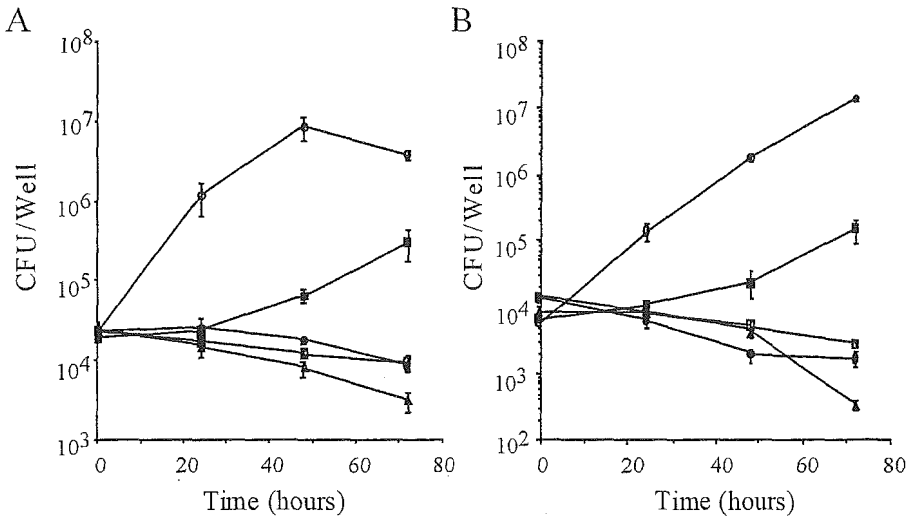


FIG. 1. Intracellular growth of *L. dumoffii* strains within J774 mouse macrophages (A) and A549 human epithelial cells (B). The formation of colonies (CFU per milliliter) was determined at the times indicated, in triplicate, for at least two independent experiments. Error bars indicate the standard deviations determined from samples taken from one experiment. Symbols: ○, *L. dumoffii* wild-type strain; ■, HOLD254; □, HOLD491; ●, HMLD4001; △, HMLD4002.

acetate followed by lead citrate and examined by electron microscopy in a JEM 2000EX instrument (JEOL, Ltd., Tokyo, Japan).

Examination of RER recruitment by transmission electron microscopy. J774 cells were plated in 90-mm-diameter petri dishes (2×10^5 cells/ml) and infected with stationary-phase *L. dumoffii* strains at an MOI of 20 for 8 and 24 h (32). Ultrathin sections were prepared as described above.

Assays for survival under stress conditions. *L. dumoffii* strains were grown for 2 to 3 days on BCYE agar plates and used to inoculate 4 ml of BYE medium. The bacteria were then grown at 37°C with aeration for at least 16 h. The initial CFU count was about 10^{10} per ml. Cells were divided into aliquots, centrifuged, and resuspended in equal volumes of 1 × M63 salts [22.0 mM KH_2PO_4, 40.2 mM K_2HPO_4, 14.6 mM $(NH_4)_2SO_4$ 500 nM $FeSO_4$ (pH6.5)]. One aliquot was used for measuring the untreated CFU. For heat shock, aliquots were transferred to 48°C and incubated for 60 min. For oxidative stress, aliquots were exposed to 10 mM H_2O_2 for 30 min. For osmotic shock, aliquots were exposed to 5 M sodium chloride for 30 min. For acid shock, aliquots were resuspended in 0.1 M citric acid (pH 3) for 5 min. Except for heat stress, the cells were incubated in a 37°C heat block. At the indicated time points, the cells were washed with 1 × M63 salts and serially diluted to determine the CFU on BCYE agar plates (29).

Detection of a *djlA* gene in other *Legionella* spp. The presence of *djlA* in 17 different strains of *Legionella* spp. was examined by PCR with the primer pair djlA-cons-1 (5′-ATAACAACCTGGTGGGGAAA-3′) and djlA-cons-2 (5′-TG GGCAATTAATTTATCTGGATG-3′), located in the transmembrane domain (TMD) and J domain within the *djlA* gene, respectively, which gave a 791-bp product. PCR was carried out by using chromosomal DNA from BCYE plate-grown bacteria as a template.

RESULTS

Isolation of intracellular growth mutants. Wild-type *L. dumoffii* Tex-KL was mutagenized with Tn*903*dIII*lacZ* as described previously (57, 68). Plasmid pLAW330 containing Tn*903*dIII*lacZ* was introduced into *L. dumoffii*, and 790 Km^r Cm^s mutants of *L. dumoffii* (HOLD strains 1 to 656 and HMLD strains 4004 to 4044 and 4048 to 4140) with various levels of β-galactosidase activity were isolated. The 790 mutants were individually screened for their ability to kill mouse macrophage-like J774 cells and human alveolar epithelial A549 cells. The mutants were grown for 2 days in 96-well tissue culture plates containing BYE medium. Then 5-μl samples of 2-day-old cultures of mutants were transferred to another 96-well tissue culture plate containing J774 cells or A549 cells. At each 24-h time point after infection, the monolayers were visually examined to determine the extent of killing of both J774 cells and A549 cells. From several assays, we isolated five mutants, based on their reproducible phenotypes. Southern blot analysis of the HindIII-digested genomic DNA of each of the five mutants probed with pLAW330 showed that four of them contained a single copy of the Tn*903*dIII*lacZ* insertion and that these insertions were distributed in distinct locations within the chromosome of *L. dumoffii* (data not shown). For reasons not yet understood, one of the mutants showed no hybridization. Therefore, the four strains were chosen for further analysis. In vitro, the growth of these four mutants in BYE broth and on BCYE agar plates was similar to that of the wild-type strain (data not shown).

Intracellular growth phenotype of the mutants within J774 macrophages and alveolar epithelial cells. We examined the four candidates for their capacity to survive and to replicate within J774 macrophages and A549 epithelial cells. Bacterial CFU were determined over 3 days. The wild-type strain multiplied over 100-fold during the 3-day incubation period within J774 macrophages (Fig. 1A). HOLD254 showed a 1-log-unit increase after 3 days of incubation, whereas HOLD491, HMLD4001, and HMLD4002 did not grow during the incubation period in J774 cells. Within A549 epithelial cells (Fig. 1B), the wild-type strain increased approximately 1,000-fold over the 3-day period, while there was a 10-fold increase in the number of intracellular bacteria of HOLD254 over 3 days. For HOLD491 and HMLD4001, the number of CFU after 3 days of infection decreased 1 log unit to the initial number of CFU, and HMLD4002 was severely defective in intracellular survival (Fig. 1B).

Sequence analysis of the junctions of Tn*903*dIII*lacZ* insertions. We cloned the HindIII fragment containing the Tn*903*dIII*lacZ* insert and the flanking sequences of the mutants (HOLD254, HOLD491, HMLD4001, and HMLD4002). Using

TABLE 3. Sequence similarities of *L. dumoffii* genes responsible for intracellular multiplication[a]

Mutant strain	Homologous gene	Organism	% Identity	% Positive
HOLD254	*djlA*	*Legionella pneumophila*	61	73
HOLD491	*icmB/dotO*	*Legionella pneumophila*	89	95
HMLD4001	17-kDa antigen gene	*Bartonella henselae*	26	43
HMLD4002	*dotC*	*Legionella pneumophila*	85	92

[a] The values are taken from a Basic Local Alignment Search Tool for amino acid comparison (BLASTX program).

the primer within Tn*903*dII*lacZ*, we partially sequenced and analyzed them to identify the genes responsible for intracellular multiplication. The results are summarized in Table 3. Sequence homology searches against the Gen Bank database were done with these genes and corresponding proteins. HOLD254, HOLD491, and HMLD4002 contain insertions within the genes homologous to known *L. pneumophila* genes. The gene disrupted in HOLD254 is the *djlA* (for "*dnaJ*-like *A*") gene, encoding a member of the Hsp40 protein family, which has not been characterized in *L. pneumophila*. HOLD491 and HMLD4002 had a transposon insertion in their sequences similar to *icmB (dotO)* and *dotC*, respectively, identified as genes essential for intracellular growth in *L. pneumophila* (5, 51). HMLD4001 had an insertion within a gene whose product showed amino acid similarity to the 17-kDa antigen, VirB5, of *B. henselae*; the gene is located within the *virB* locus, which encodes a putative type IV secretion system together with the downstream *virD4* gene (14, 49, 59). Recently, Schulein and Dehio (59) also showed that VirB4 and VirD4, encoded by the *virB* and *virD4* loci of *B. tribucorum*, were required for establishing intraerythrocytic bacteremia.

Complementation of an *L. dumoffii djlA* mutant. DjlA is known to be a heat shock protein DnaJ/Hsp40 homologue. The virulence of the *djlA* mutant was compared with that of the wild-type strain and the *djlA*-complemented mutant in J774 macrophages, A549 epithelial cells, and *A. culbertsoni*. The *djlA* mutant showed only a 100-fold increase in intracellular replication within *A. culbertsoni* (Fig. 2C). As shown in Fig. 2, bacterial growth was fully restored in the complemented strains HOLD254-1 and HOLD254-2. The restoration of the wild-type-level of multiplication of the *djlA* mutant within these cells, achieved after complementation in *trans* with the cloned *djlA* gene, is proof of the important role of *djlA* in the intracellular growth of *L. dumoffii*.

Complete sequence and genetic structure of *djlA*. Figure 3A shows the organization around the *djlA* gene and the location of the Tn*903*dII*lacZ* insertion. The transposon insertion (Tn) was located in the J domain at the C terminus of the predicted protein, which was the defined feature of the DnaJ family of molecular chaperones (16, 27). Since the two genes (*waaA* and *orf1*) which flanked *djlA* were both oriented in the opposite direction from the *djlA*, we consider the *djlA* to be transcribed

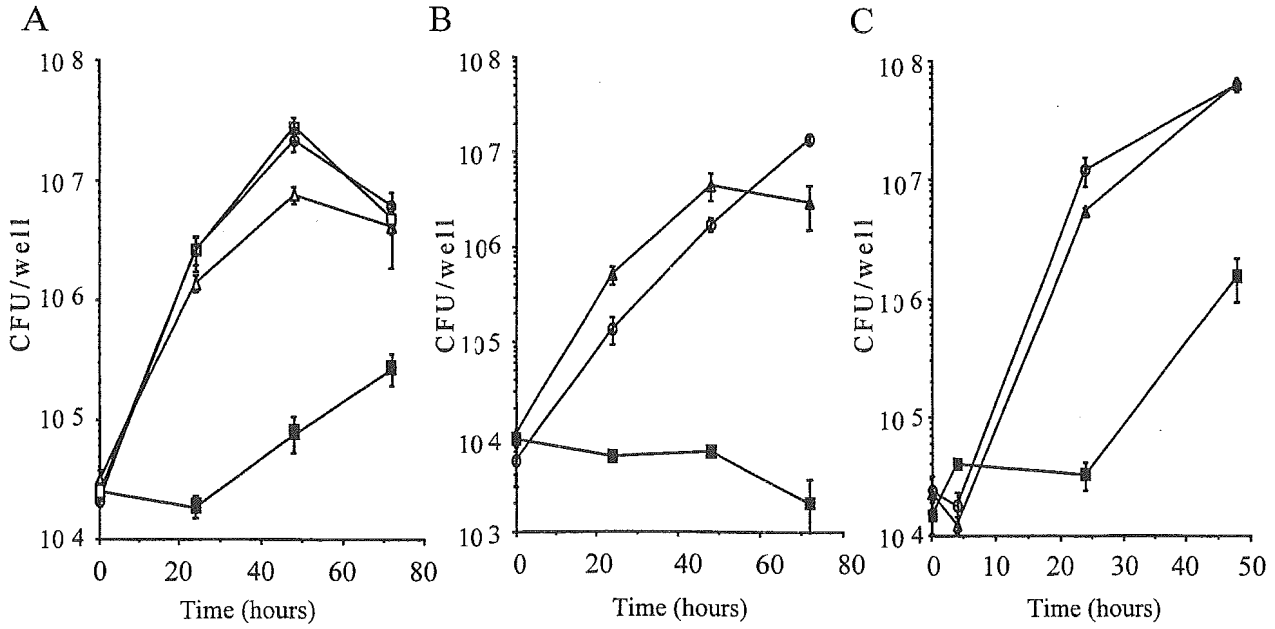


FIG. 2. Complementation of intracellular growth defects of *djlA* mutant HOLD254 in J774 macrophages (A), in A549 epithelial cells (B), and in *A. culbertsoni* (C). Growth was measured over 72 h (A and B) or 48 h (C). The data points and error bars represent the mean CFU/well for triplicate samples from a typical experiment (performed at least twice) and their standard deviations. Symbols: ○, *L. dumoffii* wild-type strain; ■, HOLD254; △, HOLD254-1 (*djlA*/pHRO18); □, HOLD254-2 (*djlA*/pHRO25).

monocistronically, and this transposon has no polar effect. The deduced amino acid sequence of *L. dumoffii* DjlA, together with *L. pneumophila* DjlA and *E. coli* DjlA, is presented in Fig. 3B. The putative *L. dumoffii djlA* gene encodes a protein of 302 amino acids with a predicted molecular mass of 35.33 kDa and an isoelectric point of 9.65. The protein size is similar to that of the *L. pneumophila* (296 amino acids) and *E. coli* (271 amino acids) proteins. *L. dumoffii* DjlA has 61% identity to *L. pneumophila* DjlA and 32% identity to *E. coli* DjlA (10, 16, 73). A potential TMD at the N terminus contains six glycines, spaced through the TMD at every three to five residues, which is similar to the structure of the TMD of *E. coli* (15, 16). There is a remarkable difference in the N terminus of DjlA protein between *E. coli* and *Legionella* spp. Clarke et al. (16) have demonstrated that *E. coli* DjlA is localized to the inner membrane and has a rare type III topology (i.e. N-out, C-in), with the N-terminal 6 to 8 residues located in the periplasm. *Legionella* spp. have longer stretches (15 residues) before the TMD structure, which are probably exposed in the periplasm. Another unique feature of *Legionella* DjlA is a glutamate-serine (QS)-rich spacer located before the J domain, instead of the glutamate-glycine (QG)-rich spacer of *E. coli* DjlA (Fig. 3B) (16). The cellular role of these QS- or QG-rich regions remain to be elucidated.

Quantification of endocytic maturation. To determine whether the *L. dumoffii* strains were able to inhibit endocytic maturation, we measured the colocalization of *L. dumoffii* phagosomes with endocytic markers LAMP-1 and LAMP-2. J774 macrophages were infected with postexponential phase *L. dumoffii* strains for 4 h (Fig. 4). The permeabilized cells were stained with monoclonal antibody 1D4B or Abl 93, specific for late endosomal and lysosomal proteins, LAMP-1 or LAMP-2. The *djlA* mutant was found in phagosomes that contained LAMP-1 (Fig. 4A), indicating that these vacuoles had fused with late endosomes, whereas, phagosomes containing wild-type *L. dumoffii* did not colocalize with LAMP-1 (Fig. 4A). When each *L. dumoffii* strain found in the phagosomes was scored for fusion with the late endosomal/lysosomal markers LAMP-1 and LAMP-2, approximately 80% of the wild-type bacteria were found in LAMP-1- and LAMP-2-negative phagosomes while 50 to 60% of the HOLD254 was found in LAMP-1- and LAMP-2-positive compartments (Fig. 4B). We also performed the same analysis for HOLD4002, the *dotC* mutant, and found that this mutant followed the same endocytic pathway as HOLD254, with 60 to 70% LAMP-1- and LAMP-2-positive (data not shown). We also conducted an assay of phagosome-lysosome fusion, at the ultrastructural level, using electron microscopy. BSA-gold was used as a pinocytic, fluid-phase marker of the endosomal-lysosomal pathway. BSA-gold was accumulated mainly in lysosomes after endocytosis of the conjugate-containing medium overnight at 37°C, followed by a chase period of 3 h at 37°C in conjugate-free medium as previously described (33). After a pulse with *L. dumoffii* strains and another chase for 4 h, electron microscopy counting of *L. dumoffii*-containing phagosomes that fused with BSA-gold-labeled lysosomes was performed to assess fusion (Fig. 5). Wild-type-strain-containing phagosomes did not fuse with BSA-gold-marked lysosomes (Fig. 5A). Quantitation showed that only 11.4% (24 of 210) of the phagosomes containing the wild-type strain fused with BSA-gold-marked lysosomes. On the other hand, 85% (187 of 220) of the phagosomes containing the *djlA* mutant strain accumulated BSA-gold (Fig. 5B). Thus, the *djlA* mutant was not able to evade phagosome-lysosome fusion.

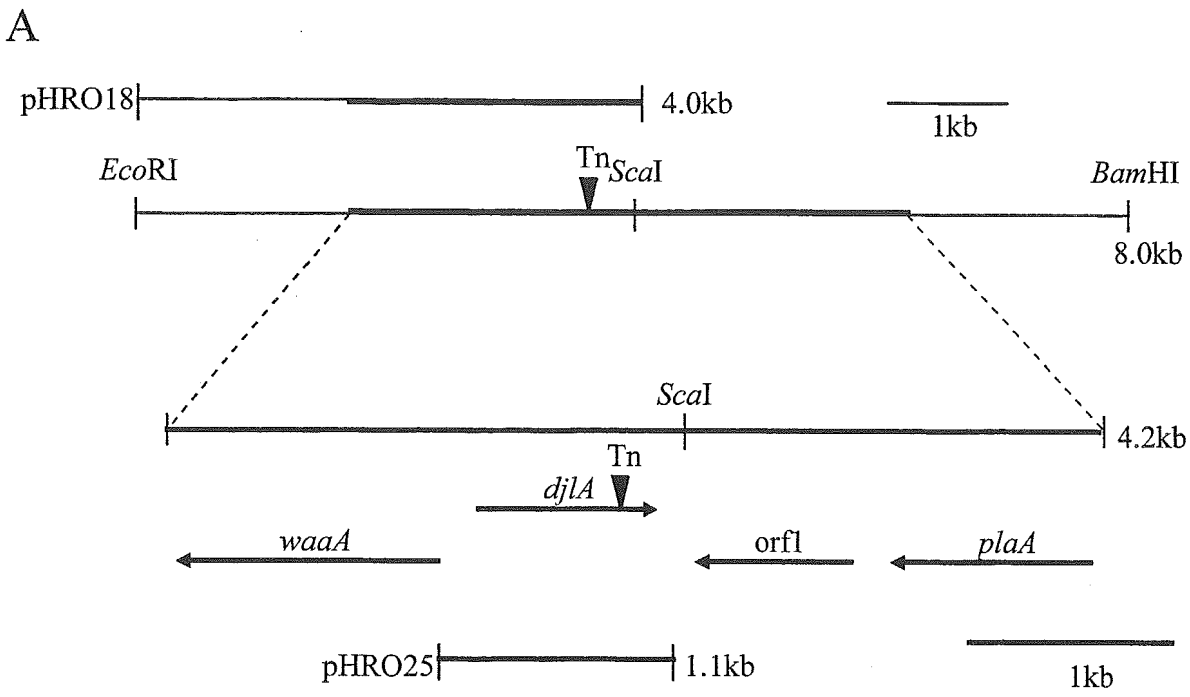
Recruitment of the RER. In mammalian macrophages and protozoa, *L. pneumophila* replicates intracellularly in specialized vacuoles surrounded by the RER of the host cells (25, 32). To determine the intracellular location of *L. dumoffii*, we examined J774 macrophages infected with wild-type and *djlA* mutant *L. dumoffii* by using transmission electron microscopy. At 8 h postinfection, the RER around 61 (37.2%) of 164 phagosomes containing wild-type strains were recruited (Fig. 6A) whereas we could not find any phagosomes containing the *djlA* mutant surrounded by RER or attached directly by ribosomes (0 of 153 phagosomes). This was also the case at 24 h (Fig. 6B and data not shown). Phagosomes containing *djlA* mutant cells appeared to harbor much debris, resulting from fusing lysosomes with these vacuoles, while phagosomes containing wild-type cells did not have any contents other than replicating *L. dumoffii* cells (Fig. 6). At 24 h postinfection, many phagosomes containing wild-type cells were broken and their inhabiting macrophages were lysed (data not shown).

Susceptibility of the *djlA* mutant to stress stimuli. In eukaryotic host cells, intracellular pathogens encounter hostile conditions such as toxic oxygen or nitrogen derivatives, intraphagosomal acidification, and harsh degradative enzymes (54, 62). As mentioned above, *djlA* is essential for intracellular growth of *L. dumoffii*. Thus, we examined whether the *djlA* mutant has an increased susceptibility to different environmental stresses. Since previous publications (12, 29) had demonstrated that *L. pneumophila* induces stress resistance in the stationary phase, *L. dumoffii* strains were grown to the stationary phase in BYE medium and subjected to acid shock, oxidative stress, osmotic stress, and heat shock (pH 3 for 5 min, 10 mM H_2O_2 for 30 min, 5 M sodium chloride for 30 min, and 48°C for 60 min, respectively). Compared to the wild-type strain, there was an elevated susceptibility to all stress conditions of the *djlA* mutant strain. There was an increase in the sensitivity of the mutant of 9.8-, 7.4-, 2.6-, and 1.6-fold on exposure to oxidative stress, osmotic stress, heat shock, and acid shock, respectively (Fig. 7). These results suggest that DjlA participated in the protection of *L. dumoffii* on exposure to environmental stress. In the *djlA*-complemented strain, in contrast, resistance to all stress stimuli was restored. The variability in the degree of complementation may result from the different expression of genes from the plasmid and the chromosome.

Presence of *djlA* in other *Legionella* spp. To determine whether *djlA* is also present in nonpathogenic *Legionella* species, PCR amplification with primers in the *djlA* gene was performed for 17 different *Legionella* strains. All the strains used in this experiment are listed in Table 2. The expected 790-bp band was observed in all *Legionella* strains tested except *L. jordanis* and *L. adelaidensis*, irrespective of whether the strain was pathogenic (data not shown). Thus, *djlA* is not unique to particular *Legionella* strains.

DISCUSSION

Legionella spp. are facultative intracellular bacteria that overcome host cell defenses. Although many studies have been



B

TMD

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L. d MSLRDFFIITTWWGKIIGAFFGYLIAGPT-GAIFGLLVGNFFDRGLYNYFSNPHWLYYTEKRRAIQKIFFEA-TFLV 75
L. p MNLRDFFVITTWWGKILGAFFGYLTAGPV-GALFGILVGNFFDRGLVSYYSNPHWLYHAEKQRIVQKAFFEA-TFSI 75
E. c          MQYWGKIIGVAVALLMGGGFWGVVLGLLIGHMFDKAR----SRKMAWF-A-NQRERQ-AFF-ATTFEV 61

L. d MGHLAKADGRVSEQELDMAR-LFMDEMRLNGEQKTLAKHLFNEGKQSRFNLDSLLENLKKT--CKDNRDLLRLFI-D 148
L. p MGHVAKSDGRVSEQEISMAKSI-MNEMKLSKGQKDLAKRLFNEGKQADFNV-SL-ALIQLQRICKDNRDLLKLFV-D 148
E. c MGHLTKSKGRVTEADIHIASQL-MDRMNLHGASRTAAQNAFRVGKSDNYPLREKMRQ-FRSVCFGRFDLIRMFLEIQ 136

L. d IQYRAAQADG-LDSKKILLLDKIFSRLGFAPLHNQYRFYEDFGRSYSEPQYNTQEQP-QQSRQSQQSDSSSHSYSSY 223
L. p IQYRAAQVDG-LSSQKIHALDNIFTHLGFAPLHKQYRFYEDFG-SYFQQEQSKQHYHNQQEYKHT---SSSQG-QQG 219
E. c IQ--AAFADGSLHPNERAVLYVIAEELGI--SRAQFDQFLRMM----QGGAQFGGGYQQQT-------GGGNW-QQA 197
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J-domain

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L. d SRYNYQPTKNNMDYAFALLEVSPKASKQEVKKAYRRLLSRNHPDKLIAQGLPQEMIKMANEKTQKIVKAYELICESKGW 302
L. p YKP-QSPPNTLA-HAFALLEVSPNANKQEVRRAYRRLLSRNHPDKLIAQGLPEEMIKLANDKTHQIMKAYELICETKGWX 296
E. c QRG---P--TLE-DACNVLGVKPTDDATTIKRAYRKLMSEHHPDKLVAKGLPPEMMEMAKQKAQEIQQAYELIKQQKGFK 271
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FIG. 3. Chromosomal arrangement of the region surrounding the *djlA* gene and sequence alignment of DjlA proteins. (A) At the top is a plasmid used for complementation studies (pHRO18) and an 8-kb region of the *L. dumoffii* cosmid clone including the *djlA* gene, along with the location of relevant restriction enzyme sites. The thick line represents the DNA region that we sequenced. Below these diagrams, the distance between the *djlA* gene and neighboring genes and the orientation and size of the transcribed genes are delineated by the arrows below the 4.2-kb sequenced region. Another plasmid used for complementation studies (pHRO25) is also shown. The site of the Tn*903*dI*lacZ* insertion (Tn) is indicated by the inverted arrowhead. The full names of the gene mapped are as follows: *waaA*, Kdo transferase gene; *djlA*, *dnaJ*-like A gene; *plaA*, lysophospholipase A gene. Orf1 is a putative open reading frame which showed no homology to known genes. (B) Sequence similarity of the predicted Dj1A protein of *L. dumoffii* (L.d, top line), *L. pneumophila* (L.p, middle line) and *E. coli* (E.c, bottom line). Amino acid residues conserved in the three sequences, appear in bold type. Gaps marked by dashes are introduced to reveal the maximal similarity among the sequences. The C-terminal J-domain and the N-terminal TMD are shown schematically above the sequences.

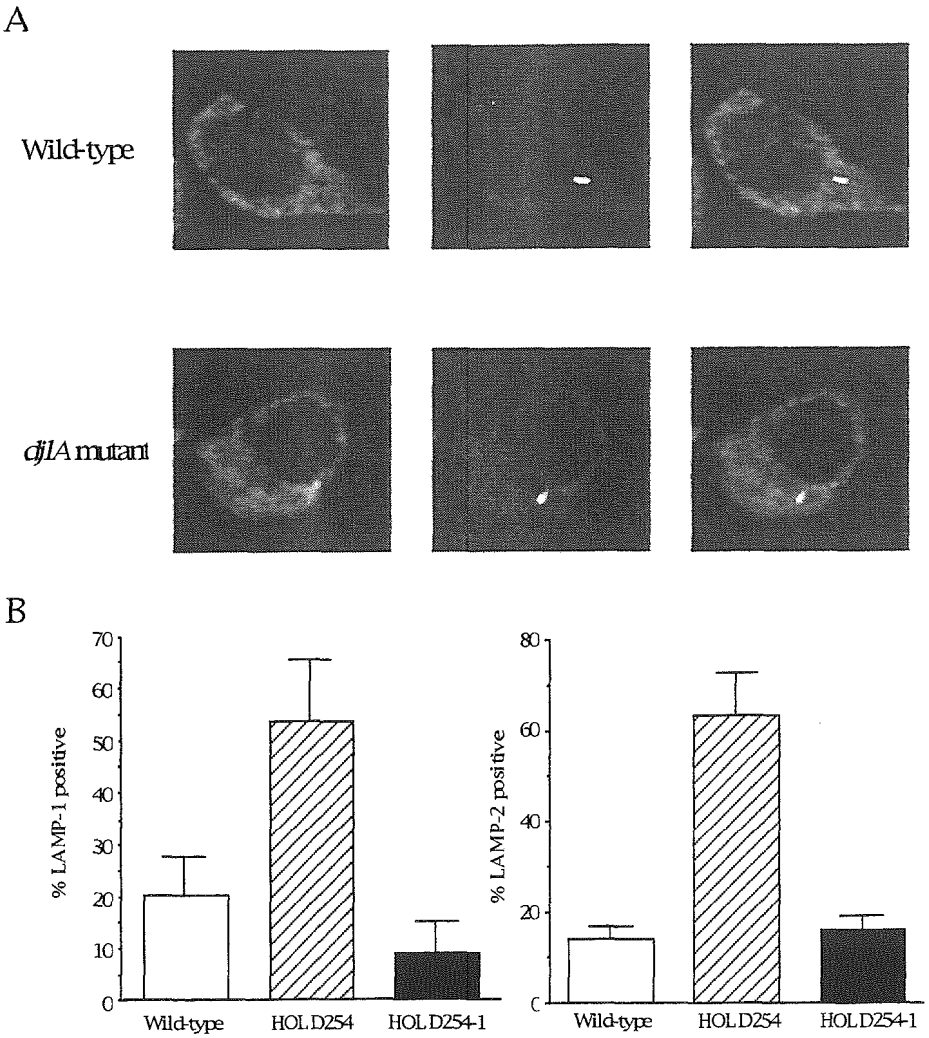

FIG. 4. Colocalization of the intracellular growth mutant with late endosomal/lysosomal marker LAMP-1 or LAMP-2 in J774 mouse macrophage cells by confocal laser-scanning microscopy. J774 macrophages were incubated with the *L. dumoffii* mutant or wild-type strain for 4 h. (A) Late endosomes and lysosomes stained with rat monoclonal antibody 1D4B, specific for LAMP-1, and Cy3-labeled anti-rat secondary antibody (red) are shown on the left. Bacteria stained with rabbit polyclonal antibody specific for *L. dumoffii* Tex-KL and Alexa488-labeled anti-rabbit secondary antibody (green) are shown in the middle. Merged images showing LAMP-1-positive bacteria (yellow) and LAMP-1-negative bacteria (green) are shown on the right. (B) Data were collected from about 100 intracellular bacteria in total. The percentage that is LAMP-1 or LAMP-2 positive was calculated by dividing the number of colocalizing intracellular bacteria by the total number of intracellular bacteria scored. The average and standard deviation described here were calculated from three coverslips per strain in two independent experiments.

undertaken to understand the intracellular life cycle of *L. pneumophila*, very few species other than *L. pneumophila* have been examined phenotypically. The aim of this study was to uncover how *L. dumoffii* survives and replicates in mammalian cells and to identify the genes of *L. dumoffii* needed for intracellular growth. We isolated 4 mutants that were defective in intracellular growth in macrophages and alveolar epithelial cells among 790 independently derived Tn*903*dII*lacZ* mutants of *L. dumoffii*. The defect in intracellular growth of these four mutants cannot be attributed to a defect in adherence or entry, because almost equal numbers of mutants and wild-type cells were present within mammalian cells at 0 h postinfection. Two of the four mutants had a transposon insertion in either the *dotC* or *icmB* homologues (5, 51, 60). The *dot/icm* genes are required for intracellular multiplication of *L. pneumophila* (5, 51, 60). Our results suggest that the *dotC* and *icmB* genes of *L. dumoffii* and *L. pneumophila* appear to perform similar functions. We propose that the *dot/icm* genes are involved in the pathogenesis of most *Legionella* species, since these genes are important in the intracellular growth of these distinct *Legionella* species.

One of the mutants defective in intracellular growth was shown to have a transposon insertion in the gene which had sequence similarity to the *djlA* gene (16). Cloning and sequence analysis of this gene revealed that the primary structure of *L. dumoffii* DjlA showed homology to other bacterial DjlA proteins (10, 16, 73). DjlA is the third DnaK cochaperone of *E. coli*, containing a J domain highly conserved in the DnaJ/

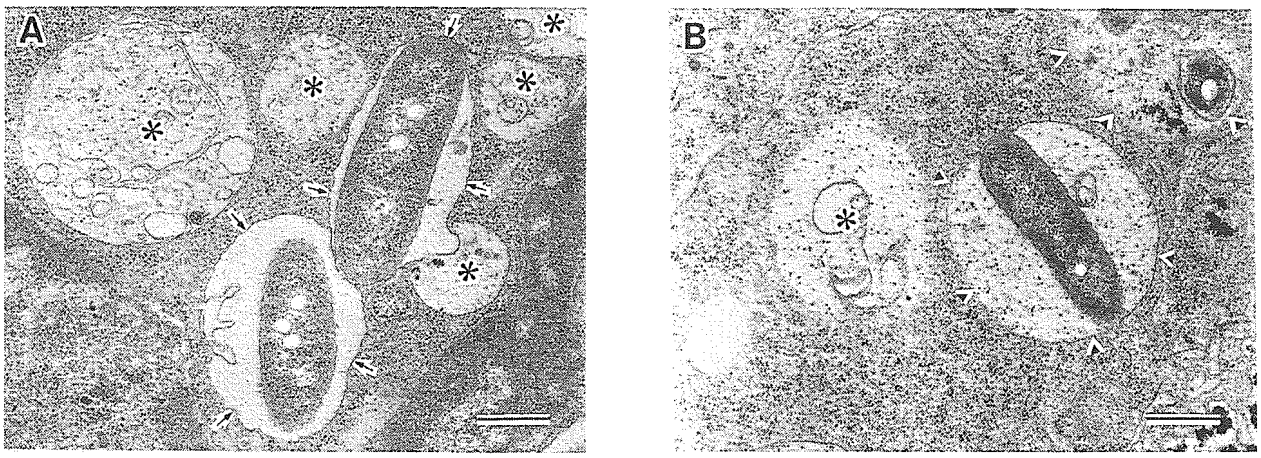

FIG. 5. Distribution of a lysosomal marker, BSA-gold, in phagosomes containing the wild-type strain or the *djlA* mutant strain. To label the lysosomal compartment, J774 cells were incubated with 15-nm BSA-gold overnight, washed, and then chased for 3 h. Cells were then infected with wild-type strain (A) or *djlA* mutant strain (B). At 4 h postinfection, the cells were fixed and processed for electron microscopy. Arrows in panel A indicate phagosomes containing no detectable gold; arrowheads in panel B indicate phagolysosomes containing BSA-gold; asterisks indicate, lysosomes containing BSA-gold. Bar, 0.5 μm.

Hsp40 family of molecular chaperones, including DnaJ and CbpA (16, 27, 65). CbpA is 39% identical to DnaJ along its entire length (64), while DjlA does not have any sequence similarity other than the J domain to DnaJ and CbpA in *E. coli* (26, 37). DjlA is unique in its structure and location in the DnaJ family. The J domain resides in the C terminus of DjlA but in the N terminus of other DnaJ family proteins. The N terminus of DjlA is integrated into the inner membrane through the single TMD, and the C-terminal J domain is located in the cytoplasm (16), while the whole of DnaJ and CbpA is localized in the cytoplasm. Moderate overproduction of *djlA* can trigger the synthesis of the colanic acid capsule in *E. coli*, mediated by the two-component regulatory system RcsC-RcsB, cooperating with DnaK and GrpE, but not DnaJ (15, 27, 37, 73). Unlike CbpA, DjlA could not adequately complement bacteriophage λ growth in a DnaJ-null background or restore bacterial growth above 40°C or below 16°C in the *dnaJ cbpA* null background in *E. coli* (15, 26, 37). The DjlA deletion mutant exhibits no apparent growth phenotype in *E. coli* (15, 16, 26). Thus, the true role of DjlA has been unclear.

We demonstrated that the *djlA* mutant of *L. dumoffii* exhibited a defective growth phenotype in mammalian cells and protozoan hosts. Phagosomes containing wild-type *L. dumoffii* excluded the late endosomal/lysosomal markers LAMP-1 and LAMP-2 and a lysosomal marker, BSA-gold, and were surrounded by RER in J774 macrophages, while *djlA* mutant-bearing phagosomes contained LAMP-1, LAMP-2, and BSA-gold and were not surrounded by RER (Fig. 4 to 6). It has been

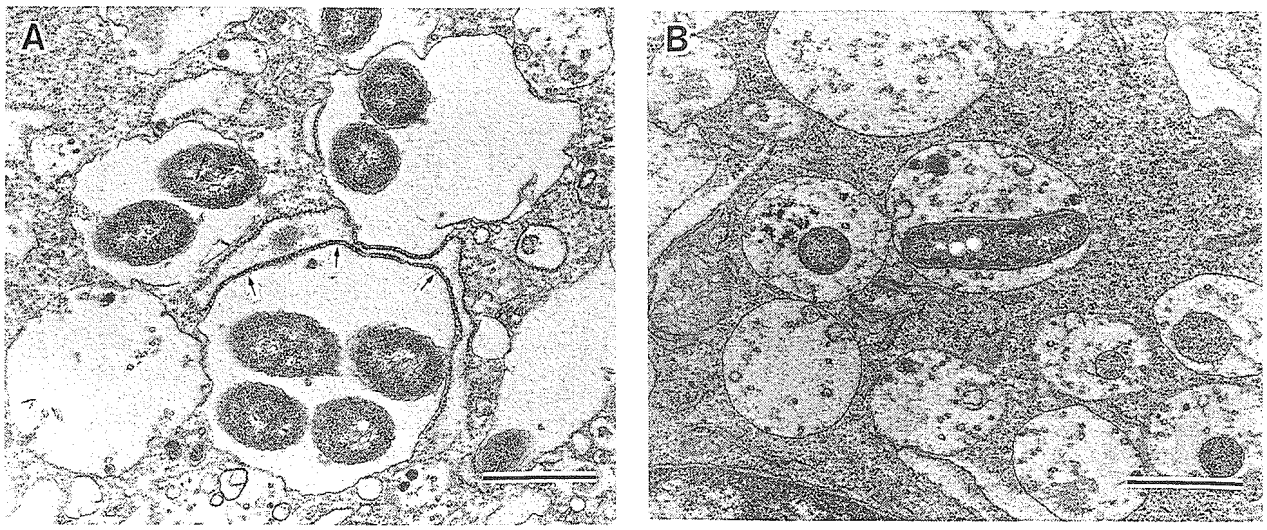

FIG. 6. Transmission electron micrographs of J774 mouse macrophages infected by the wild-type *L. dumoffii* (A) and the *djlA* mutant HOLD254 (B) at 8 h after infection. (A) Wild-type *L. dumoffii*-containing phagosomes were surrounded by RER (arrows). (B) HOLD254-containing phagosomes appeared to harbor much debris resulting from fusing lysosomes. Bar, 1.0 μm.

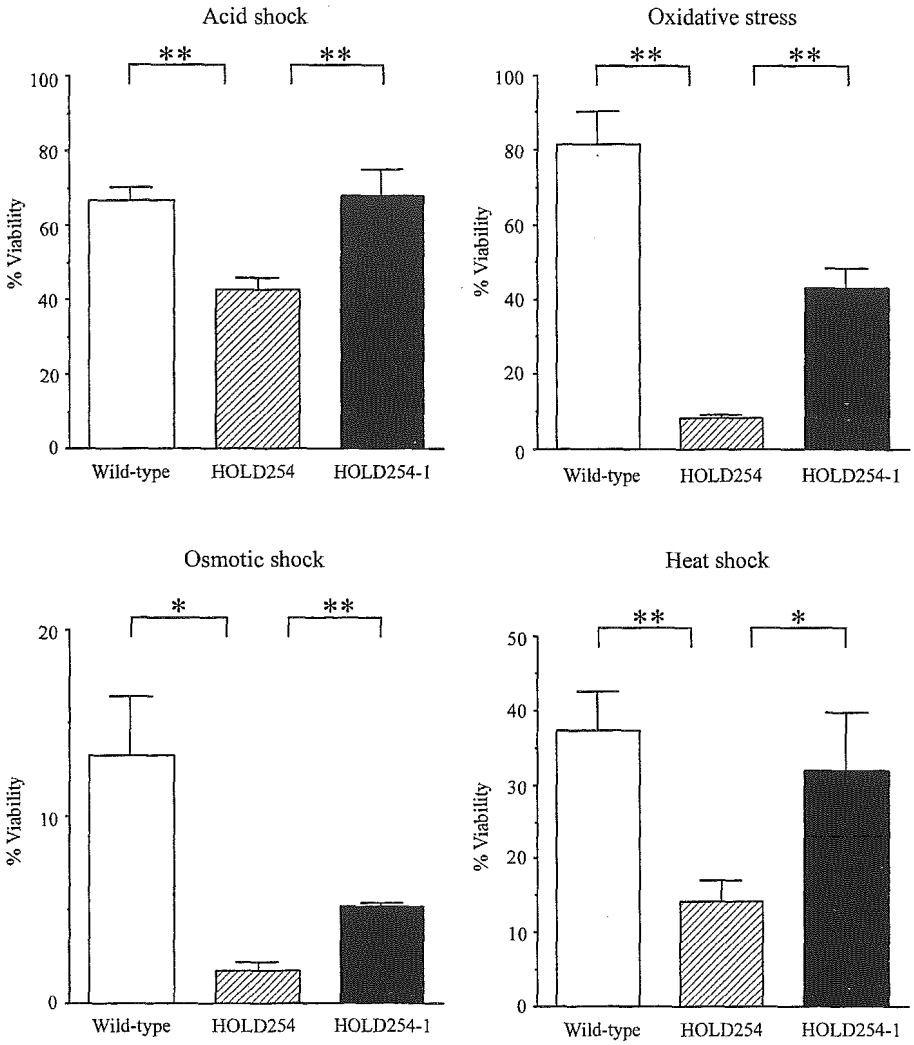

FIG. 7. Sensitivities of in vitro-grown stationary-phase wild-type *L. dumoffii* (open bars), the *djlA* mutant strain (hatched bars), and the *djlA* complemented strain (solid bars) to oxidative stress, osmotic stress, acid stress, and heat shock (10 mM hydrogen peroxide for 30 min, 5 M sodium chloride for 30 min, pH 3 for 5 min, and 48°C for 60 min, respectively). Stationary-phase cultures were exposed to each stress as described in Materials and Methods. The percentage of viable bacteria was calculated by dividing the CFU obtained from plating the bacteria onto BCYE agar plates following exposure to the indicated stress by the CFU of the bacteria obtained from plating the bacteria onto BCYE agar plates prior to exposure to the stress and multiplying by 100. Experiments were performed at least three times, and the results represent the mean and standard deviation. Results were analyzed for significance by analysis of variance and by a two-tailed, unpaired *t* test. Asterisks indicate significant differences between the *djlA* mutant and two other strains. (*, $P < 0.01$; **, $P < 0.001$).

reported that *L. pneumophila* is targeted into RER-surrounding phagosomes that do not fuse with lysosomes in mammalian cells (25, 33), while *L. micdadei* is targeted into RER-free phagosomes that are thought to fuse to lysosomes in mammalian cells (3, 36). Doyle et al. (20) reported that virulent *L. longbeachae*-containing phagosomes were surrounded by RER but avirulent *L. longbeachae*-containing phagosomes did not have RER. Our observations suggest that *L. dumoffii* might replicate in phagosomes which have not fused with lysosomes and are able to recruit host cell organelles, similar to that reported for *L. pneumophila*. The *djlA* mutant seemed to be intact (Fig. 5B), and no loss of CFU was observed during the infection (Fig. 1A and 2A). It is possible that the mutant bacteria are in either a late endosomal or a nondegradative lysosomal compartment, as described by Joshi et al. (35). The frequency of recruitment of *L. dumoffii* RER at 8 h is lower than that reported for *L. pneumophila* (32). We suspect that association with ER and avoidance of lysosomes by *L. dumoffii* is temporary, as shown for *L. pneumophila* (63).

Although the precise function of DjlA is unclear, it does not seem to play a direct role in intracellular trafficking. DjlA might contribute to folding or transportation of the proteins, such as Dot/Icm proteins, which play an important role in intracellular survival and growth. Most of the Dot/Icm proteins are located in the bacterial membranes, where they may associate to form a large transport complex, the type IV secretion apparatus (17, 43, 51, 60, 61). DjlA might cooperate with Dot/Icm proteins through their interaction in the membranes, since the N-terminal portion of DjlA is located in the cytoplastic membrane (16, 37). It has been reported that the two-

component regulatory system, PhoP-PhoQ, of *Salmonella enterica* serovar Typhimurium plays an essential role in survival within macrophages (28). It is possible that DjlA promotes *L. dumoffii* to adapt to intracellular environments and to coordinate with the two-component signal transduction systems. In vitro, DjlA-deficient mutants showed an increased susceptibility to several stresses, including oxidative stress, that might be encountered by bacteria in mammalian cells. DjlA might protect the genes or proteins, including Dot/Icm and catalase-peroxidase (7), that are important for intracellular growth, from harmful stress in a direct or indirect manner. Several lines of evidence for the important role of stress proteins in intracellular growth and virulence have been reported for intracellular pathogens; these include DnaK of *Brucella suis* (38), ClpC and ClpP of *Listeria monocytogenes* (24, 54), Lon of *B. abortus* (53), and GsrA of *Yersinia enterocolitica* (69). In *L. pneumophila*, at least 30 proteins are included during the intracellular infection of macrophages and at least 13 of these proteins, including GroEL (Hsp60), GroES, and GspA, are also induced by several stress conditions in vitro (1, 2, 21). Recently, Pedersen et al. (50) demonstrated direct evidence for the role of the stress protein of *L. pneumophila*, HtrA, during intracellular growth in mammalian cells but not in protozoan cells. Our data indicated that DjlA plays an important role during intracellular growth in both mammalian and protozoan cells. Besides Dot/Icm proteins, stress proteins or molecular chaperones might play an important role in the intracellular growth of the *Legionella* species.

In conclusion, we showed the essential role of *L. dumoffii* Dot/Icm homologues and DjlA during the intracellular infection of mammalian cells and protozoa. The precise mechanism of DjlA involvement in intracellular multiplication, including interaction with DnaK, remains to be elucidated. Further investigation of specific substrates with which DjlA interacts will lead to a better understanding of the intracellular survival mechanism in the *Legionella* species.

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IV. 研究成果の刊行物・別刷

４．環境中のレジオネラとレジオネラ症の迅速かつ網羅的
遺伝子診断法に関する研究

ゲノム情報を使った感染症の網羅的診断法

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これまでに、微生物のゲノム情報をどう解析するかという話が続きましたが、私は、現在までに分類学上正式に記載されている約5,500種の細菌の情報を、どのように感染症の診断に利用するかという話をします。

感染症の診断と病原細菌の同定

5,500種の細菌のなかでヒトに病気を起こす細菌性病原体は約900菌種で、そのうちもっとも危険度の高いレベル3の菌種としてチフス菌、炭疽菌、ペスト菌および結核菌などに代表される33菌種が、レベル2およびレベル1で、日和見病原体といって通常は病気を起こさず免疫力が落ちたときに感染を起こすものをいれて約900種に達します。そのなかで、菌類では50類ほど、病原性ウイルスとしては110種ほどを対象とした感染症診断法を開発することを目標に、私どもは研究を進めています。

医師が発熱した患者さんを前にして診察をするとき、これだけの病原体が対象となるため、原因菌を同定することは、かなりの経験を積んだ医師でも容易ではありません。そこで、従来は検体を採取して検査をしていました（図1）。下痢をしているのであれば、下痢便を採取して1日から数日かけて培養し、それをもとに同定作業を行います。この同定作業も1日から数日かかります。結核菌だと培養に1か月ほど、同定作業にさらに数週間かかります。このように長い時間がかかると、患者さんの病気が治った後に結果がでたり、運が悪いと重篤化して初めてわかるため、感染症の診断・治療にはあまり役立ちません。

いち早く病原体を特定し、その病原体に特異的に効く抗生物質を最初から選択することができれば、治療が的確になり、無駄な抗生物質を使う高額な治療費の浪費を抑えることができます。

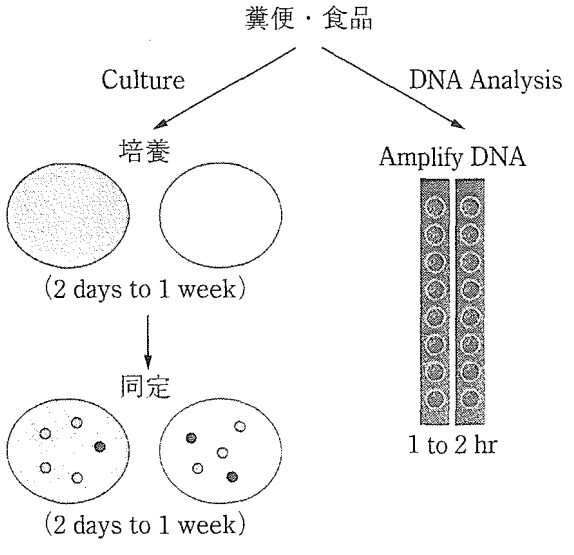

図1　培養検査と遺伝子検査の比較

このような視点に立って、私どもは主要なヒト感染症のうち、下痢症、気道感染、敗血症、髄膜炎、性感染症、眼科領域、皮膚感染などにわけ、これらの病気を起こす病原体を網羅的に迅速に解析する方法を作成しています。

感染症診断法の問題点

ところで、病原体のなかで通常の培地で培養できるものは、ごくかぎられています。たとえば、呼吸器感染症の場合、通常の培養で引っかかる病原体と、熟練した検査技師が特殊な培地を使って初めて同定できるものがかなりありますが、それでも通常の培養では培養できない病原体が多くなっています。ウイルスになると、インフルエンザウイルスのような特殊なごく一部の抗原検査が外来などでできるようになっていますが、通常は組織培養というやっかいな方法でなければ分離できないため、病院などではそのような検査をまったく行っていません。

皆さんが熱がでて病院にいったとき、検体をだすと、すべての感染症の病原体が調べられているものと考えがちですが、現実には、すべてを検査できないのが実情です。保険診療ではすべてを調べることはできません。医師が指定したときは特殊な検査を行いますが、それ以外は通常の数少ない培地を使って、頻度の高いものだけが検査対象となります。頻度の低い疾病、たとえば1年に1回しか見つからないような疾病や、外国にしかないような疾病、ウイルスは通常検査の対象外です。

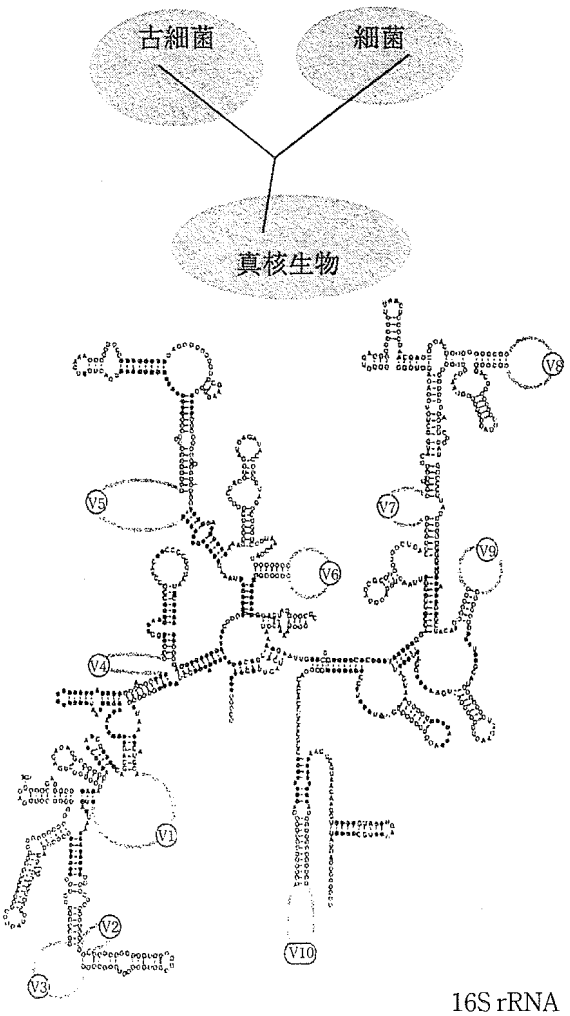

図2　生物のリボソームによる系統分類　図中のVは配列が菌種によって異なる

階級に応じた細菌の同定

新しい感染症の診断法には遺伝子情報を使います。大腸菌の場合、ゲノムは460万塩基対ほど、ヒトはその1,000倍ほどあります。そこで、すべてのゲノムを対象に使うことは無理があるため、大腸菌のゲノムのなかの、どの情報を使うかが問題になります。大腸菌の菌株によってもゲノムサイズは違いますし、大腸菌以外の菌種によってもさまざまです。また、遺伝子の数も数百から数千とかなりのひらきがあります。

15年ほど前から、真核生物に共通な16Sリボソーム RNA（16S rRNA）の遺伝子配列を使って微生物を分類する方法がさかんになり、そのデータが蓄積されてきました。そのデータをもとにすると、地球上の生物は3つのドメインに大別することができます（図2）。細菌（Bacteria）と古細菌（Archaea）、真核生物（Eukaryotes）です。この真核生物のなかに、原虫、カビ、動物、植物が分類されます。この16S rRNA(真核生物は18Sリボソーム RNA）の配列情報をもとに作成した系統樹を使うと、細菌性の病原体をほぼ同定することができま

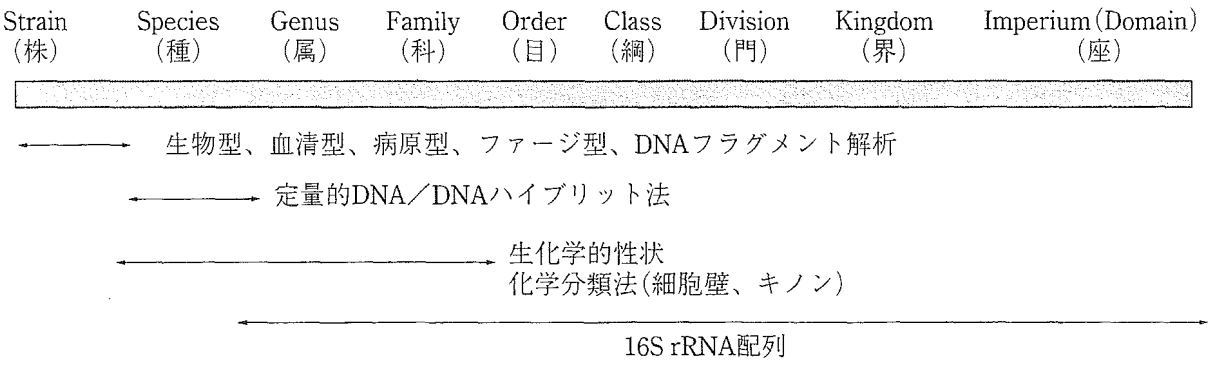

図3　分類階級と使用される指標

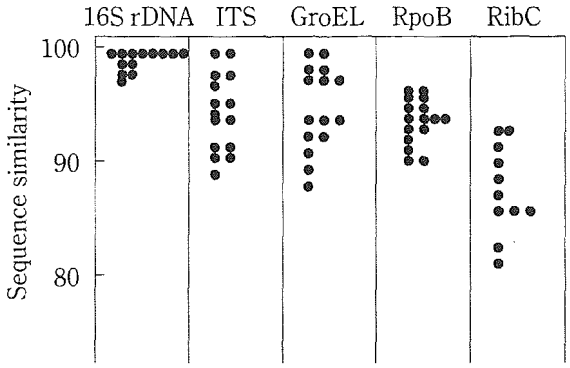

図4　Bartonella 属菌種の遺伝子の多形

表1　菌種の定義の変遷

- 少ない生化学的性状で病原菌を同定し分類した
- 血清型による分類：
  連鎖球菌、サルモネラ
- 数値分類：
  多数の性状を調べて分類同定
- 化学分類：
  安定な細胞壁、Quinone、GC %、脂質
- リボソーム配列と DNA／DNA ハイブリッド法

す。

微生物の分類は、分類段階によって使う情報が違いますが、現在、16S rRNA 配列で微生物の高次分類から菌種に近いところまで分類するシステムが確立しています（図3）。そして、この15年間の研究の過程で、この方法論の利点と欠点も明らかになっています。図4は、Bartonella 属の菌種の16S rRNA 配列を比較したものですが、ほとんどが同じ配列となります。つまり、16S rRNA 配列では、Bartonella 属の菌種を識別することはできません。この16S rRNA にかわるものとして、23S リボソーム RNA の配列と、タンパク質、RNA ポリメラーゼなどが候補にあがってきています。他の遺伝子配列をみると16S rRNA より配列に多様性があることから、識別能力があります。GroEL でも同じです。

病原細菌の同定法

これまでに、菌種の定義が変遷してきました（表1）。現在では、種の同定にリボソーム配列と DNA／DNA ハイブリッド法を使っています。この定義に基づいて病原細菌を同定する場合、16S rRNA だけでは無理で、さらに細かい候補遺伝子、特に病原菌の場合は病原性因子、毒素をつくるかつくらないか、組織に侵入するかしないかなどを検出のターゲットにして検出系を組むようにします。私たちが目標としているのは、患者さんが来院して検体を採取して、あるいは入院患者さんから朝の検査で検体を採取して、その結果を次の日、理想をいえば2～4時間でだせるようなシステムを開発することです（図5）。そのためには、遺伝子を抽出し、その遺伝子を特異的にふやす方法が必要になります。

図5に示す方法であれば、臨床材料から病原体の遺伝子を抽出して遺伝子を増幅するまでに約1時間です。抽出作業に約30分、もっとも早い遺伝子の増幅方法だと、1個の遺伝子を100万個までふやすのに10～15分ですみます。そして、病原体を決める際に、約1,000種の病原体すべてを対象とすることは物理的

に不可能であることから、病原性の強いものは遺伝子増幅方法でいっきに確定し、病原性の弱いものは属レベル、ファミリーレベルで捕まえるようして、遺伝子を増幅する試薬の種類を極力減らす工夫をしています。

ここで遺伝子をグループで増幅する方法を使った場合、識別が必要となりますが、それにはDNAチップを使います。DNAチップには病原体のリボソームの配列と病原因子を固定します。それと1時間ほどグリッドをつくらせ、レーザースキャナーでみたり、蛍光顕微鏡、CCDカメラで捕まえる方法を使っています。

DNA増幅キットの開発

喀痰の病原体を調べるとき、普通は、細菌とカビを対象にしますが、ウイルスの場合はRNAウイルスとDNAウイルスが混在しているため、抽出方法、増幅方法がかわります。ここにいろいろな病原体があり、この数をみると20いくつかしかはいっていませんが、ここに遺伝子を増幅するための試薬セットが16個はいっています。ただし、病原体には900菌種もあるため、これではすべてを増幅できないのではないかと考えるかもしれませんが、ひとつの属の菌種をまとめて増幅するような試薬をいれています。たとえば、Chlamydiaのプライマーはクラミジア属の菌なら何でも増幅することができます。さらに、ひとつのなかに何種類かの遺伝子増幅試薬をいれることで、ウエル数を減らします。このなかには3種類ほどの試薬がはいっていますが、これを増幅した後、電気泳動にかけると増幅したDNAフラグメントの違いがでて、その長さを測定することで、どの病原体の遺伝子が増幅したかがわかります。

ただし、このようなグループプライマリーで増幅したものは、さらにマイクロアレイなどを使って細かな種の識別をするようにします。

下痢でも同じようなことがいえます。その場合、呼吸器疾患とは違って16S rRNAをほとんど使わず、病原因子を使って検出します(図6)。

病原体遺伝子の抽出法

ほとんどの細菌検査室では、検査費削減のために遺伝子の増幅機を使い、電気泳動で得たフラグメントで識別しています。費用に少し余裕がある施設では、フラグメントをさらに細かくDNAチップで読みとったり、さらに余裕のある施設ではリアルタイムで増幅をモニタリングして病原体数を測定しています。

このような方法でもっとも重要な点は、病原体の遺伝子の抽出法です。細菌の場合、グラム陽性菌とグラム陰性菌でDNAの壊れ方が違います。芽胞をもつ菌は壊れにくいため、

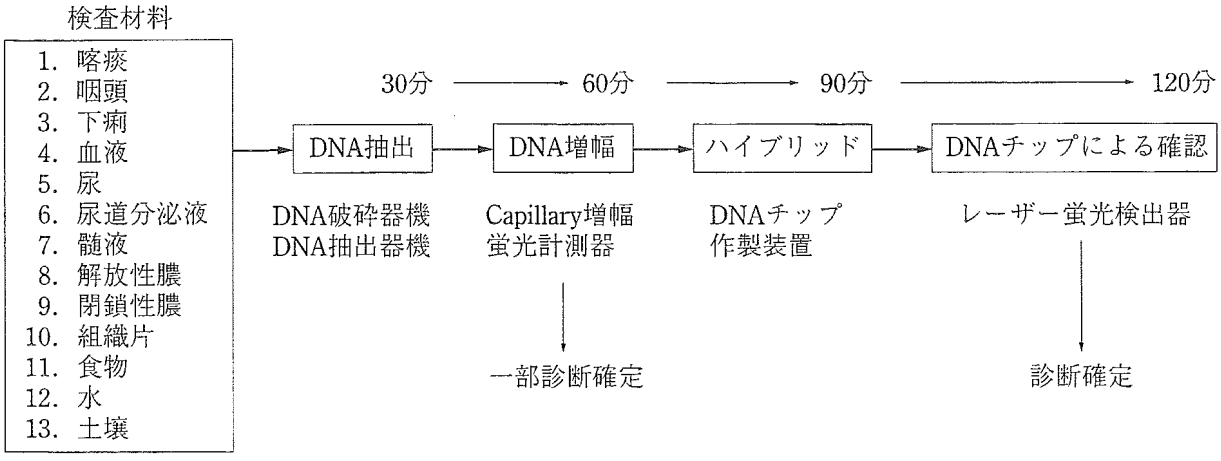

図5　迅速な遺伝子診断

どの病原体も同じレベルで破砕しなければなりません。私たちはジリコニアビーズというガラスビーズより密度が2倍ほど高いビーズを使って、菌体を物理的に破砕しています(表2)。また、タングステンビーズはジリコニアビーズの約2倍の密度があるので、さらに強力な破砕ビーズになります。これを使うと、ほとんどの菌がほぼ100％に近い状態で壊れます。

ちなみに、タングステンビーズがもっとも破砕効率がよいのですが、純度の高いタングステンビーズの入手は容易ではありません。純度のよいものは高価で、臨床の場ではなかなか使えません。現実的には、ジリコニアビーズを使って物理的に破砕してDNAを抽出しています。

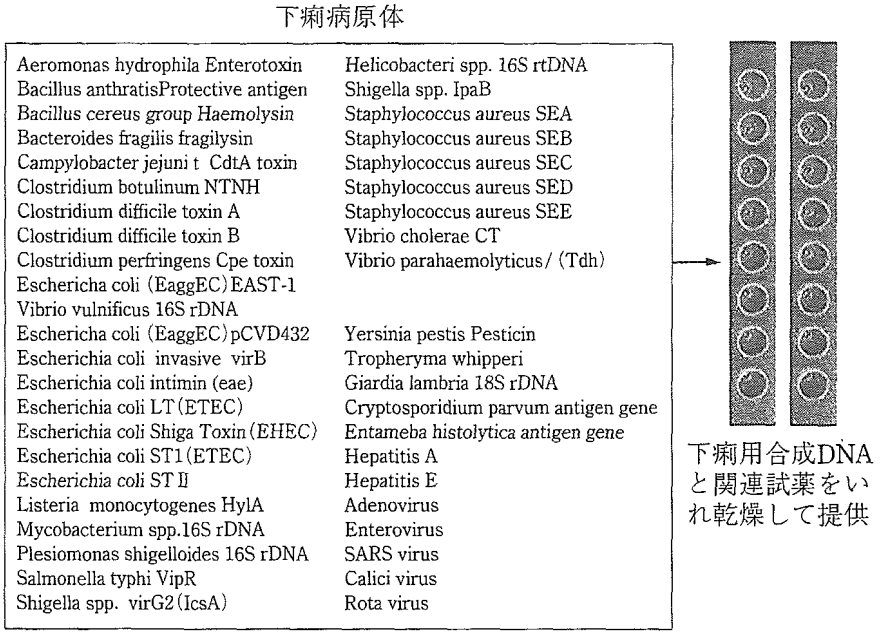

図6　多数の下痢病原体の遺伝子検査

表2　細菌を破壊する共通のプロトコール

	酵母菌 (*Saccharomyces cerevisiae*)	コメコウジカビ (*Aspergillus oryzae*)	枯草菌 (*Bacillus subtilis*)
ガラスビーズ	38％	29％	42％
ジルコニアビーズ	88％	66％	81％
タングステンビーズ	99％	92％	96％

尿道炎の病原菌の同定

しかし、検体によっては簡単な方法で病原体遺伝子の抽出が可能です。たとえば、尿道炎で性病科にきた患者さんの尿道分泌物を蒸留水に浸して3分間ほどボイルした液の一部を遺伝子増幅試薬に加えると、約15分後には、遺伝子が増幅したものをモニタすることができます(図7)。20～40サイクルで遺伝子が増幅するため、サンプリングしてから30分以内に答えがでます。そのため市中の医院でも使えると考えています。

実際の尿道炎では、病原体の種類は非常に少ないため、15種類の病原体をふやす試薬セットをつくって、すべての性病の病原体をモニタする方法を開発しています(表3)。

すべての菌を増幅するようなプライマリーをつくって、まだ尿道炎を起こすことが学問的に確定されていない細菌の遺伝子を増幅し、それをさらにチップで解析することで、新たな尿道炎の病原体を同定する方法論を構築しています。

ただし、ひとつのウエルのなかに何種類もの試薬をいれているので、遺伝子が増幅したことがモニタリングでわかっても、電気泳動でそのサイズを調べる必要があります。たとえば、図8の場合、340塩基のDNAフラグメントがふえただけでは、どの細菌の遺伝子が増幅したのかはわかりません。これをマイクロアレイと反応させて細かく識別するように

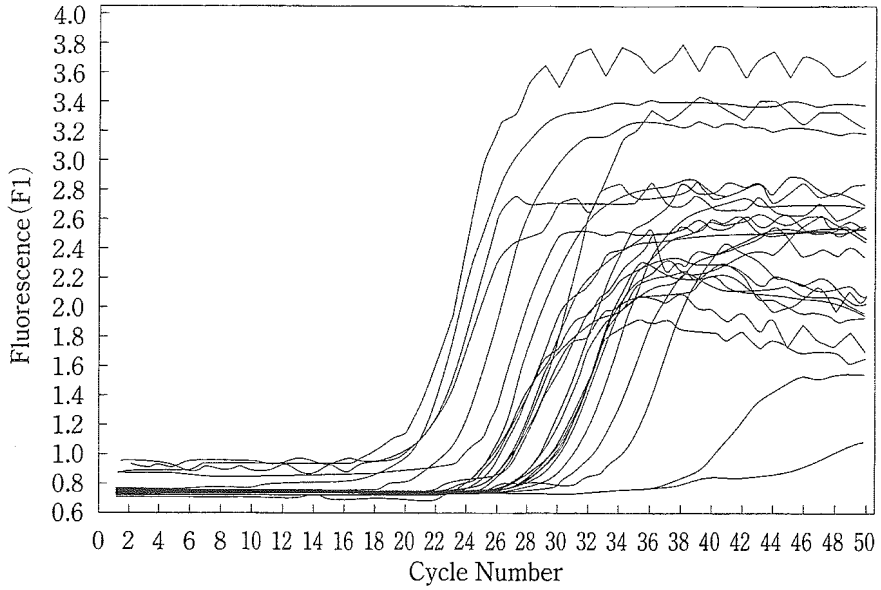

図7　尿道炎の Real Time PCR

表3　尿道炎を起こす病原体の遺伝子検査法の標的遺伝子

	病原体	遺伝子
1.	*Bacteroides ureolyticum*	16S rDNA
2.	*Chlamydia trachomatis*	MOMP
3.	*Haemophilus ducreyi*	16S RNA
4.	*Haemophilus influenzae*	16S rDNA
5.	*Mycoplasma fermentans*	IS like element
6.	*Mycoplasma genitalium*	Adhesin antigen
7.	*Mycoplasma hominis*	16S rDNA
8.	*Mycoplasma pneumoniae/genitalium*	16S rRNA
9.	*Neisseria gonorrhoae*	16S rDNA
10.	*Haemophilus parainfluenzae*	16S rDNA
11.	*Treponema pallidum*	47KDa gene
12.	*Treponema pallidum*	16S rDNA
13.	*Trycomonas vaginalis*	B-TUBULIN
14.	*Ureaplasma ureolyticum*	16S rDNA
15.	Herpes simplex Ⅰ-Ⅱ	DNA polymerase

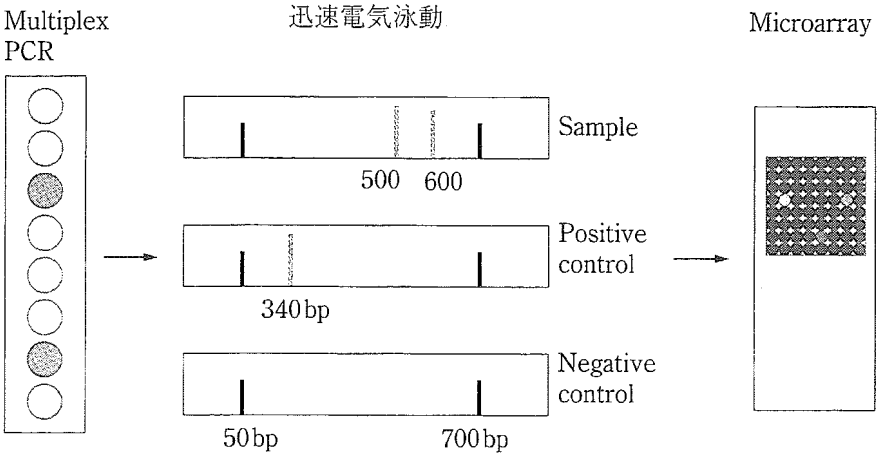

図8　遺伝子増幅と迅速電気泳動

します。

マイクロアレイは、ひとつの細菌の全ゲノムのマイクロアレイと違って、菌種に特異的な配列がはいっていて解析が楽です。図9の一番左は、Mycobacterium アレイの *M. bovis* で、同じスポットが3つ特異的に反応しています。ところが、右の *M. kansasi* や *M. gordoni* は反応せず、16S rRNA の配列が非常に近いものはときどき識別が難しくなりますが、スポットの強さをみれば定量が簡単であることがわかると思います。

さらに幅広く900種類ほどをマイクロアレイに固定したときのデータを図10に示します。性病を起こすクラミジアの場合、特異的に反応します。

図11は、白血球のなかの細菌の遺伝子を増幅してマイクロアレイと反応させたところ、*Ehrlichia sennetsu* のシグナルがでてきました。通常は培養できないため培養検査の対象にはなりませんが、ユニバーサルプライマーという、微生物を何でもふやす遺伝子増幅試薬を使うと、ときどき引っ

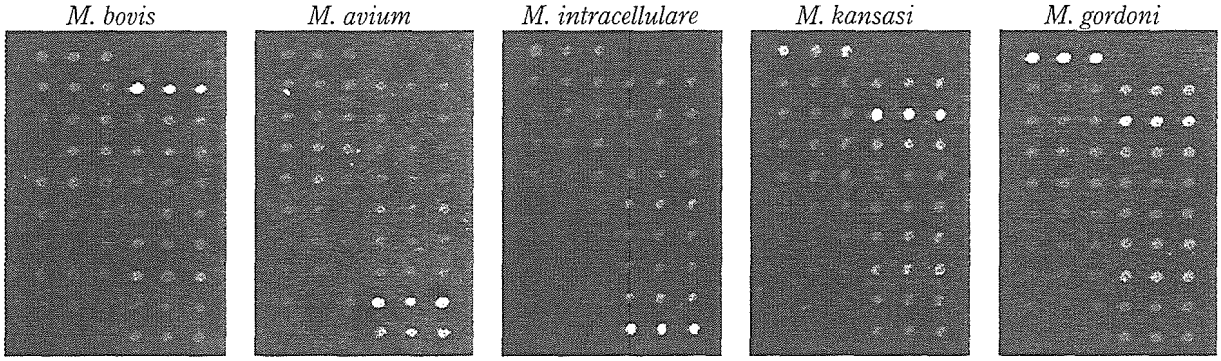

図9　Mycobacterium 属菌の DNA アレイ

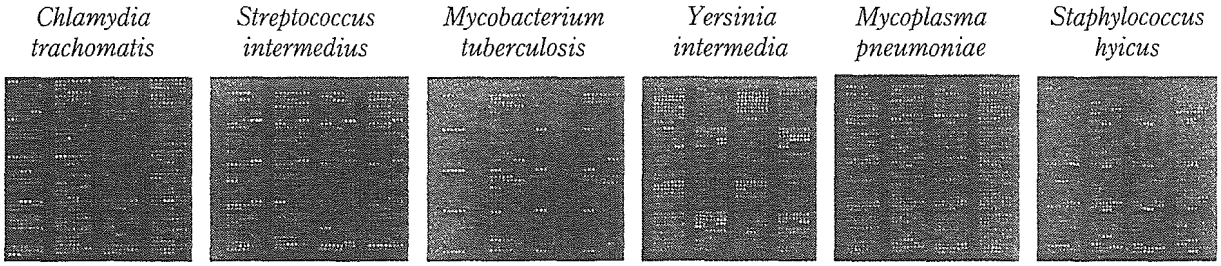

図10　リボソームを使ったマイクロアレイ（口絵カラー参照）

かかることがあります。

新しいシステムの構築

もう一度振り返ってみます。菌体を破砕して DNA を抽出する操作、遺伝子をふやすための試薬セットを準備しリアルタイムで遺伝子が増幅するのをモニタリングする、増幅したものをマイクロアレイに反応させて、それをレーザースキャナーでスキャンする工程があります。おのおのの工程で高価な機械を使います。たとえば、リアルタイムでモニタリングするような機械は高価ですし、レーザースキャナーも高価です。貧乏な細菌の検査室ではなかなか、このような装置を導入してもらえません。

そこで、私たちがもっとも力をいれているのは、もう少し安価で実用的なシステムを開発することです。私どもは、縦横 8mm 角のファイバに約 100 種類の遺伝子をのせるようにしています（図 12）。中空ファイバのなかに DNA を埋めこんで、それを 100 本なら 100 本のファイバを束ねた後、金太郎飴のように切断して、そのひとつを DNA チップとして使うようにします。シリコンアレイとしては 3mm 角のものを使っています。

図 13 は、実際にファイバアレイでだしたデータで、下痢の病原体です。これがそのときの実際の画像です。同じものを 2 つのせてあります。

この方法で、赤痢菌の virG やエロモナスと

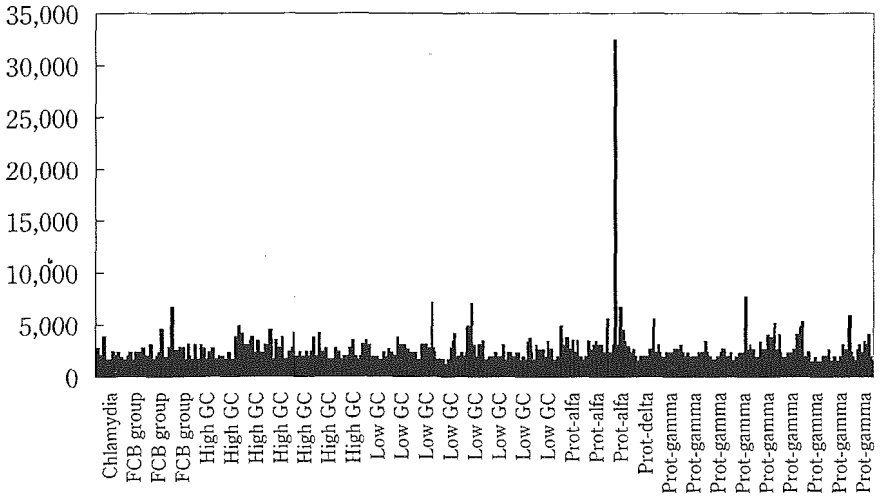

図11　白血球中の *Ehrlichia sennetsu*

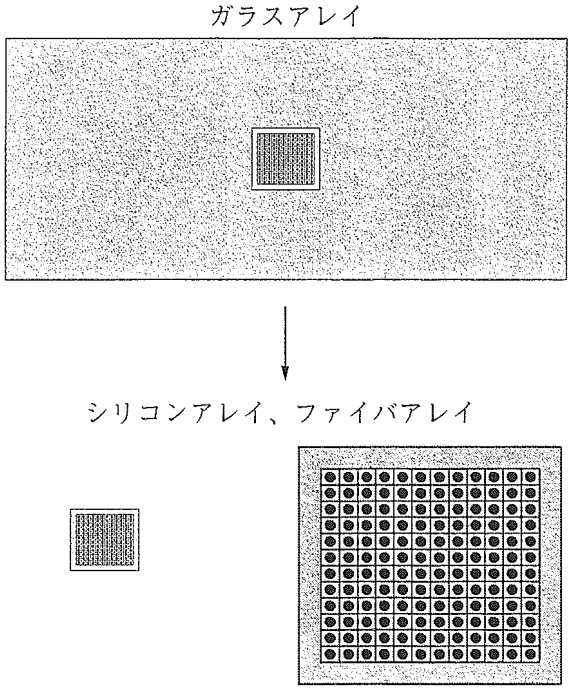

図12　迅速で安価なマイクロアレイ

いう下痢を起こす病原体も、簡単に識別することができます。リボソームRNAではときどき類似した配列をもっている菌のシグナルがでてきますが、それも簡単に識別することができます。

マイクロアレイというと、たくさんのシグナルを定量する技術がたいへんですが、この場合、簡単に定量することができます。

常在菌を固定したマイクロアレイの利用

これを進めて、環境中の微生物を網羅的に調べるマイクロアレイを開発しています。環境中の微生物として5,000種ほどが記載されていますが、実際には数百万菌種はいると推定されています。それらを解析するには、網羅的に既知の微生物の遺伝子を固定したマイクロアレイを作製してスクーニングするシステムが不可欠です。私たちはこれを土壌や健康者の糞便内の菌のモニタリングに使っており、現在データを蓄積しています。健康なときと病気になったとき、菌相の変化をマイクロアレイで網羅的に調べることが、これからの目標になっています。

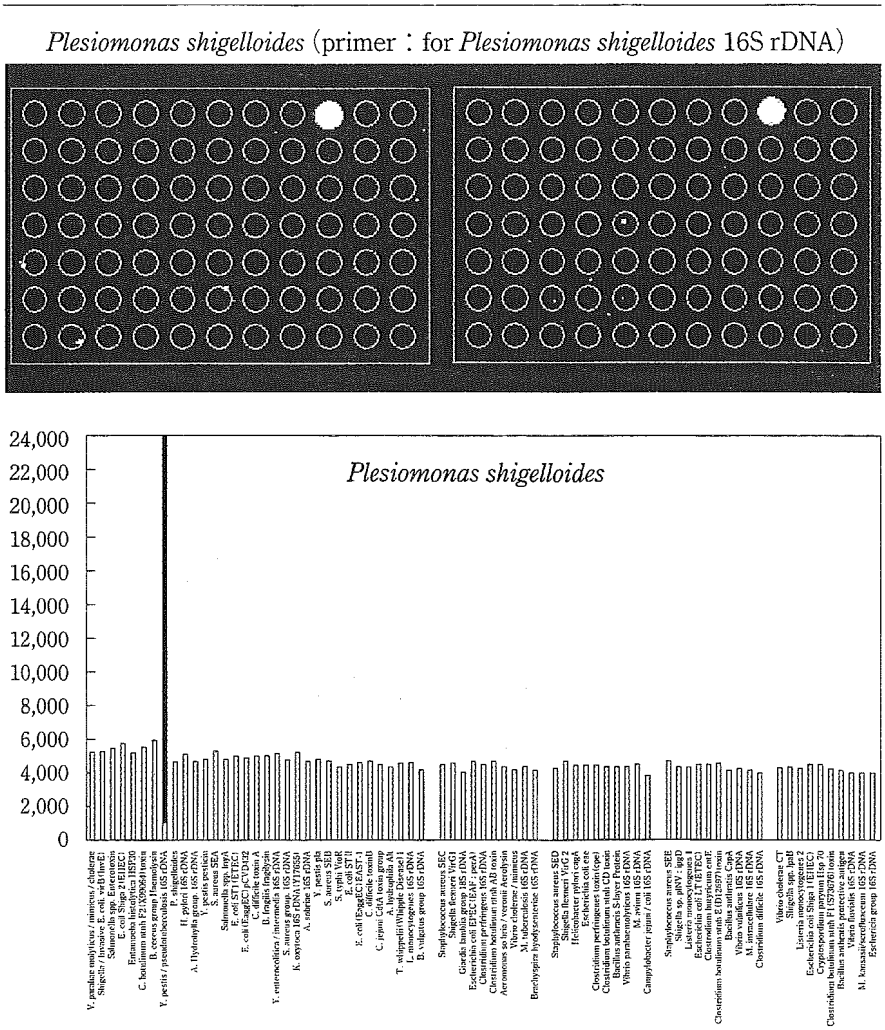

図13　ファイバーアレイで識別した下痢の病原体

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Q&A

■Q■

薬剤感受性にゲノムを使った診断について、薬剤感受性のデータが得られませんがどうしていますか。

●A●

感受性の情報をすべてゲノムの情報だけで予測することはできないので、当分の間、培養検査は生き残ると思います。診断で使っているDNAチップは、病原菌の場合はほとんどがオリゴを使っています。すべてオリゴにしたいのですが、環境中にはフローラの解析が未知の菌がたくさんいるため、それを捕まえるためにcDNAも使っています。

■Q■

高額な医療費を抑えることが可能となれば、患者にしてみれば喜ばしいものの、それで利益が下がれば新しい研究ができなくなります。そのバランスはどうしていますか。

●A●

今後、定額医療が定着すると、いかに薬を少なく使って、早く患者さんを治療するかが、医療上、重要になります。その意味では貢献できると考えています。

■Q■

ユニバーサルプライマーを使うと、メジャーなものだけしか捕まらないのではないのでしょうか。

●A●

実際、糞便などでユニバーサルプライマーを使うと、うまくいって糞便全体の0.1％ほどまでのフローラしか捕まえられません。ユニバーサルスペシフィックグループプライマーを組合わせて、フローラの解析に使っています。

■Q■

シリコンアレイやファイバアレイの検出法はどうするのですか。

●A●

グラスアレイの場合、レーザースキャナーを使います。シリコンアレイとファイバアレイの場合、CCDカメラを使うか、一番安く使える蛍光顕微鏡での観察を考えています。

■Q■

DNAチップを、電気泳動する前に用いれば早くなると思いますが、できない理由は何ですか。

●A●

リアルタイムでモニタリングできるところは、そのままDNAチップを使います。リアルタイムの機械をもっていない施設は、電気泳動をして、そのフラグメントをみてからチップにあてるようにしています。

■Q■

ゲノム解析によって、種をあらためてわけるとか、一緒にするようなことはないのでしょうか。

●A●

現在、全染色体の遺伝子の30％以内の多様性であれば、その株の集団は、ひとつの種と定義されています。それにかわるような定義はまだできていません。現在、5～10個のハウスキーピング遺伝子を数個選んで、その多様性を比べることで種を決める動きがでてきています。5個でよいのか10個でよいのか、まだコンセンサスが得られていません。ただし、ゲノムの情報が蓄積されれば、種を決める新しい概念ができると思います。16S rRNAデータが蓄積して種の系統の概念ができあがるのに15年ほどの年月がかかりました。これからさらに蓄積していけば、10年後、20年後には新しい種の概念ができるかもしれません。

診　　断

細菌の迅速診断システム

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Rapid diagnostic system of bacterial infection

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Abstract

Approach to develop genetic detection system for bacterial infection in blood is a demanding subject because number of bacteria in blood stream is very few and in most cases the number of bacteria is less than the detection limit of gene amplification methods. At the first stage of blood sampling, we only applied gene amplification method against fastidious organisms to culture and organisms causes severe infection. Organisms which are usually found very few in blood are subjected to blood culture. After monitoring system of blood culture gave positive signal of bacterial growth, DNA or RNA in culture media were extracted and amplified with universal primers for bacteria and fungi.

Amplicons were applied to a rapid sequencer. However, in case of mixed infection, the sequencing is not successful. In such cases, the amplicons are analized with DNA microarray, immobilized 16S rDNA and 28S rDNA of 1,000 human pathogenic bacteria and fungi.

Key words: bacterial infection, microarray, sepsis, bacteremia, molecular diagnosis, blood culture

はじめに

菌血症や敗血症を疑う診察指標が出揃っても，通常，病原体の抗体検査を行うためには，抗体が上昇するまでの日数が必要で，疾病を絞り込むための更なる診察情報が必要になる．手がかりのない感染初期の状況では，血液培養が唯一の感染症の病原体を特定する積極的な検査法になる．感染症を疑って培養を行っても現状では血液培養が陽性になる確率は2–3割で，臨床医の診断治療に役に立つ情報を提供できるケースは少ない．このことは血液培養に期待する臨床医の意欲を低下させる原因にもなっている．この培養陰性の背景には発熱の原因が感染症以外にある場合も含まれているが，感染症であった場合も，ウイルス，培養困難な細菌，抗生物質治療後の検査などが隠されている．血液培養の培地は頻度の高い一般細菌の検査に照準を合わせて作成されており，培養困難な細菌で頻度が少ない病原体検査は特別な場合を除いて，忘れ去られてきた．

そこで遺伝子検査が現在の血液培養に全部か

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