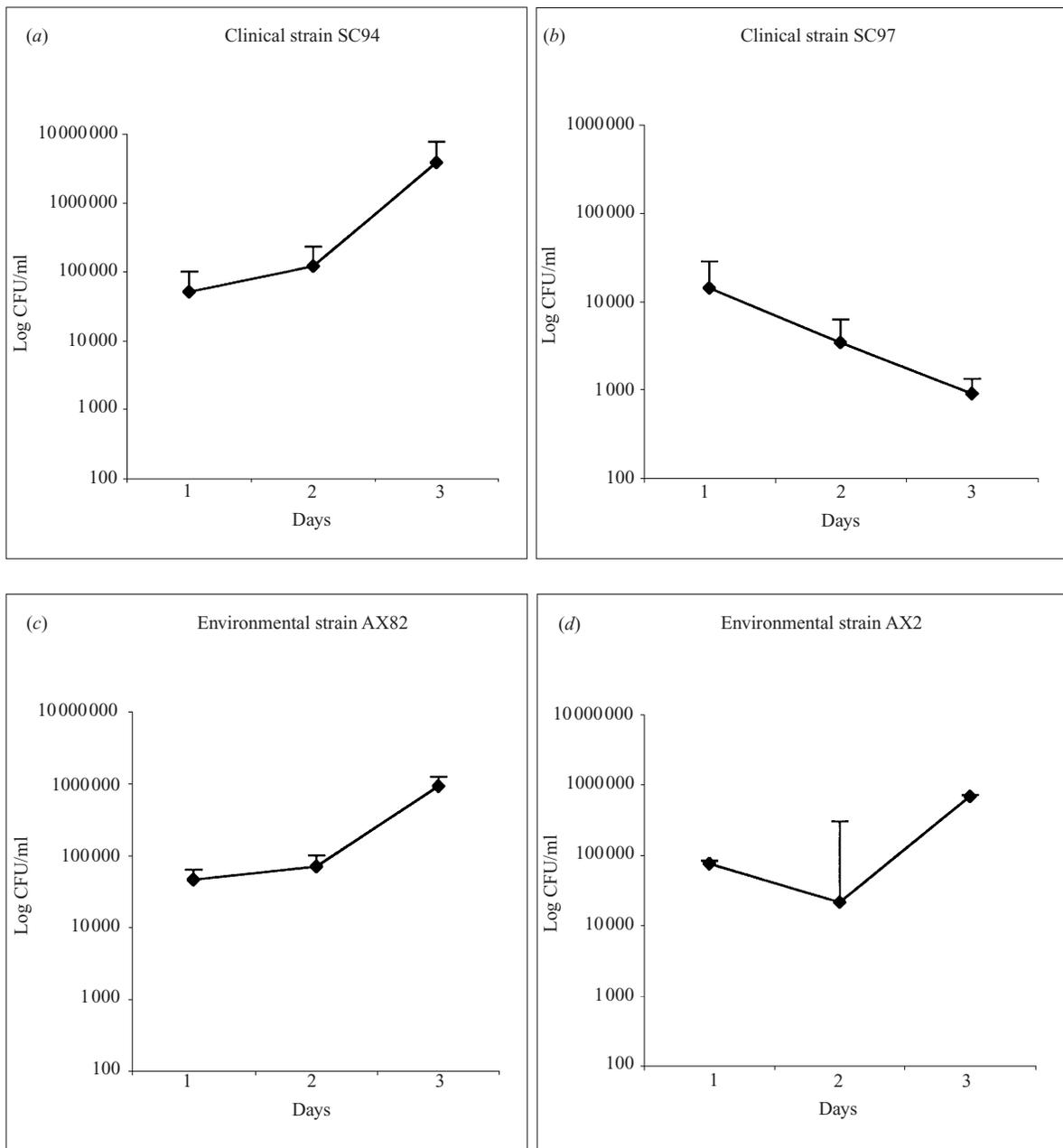
**Fig. 4.** Growth kinetics of clinical and selected environmental isolates in acanthamoeba. Panel (*a*), clinical strain SC94; Panel (*b*), clinical strain SC97; Panel (*c*), environmental isolate of serogroup 1 (AX82); Panel (*d*), environmental isolate of serogroup 1 (AX2). Results are given in log c.f.u./ml, with mean type errors.

differed by only one band, possibly corresponding to a genomic domain involved in virulence. Cloning and sequencing of this 0·6 kb RAPD fragment revealed at least one significant 124-amino-acid open reading frame with homology with *Thiosphaera pantotropha* nitrite reductase [identities = 12/49 (24%), positives = 25/49 (50%)] (http://www.ncbi.nlm.nih.gov/BLAST/) (data not shown). This potential gene will now be expressed in legionella hosts to test its effect in the amoebic co-culture model.

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