Research letters

Genome-based design of a cell-free culture medium for *Tropheryma whipplei*

Patricia Renesto, Nicolas Crapoulet, Hiroyuki Ogata, Bernard La Scola, Guy Vestris, Jean-Michel Claverie, Didier Raoult

Empirical approaches have guided the development of bacterial cultures. The availability of sequenced genomes now provides opportunities to define culture media for growth of fastidious pathogens with computer modelling of metabolic networks. A key issue is the possibility of growing host-dependent bacteria in cell-free conditions. The sequenced *Tropheryma whipplei* genome was analysed to identify specific metabolic deficiencies. We used this information to design a comprehensive medium that allowed three established *T whipplei* strains from culture with human cells and one new strain from a clinical sample to grow axenically. Genomic information can, therefore, provide sufficient clues for designing axenic media for fastidious and uncultured pathogens.

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Tropheryma whipplei, the agent of Whipple's disease, has proved particularly recalcitrant to cultivation and has been grown only in association with eukaryotic cells. Nearly a century elapsed between the first description of the disease in 1907 and the reproducible propagation of this micro-organism within a fibroblast cell line (HEL) in 2000, despite numerous efforts. The bacterium has, therefore, been thus far poorly characterised. Although the obligate intracellular nature of *T whipplei* has been questioned,[1] all attempts to subculture *T whipplei* in axenic medium have remained unsuccessful. Independent studies by Bentley and colleagues[2] and by Raoult and colleagues[3] have reported the small 0·9 megabase genomes of two *T whipplei* strains. The analysis of the genomic sequence revealed specific deficiencies in the predicted metabolism of *T whipplei* (figure 1). This information was successfully used to design a comprehensive axenic medium.

Computer modelled functional analysis of *T whipplei* genome revealed that the bacterium is well equipped for energy metabolism, nucleotide biosynthesis, and regulatory processes compared with other microbes of similar genome size.[3] However, many deficiencies were predicted in the biosynthesis of aminoacids. The synthetic enzyme pathways were entirely missing (absence of genes) for nine aminoacids (histidine, tryptophan, leucine, arginine, proline, lysine, methionine, cysteine, and asparagine). In addition, de novo synthesis of glutamate and glutamine is impossible because of the absence of the tricarboxylic-acid cycle. As a consequence the glutamate-dependent biosynthesis of aspartate, threonine, valine, and isoleucine become impossible. Finally, prephenate dehydratase, an enzyme for phenyalanine synthesis, is also missing. These metabolic deficiencies suggest that *T whipplei* acquires aminoacids or precursors from its host through membrane transport systems. This conclusion guided our rational design of an axenic medium for culturing *T whipplei*. As a basis, we chose a cell-culture medium that provided missing aminoacids (DMEM/F12 medium, Invitrogen Life Technologies, Carlsbad, CA, USA). This medium was supplemented with 10% fetal calf serum (Invitrogen Life Technologies), 1% L-glutamine (stock solution 200 mmol/L, Invitrogen Life Technologies) and 1% human non-essential aminoacids (stock solution 100×, Invitrogen Life Technologies).

Cell-free supernatant of a 25 cm^2 flask containing *T whipplei*-infected fibroblasts[1] was used to inoculate 30 mL of this medium in a 25 cm^2 flask maintained vertically at 37°C in a 5% carbon dioxide atmosphere. Kinetic growth was monitored daily by Gimenez staining and by flow cytometry counting, which permits quantitative and qualitative analysis of a microbial population.[4] The plots for *T whipplei* were devoid of any small particle background, confirming the absence of eukaryotic cell debris (webappendix; http://image.thelancet.com/extras/03let5174webappendix.pdf). The plots exhibit a significant increase in the number of particles over 8 days—ie, from $2{\cdot}43\times10^7$ to $1{\cdot}37\times10^9$/mL. These values were obtained during the third passage. To check for possible contamination, petri dishes were prepared with the same medium supplemented with agar and inoculated with the *T whipplei* suspension used in the primary culture. After 40 days of incubation, we saw no colonies. The growth of *T whipplei* in this newly designed medium was confirmed by immunofluorescence assays (not shown) and by quantitative PCR (passage three, figure 2) coupled with sequencing of the obtained amplicons (not shown). To date, this strain has been propagated through 14 passages in such host-independent conditions. The doubling time (28 h) of the strain (Twist, Marseille, France) was unchanged from passage three and up to now. All values obtained by cytometry counting were verified by quantitative PCR. To ensure its continued propagation, this axenic culture is subcultured each week by one part to 20 dilution in fresh medium.

T whipplei viability in the axenic culture was assessed by acridine orange staining and its ability to invade HEL cells was unchanged. The morphology of the bacterium grown in our medium for more than three passages showed only isolated bacteria. Bacteria forming rope-like structures are not seen in axenic cultures (figure 2), in contrast to fibroblast-associated cultures.[3] *NotI* and *SpeI* restriction profiles obtained by pulse-field gel electrophoresis of genomic DNA from these two cultures were indistinguishable (figure 2), whereas experimental restriction profiles of various strains of *T whipplei* were distinct (not shown). This difference was also predicted by computer modelling analysis of the two sequenced strains. After this first result, we successfully propagated two other isolates (endo 5 and slow 2, 11 passages each) obtained primarily on cell culture and one directly

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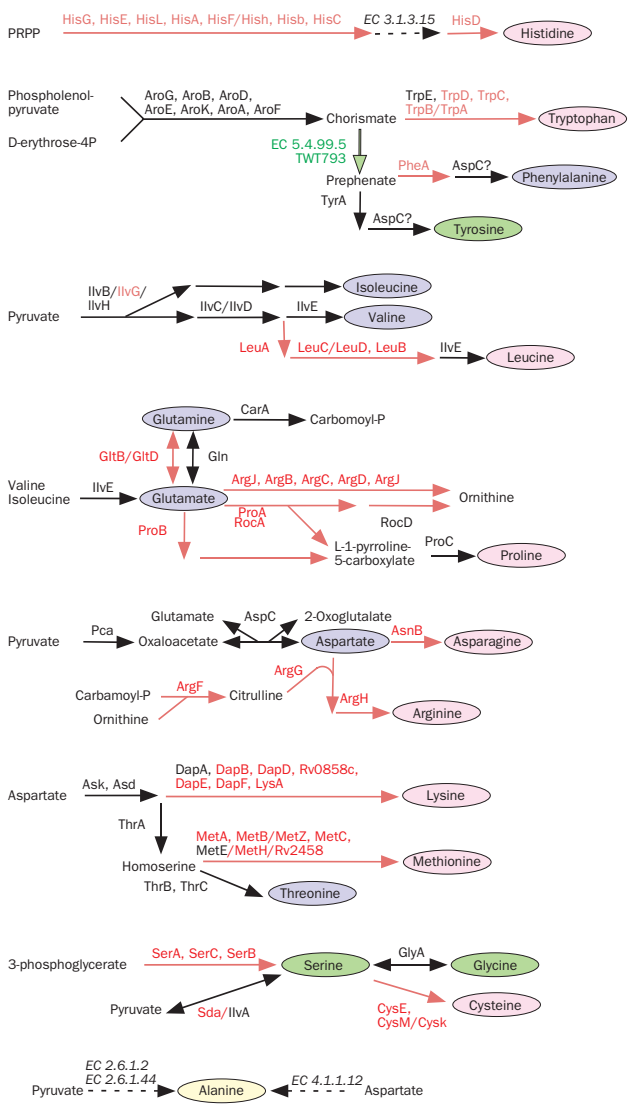

Figure 1: **Predicted aminoacid metabolisms of *T whipplei* based on *M tuberculosis* metabolisms**
Metabolic pathways automatically reconstructed in computer model by entering *T whipplei* genome annotation (web tool available at http://www.genome.ad.jp/kegg-bin/mk_point_html). Proposed pathways individually assessed for completeness in reference to enzymatic reaction data. Pink oval=metabolic pathways seem to be entirely lost for 9 aminoacids. Lilac oval=metabolic pathways seem deficient for 7 additional aminoacids. Green ovals=3 aminoacids with intact metabolic pathways. Yellow ovals=enzymes for alanine biosynthesisnot identified in *M tuberculosis* and *T whipplei.* Black writing=enzymatic steps predicted to be present in both *M tuberculosis* and *T whipplei*. Red and green writing=enzymatic steps present only in *M tuberculosis* or in *T whipplei*, respectively. Dashed black line=enzymatic steps absent in *M tuberculosis* and *T whipplei*. Second lilac oval=addition of L-glutamine predicted to unblock biosynthesis of glutamate, aspartate, threonine, isoleucine, and valine. PRPP=5-phosphoribosyl diphosphate.

from a heart-valve sample (four passages). For these strains, growth was first estimated by flow cytometry counting and confirmed by quantitative PCR. The identity of all the strains grown in axenic conditions was positively assessed by sequencing PCR amplicons of 16S–23S ribosomal DNA intergenic spacer. All sequences were 100% identical to the original inoculum.

The challenge of growing uncultured micro-organisms is receiving increasing attention and various strategies are being investigated.[5] The growth in pure culture of previously uncultured bacteria has been achieved by simulating their natural environment. Diffusion chambers

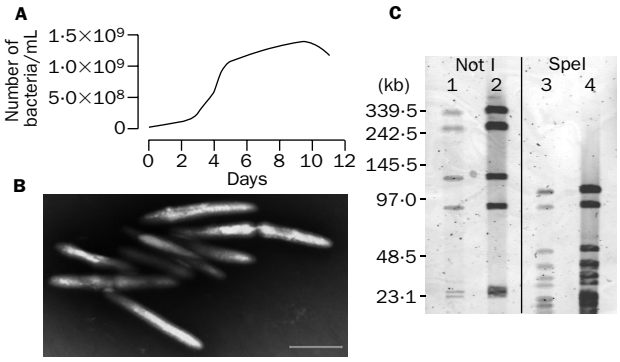

Figure 2: **Time-course assessment of *T whipplei* (strain Twist-Marseille CNCM I-2202) growth under axenic conditions**
A: Kinetics curve established at third passage from real-time PCR done with primers tws3f and tw4r targeting 16S-23S ribosomal DNA intergenic spacer of *T Whipplei* (webappendix). Each point done in duplicate and measurements done twice. B: Electronic microscopy, negative staining, bar 500 nm. C: Restriction endonuclease profiles of genomic DNA from *T whipplei* cultivated under different conditions and analysed by pulse-field gel electrophoresis. Lanes 1 and 3, culture on HEL cells; lanes 2 and 4, axenic culture (webappendix).

allowing the exchange of uncharacterised chemical components from seawater have been successfully used to grow marine micro-organisms. The culturability of such micro-organisms is also investigated with high-throughput screening methods and miniaturisation procedures. Sonication of the initial sample, extended incubation periods, and the use of gellan gum supplemented with calcium chloride instead of agar, has also permitted extension of the range of culturability among soil bacteria.

Metabolic analysis was previously applied to the design of media for *Escherichia coli* to obtain optimum growth.[6] Our hypothesis was that the genome information of poorly characterised bacteria, especially parasitic ones with reduced genomes, could also be used to define axenic culture conditions, opening the way to much easier microbiological studies. Genome reduction is associated with the loss of genes the functions of which are complemented through a host-dependent lifestyle providing them with many organic molecules. Missing genes are thus expected to readily suggest which metabolic pathways have to be complemented. For *T whipplei*, the supplementation of the medium with aminoacids proved to be the key factor for growth. Culture conditions could be improved further by varying the concentrations of the medium components. For instance, we have preliminary evidence that the growth rate is strongly affected by the glutamine concentration. This finding is consistent with our analysis of *T whipplei* metabolism in which glutamine is predicted to be essential for the synthesis of nucleotides and several aminoacids.

The development of an axenic culture for *T whipplei* opens the way for similar attempts with other bacteria hitherto thought strictly intracellular.

Contributors
D Raoult supervised the work done in the study. P Renesto was involved in the elaboration of experimental protocols in the overall data analysis and in the restriction fragment length polymorphism analysis. She was also responsible for preparing the results for publication. N Crapoulet and G Vestris propagated *T whipplei* in axenic medium. N Crapoulet did flow cytometer analysis, quantitative PCT, and immunofluorescence assays. B La Scola contributed knowledge and experience with *T whipplei*, and was involved in the elaboration of experimental protocols. H Ogata and J-M Claverie did the metabolism prediction for the study. All researchers saw and approved the final version of the paper.

Conflict of interest statement
None declared.

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Unité des Rickettsies, CNRS UMR 6020, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseille, France (P Renesto PhD, N Crapoulet, Prof B La Scola PhD, G Vestris, Prof D Raoult PhD); **Information Génomique et Structurale, UPR 2589, Marseille** (H Ogata PhD, J-M Claverie PhD)

Correspondence to: Prof Didier Raoult (e-mail: Didier.Raoult@medecine.univ-mrs.fr)

Risk of relapse after meningococcal C conjugate vaccine in nephrotic syndrome

A S Abeyagunawardena, D Goldblatt, N Andrews, R S Trompeter

In November, 1999, all children under age 18 years in the UK were offered immunisation with the newly introduced meningococcal C conjugate vaccine (MCCV). In a cohort of 106 patients with nephrotic syndrome, there were 63 relapses during the 12 months before vaccination, and 96 during the equivalent period postvaccination (p=0·009). The relapse rate of nephrotic syndrome increased significantly after administration of MCCV. Whether to vaccinate such children needs to be carefully considered.

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Most cases of primary nephrotic syndrome in childhood respond to steroid treatment.[1] However, many of these children subsequently relapse, necessitating long-term steroid or cytotoxic treatment. Immunogenic stimuli such as viral infections are implicated in triggering a relapse of the syndrome.[2] Apart from anecdotal evidence, in which vaccinations have been associated with development of nephrotic syndrome, the effect of vaccination on the stability of the syndrome has not been investigated.

In the 8 months after the launch of the national meningococcal C conjugate vaccine (MCCV) programme, nine children with nephrotic syndrome who were outpatients at Great Ormond Street Children's Hospital NHS Trust, London, UK, presented with relapse after vaccination. To explore the possible relation between relapse and vaccination, the relapse rate during the 12 months before and after vaccination were studied in children with steroid-sensitive nephrotic syndrome, who were vaccinated between November, 1999, and October, 2000.

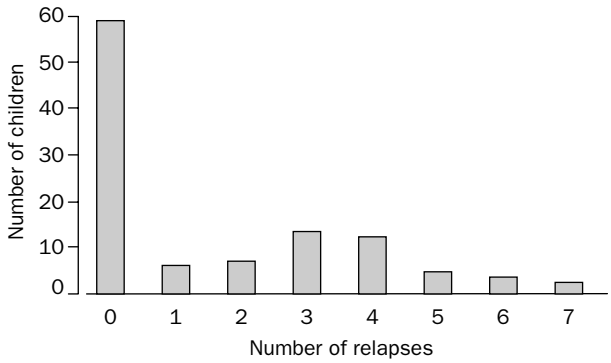

Figure 1: **Frequency of relapses in 106 vaccinated children**

Of 224 patients who regularly attended the outpatient clinic at Great Ormond Street Hospital, 106 received MCCV during the first 12 months of the campaign (74 [70%] boys, 32 [30%] girls). Informed oral consent from the parents and local ethics committee approval were obtained. Vaccination status was initially determined by telephone, and subsequently confirmed with vaccination records. Immunosuppressive treatment, medical advice, and parental concerns about a newly introduced vaccine contributed to the low rate of vaccination in this cohort. The occurrence of relapses (proteinuria 3+ per 24 h, for 3 consecutive days) was recorded for 12 months before and after vaccination. Details of relapse were obtained from patient-held records and case notes. The episodes were defined as independent if they took place at least 1 month apart. Therefore, within any 1-month period, a maximum of one relapse could occur for an individual. Of the 106 children identified, 59 had no relapses whereas 47 had at least one relapse in the 24 months analysed (figure 1). There were a total of 159 relapses (mean 3·4), and for subsequent analysis of prevaccination and postvaccination periods only these 47 children were included.

Figure 2 shows the comparison of the number of relapses in the two periods. Overall, 63 relapses happened in the 12 months before vaccination, compared with 96 in the 12 months postvaccination. We used Poisson regression to calculate the relative incidence (RI), and to test for the significance of the difference between the relapse rates in the two periods as well as the effect of sex. We compared the number of individuals with a decrease or increase in relapse rate after vaccination using McNemar's test.

With the prevaccination period as the reference, an RI of 1·52 (95% CI 1·10–2·11) was noted postvaccination (p=0·009). There was no evidence that this effect varied between boys and girls (p=0·73). When the 12-month postvaccination period was split into 1–6 months (58 relapses) and 7–12 months (38 relapses), the RI estimates for these two periods compared with the prevaccination period were 1·84 (95% CI 1·27–1·65) and 1·21 (95% CI 0·80–1·82), respectively. Thus, only in the 6-month postvaccination period was the risk significantly raised. By contrast, there was no evidence of a reduction in the number of prevaccination relapses with time, with 30 relapses in the 1–6 months and 33 in the 7–12 months before vaccination.

Although some individuals had as many as seven relapses, the difference between the number of prevaccination and postvaccination relapses did not seem to be related to the high numbers in a few individuals postvaccination. For example, when we analysed only individuals with at least four relapses, the RI for the postvaccination period was 1·56 compared with 1·46 in those with between one and three relapses. Additionally,

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Material and methods

To assess growing of *Tropheryma whipplei* under axenic conditions, three samples (100 μL each) of the bacterial culture were taken daily and stored at –80°C. These samples were further used for flow cytometry counting and quantitative PCR assays. Two others samples were used for immunofluoresence assays with a specific antibody[1] or Gimenez staining.

Counting of *T whipplei* by flow cytometry

Before counting, bacteria were fixed for 1 h in 0·4% formol. We did analyses with the Microcyte portable flow cytometer based on 635 nm diode laser technology (Optoflow, Oslo, Norway). The flow cytometer was set up according to the manufacturer's manual. The sheath fluid was stored at 4°C but was allowed to reach room temperature before use. Total particle density (bacteria/mL) was assessed directly by the instrument with the forward scatter light signal. Gating, or selection of the region of interest, is accomplished by use of a cursor to define a line representing that region on a one-parameter histogram, analogous to setting a linear analysis gate on a Coulter instrument. Gates were set empirically, as would be the case with a conventional cytometer. Data analysis was carried out by techniques of multiple gating (FCS Express, De Novo Software, Thornhill, ON, Canada).

Real-time quantitative PCR

DNA was extracted from 100 μL and 10 μL cell suspension with the MagNA Pure LC DNA Isolation Kit III (Roche Diagnostics, Mannheim, Germany). Amplification and quantification were done with the Light Cycler PCR and detection system (Roche Diagnostics).

Mixes were prepared following the manufacturer's instructions (FastStart DNA Master SYBR Green, Roche Diagnostics) and with primers tws3f (5′-CCGGTGACTTAACCTTTTTGGAGA-3′) and tws4r (5′-TCCCGAGGCTTATCGCAGATTG-3′), which target a 489 bp fragment of the 16S–23S ribosomal DNA intergenic spacer.[2] The PCR programme was one cycle of denaturation at 95°C for 1 min followed by 35 cycles of 95°C for 15 s, annealing at 56°C for 5 s and extension at 74°C for 10 s. The Light-Cycler was then programmed to do a melting cycle to measure the melting temperature of the amplicon and probe. This programme consisted of a denaturing step at 95°C for 15 s, after which the Light-Cycler cooled to 66°C for 30 s, and then increased the temperature at a rate of 0·1°C/s until the temperature reached 95°C. During this stage, the Light-Cycler was continuously measuring the fluorescence. We did quantification by online monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). Serially diluted samples of genomic bacterial DNA obtained from *T whipplei* cultures enumerated with the Microcyte were used as external standards in each run. The cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of template DNA. The concentrations of bacterial DNA in the culture samples were calculated by comparing the cycle numbers of the logarithmic linear phase of the samples with the cycle numbers of the external standards.

Enzymatic digestion and pulse-field gel electrophoresis analysis

After a 3 h equilibration in appropriate restriction enzyme buffer, plugs (*T whipplei* suspension mixed with 1% melting point agarose) were incubated overnight with 50 U of restriction enzyme, placed into wells and sealed with 1% agarose. Electrophoresis was done in a contour-clamped homogenous electrical field (CHEF DR III, Biorad, Richmond, CA, USA) with a 1% agarose gel (Sigma Chemical, Uppsala, Sweden) 0·5×running buffer (45 mmol/L Tris, 45 mmol/L boric acid and 1 mmol/L EDTA) and run at 14°C with a pulse time of 60 s for 15 h at 200 V. A bacteriophage λ ladder pulsed-field gel marker of 48·5 to 1018 kb and a low-range pulse-field gel marker of 0·1 to 200 kb (New England Biolabs, St Quentin Yvelines, France) were used to check the band sizes. The gel was stained in a solution of ethidium bromide for 30 min before being visualised.

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