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Effect of AL072, a Novel Anti-*Legionella* Antibiotic, on Growth and Cell Morphology of *Legionella pneumophila*

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Abstract AL072 is a potent anti-*Legionella* antibiotic produced by *Streptomyces* strain AL91. The minimum inhibitory concentration (MIC) of AL072 against *Legionella pneumophila* was 0.2 μg/ml. Bacterial growth was rapidly inhibited at the dose range between the MIC and 20 times of the MIC when the antibiotic was added at the mid-exponential phase. Ultrastructural changes in *L. pneumophila* were observed upon treatment with AL072. Under electron microscopical observation, the organisms treated with AL072 exhibited characteristic morphological changes in the cellular outer coat. Also irregular morphological changes, such as the formation of filamentous materials in the cytoplasm, an increase in the size and number of cytoplasmic vacuoles, the extruding of cytoplasmic contents, the formation of spheroplast and ghost cells, and blebbings in the cell wall were observed. Furthermore, immunoelectron microscopical observation of the group treated with the MIC showed that the immune complex attached mainly to the cell wall. The results of these experiments indicate that AL072, like the inhibitors of cell wall synthesis, act selectively on the cell wall of *L. pneumophila*.

Key words: AL072, electron microscopical observation, *Legionella pneumophila*

Legionella pneumophila is a gram-negative bacterium and the causative agent of Legionnaires' disease or Pontiac fever [6]. This bacterium multiplies in biofilms that are formed in water plumbing systems and subsequently often spreads by aerosol from a shower [12]. Therefore, cooling towers are excellent amplifiers for *Legionella*, and also efficient disseminators of these bacteria [1, 4, 7, 8].

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The most logical way of preventing *Legionella* infections is to eliminate it from its breeding source, *e.g.* cooling towers, which could produce aerosols susceptible to human inhalation [18]. However, this is difficult to accomplish in practice because of the resistance of bacteria to biocides [13, 22]. Hot water (60°C) flushed through pipes and UV light can kill *Legionella* spp., yet these methods are too expensive to treat large amounts of water [5, 16, 18, 20]. Moreover, some antiseptics have a corrosive effect on cooling systems [21]. Accordingly, a renewed effort is being made to identify biocides that are safe for humans but kill *Legionella* spp. Several antimicrobial agents have been investigated as potential alternatives for clinical use in the treatment of legionellosis [11]. Studies on the effects of several antibiotics on the cell morphology of *L. pneumophila* have been previously reported [2, 17]. *L. pneumophila* is sensitive to erythromycin, rifampicin, aminoglycosides, and other antibiotics in *in vitro* tests. However, only erythromycin combined with rifampicin is found to be effective *in vivo* [13]. *L. pneumophila* is an organism that possesses a unique cellular lipid composition with a high proportion (>68%) of branched-chain fatty acids. This unusual chemical make-up may affect the organism's morphological response to certain antibiotics [14, 15]. The inhibitors of cell wall synthesis, ampicillin, cefotaxime, and methicillin, display bactericidal activity and induce extensive morphological changes, which include the formation of membranous lesions through the extrusion of cytoplasmic contents, disruptions in cell wall, development of vacuole-like lesions in cell wall, and cell lysis [17]. In terms of ultrastructural damage and loss of viability, the protein and DNA synthesis inhibitors are less effective than the antibiotics that acted on the microbial cell wall [17].

Recently, a novel potent anti-*Legionella* antibiotic, AL072, produced by *Streptomyces* strain AL91 was identified and

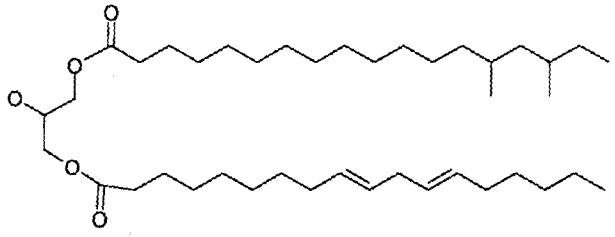


Fig. 1. The structure of AL072.

isolated [23]. Its molecular weight was determined by FAB-MS (m/z 648), and the compound was identified as a novel 1,3-diacylglycerol with a molecular formula of $C_{41}H_{76}O_5$. One of the two acyl groups is linoleyl and the other is 3,5-dimethyl octadecanoyl (Fig. 1). The MIC value for AL072 against *Legionella* spp. was 0.2 μg/ml. The compound was extremely specific towards the *Legionella* species (Table 1). Interestingly, no antibacterial activity was demonstrated against any other bacteria tested.

This study presents the ultrastructural changes in the cell morphology of *L. pneumophila* after treatment with AL072.

Growth Pattern and Viable Counts

To investigate the growth pattern of *L. pneumophila* ATCC 33152, a colony was inoculated into a BCYEα [23] liquid medium and the mid-exponential phase of growth was determined. Bacterial viable counts were performed at 6, 12, 15, 18, 22, 25, 27, 30, 35 and 40 h of incubation in triplicates by inoculating 100 μl volumes of 10-fold serial dilutions of the cultures in phosphate-buffered saline onto a BCYEα agar medium. The resulting colonies were counted and viable counts (CFU/ml) were determined from plates containing 30-100 colonies. As shown in Fig. 2, the number of organisms in a BCYEα liquid medium increased steeply during 6-22 h of culture, and there was no further increase after 22 h. To investigate the growth inhibitory effect of AL072, the antibiotic was added at the 26 h time point. The viable counts for *L.*

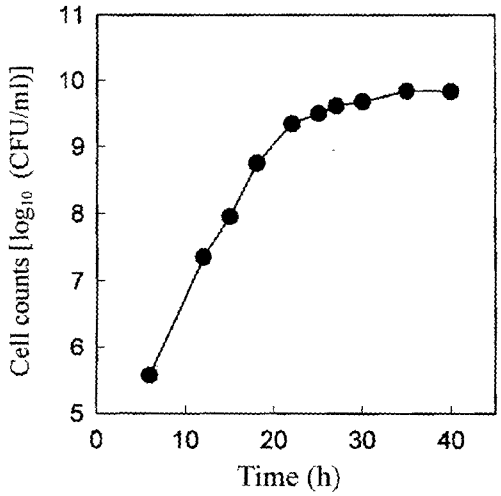


Fig. 2. The growth pattern of *L. pneumophila* ATCC 33152 in a BCYEα liquid medium. The mid-exponential phase of growth was observed at 12-15 h.

pneumophila ATCC 33152 in a BCYEα solid medium showed a slight decrease in the MIC dose. However, with 20 times of the MIC dose, the viable counts reduced rapidly (Fig. 3).

Production and Titration of the Antibody against the AL072/BSA Conjugate

AL072 was conjugated with bovine serum albumin (BSA) according to the method of Dixon-Holland and Katz [3]. The conjugated antibiotic was then subcutaneously inoculated into two New Zealand White rabbits (3.5 kg) for the production of the antibody. After three consecutive booster injections, whole blood was collected from the heart. The antibody titer was measured by an enzyme-linked immunosorbent assay according to the method of Kim and Lim [10]. Employing the Criss-cross reaction method of antigen and antibody, the optimal antigen concentration was calculated as 17 μg/ml. The titer of the antibody against the AL072/BSA conjugate was 1:2048 (Fig. 4).

Table 1. The MIC values of AL072 against the various microorganisms.

Microorganism	MIC (μg/ml)	Microorganism	MIC (μg/ml)
Legionella pneumophila ATCC 33152	0.2	*Legionella micdadei* ATCC 33218	0.2
Legionella bozemanii ATCC 33217	0.2	*Legionella dumoffi* ATCC 33229	0.2
Corynebacterium diphtheria (1)[a]	>100	*Pseudomonas aeruginosa* [7]	>100
Staphylococcus aureus (4)	>100	*Klebsiella pneumoniae* [5]	>100
Listeria monocytogenes (1)	>100	*Enterobacter cloacae* [5]	>100
Streptococcus spp. (6)	>100	*Serratia marcescens* [4]	>100
Salmonella spp. (2)	>100	*Citrobacter freundii* [1]	>100
Escherichia coli (10)	>100	*Shigella flexneri* [1]	>100
Bacillus spp. (2)	>100	*Proteus* spp. [9]	>100

[a]number(s) of the different strains tested.

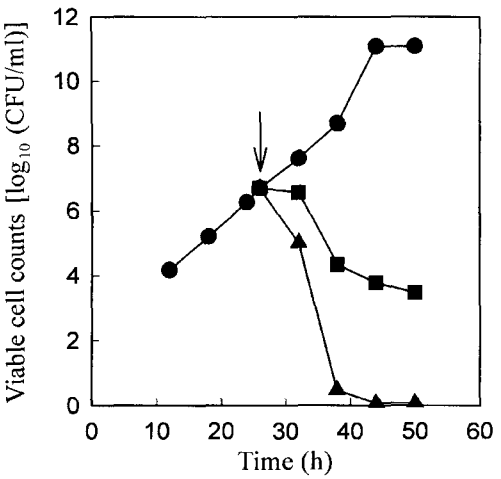


Fig. 3. Viable cell counts of *L. pneumophila* ATCC 33152 with and without AL072 in a BCYEα solid medium.
AL072 was added at the mid-exponential phase of growth (arrow, at 26 h point). ●—●: no antibiotic treatment, ■—■: MIC treated, ▲—▲: 20 × MIC treated.

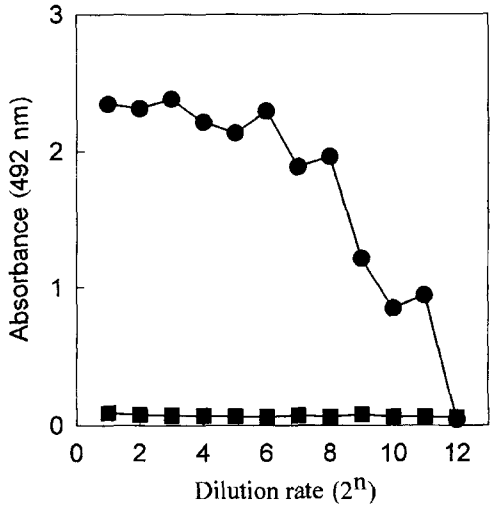


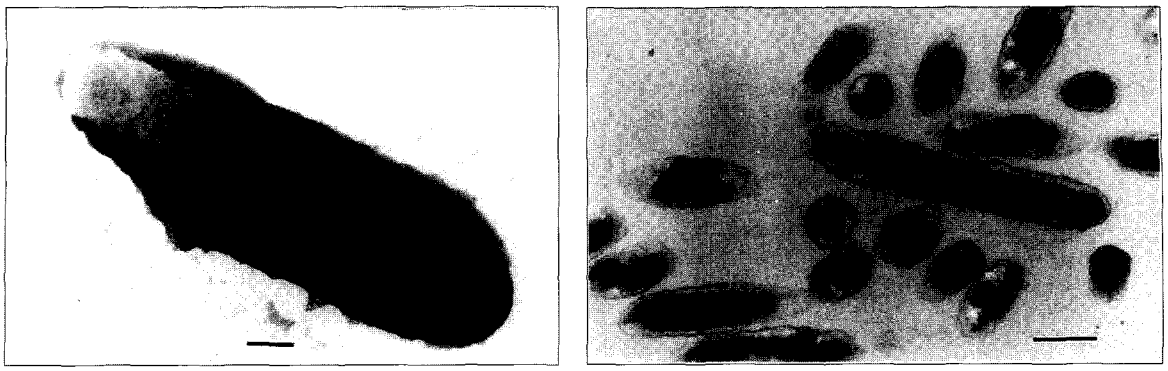
Fig. 4. Titration of antibody against the AL072/BSA conjugate at 17 μg/ml.
The titer of the antibody was 1:2048 (2^{11}). ■—■: control, ●—●: diluted AL072 (1:100).

Electron Microscopical Observation

For negative staining, samples were stained with 2% (w/v) phosphotungstic acid (pH 7.4) for 5 min. AL072 was mixed with the test strain in the mid-logarithmic phase to make final concentrations of 0, 1, and 20 times of the MIC. After incubation for 6 and 24 h in the presence of AL072, 10 ml samples were collected from each of the cultures. The organisms were harvested by centrifugation at 600×g for 10 min. The pellets were washed 3 times with Phosphate-buffered saline (PBS) and prepared for electron and immunoelectron microscopy. For electron microscopy, the cultured bacterium was fixed in 3% (v/v) glutaraldehyde in a cacodylate buffer for 100 min. The organisms were washed 3 times with the cacodylate buffer and embedded in 2% agar. Then the pellets were dehydrated and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined at 80 KV with an electron microscope (JEOL100/CXII, Japan). For immunoelectron microscopy, *L. pneumophila* ATCC 33152 treated with AL072 were harvested. The bacteria were reacted with the antibody against the AL072/BSA conjugate at 37°C for 1 h. After centrifugation at 600×g for 10 min, the pellets were washed 3 times with PBS. The bacterial pellet was then reacted with gold conjugated protein A (Accurate Chemical and Scientific Corp., West Bury, U.S.A.) at room temperature for 2 h. Thereafter, the bacteria reacted with AL072, with antibodies against the AL072/BSA conjugate, as well as with gold conjugated protein A were fixed with 3% glutaraldehyde followed by embedding in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate, and examined at 80 KV with an electron microscope.

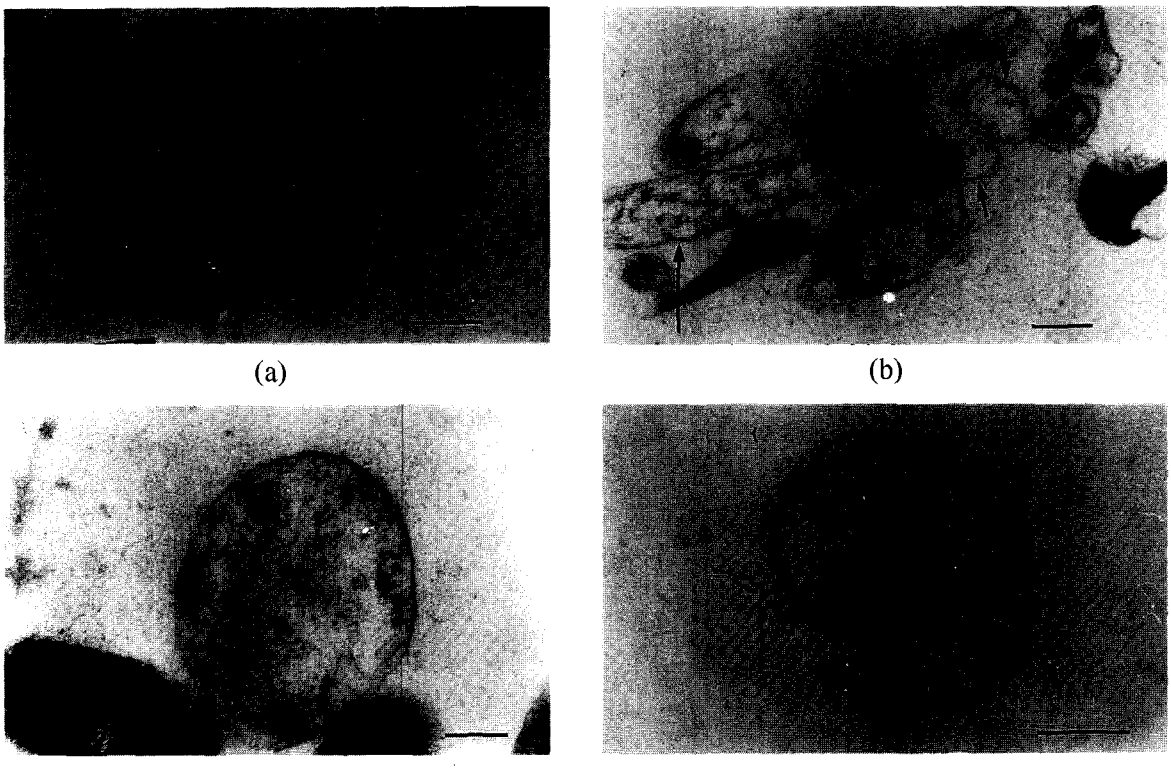
As shown in Fig. 5a, the cell surface of *L. pneumophila* ATCC 33152 was wavy and ruffled and its size was 2-10 μm in length and 0.25-0.5 μm in width. The structure of the cell wall seemed to be consistent with that of gram-negative bacteria. In the cytoplasm, a small number of intracellular vacuoles and a fine skein of nuclear elements were also observed. The latter was distributed evenly throughout the cytoplasm. Several organisms exhibited a vacant space between the cell wall and cytoplasm (Fig. 5b). As shown in Fig. 6, the organisms treated with AL072 exhibited the characteristic morphological changes. The cell wall was partially breaken down so that the cell sustained only with cytoplasmic membrane could be observed. The formation of a clear space between the cell wall and cytoplasm that was detected in normal control could not be observed in the cell treated with AL072 due to the filling with electron-dense globular materials (Fig. 6a). The most characteristic changes, such as the formations of filamentous materials and vacuoles in the cytoplasm as well as blebbings of the cell wall, were occasionally observed (Fig. 6b). Large spheroplasts lacking a cell wall, contained several electron-dense globular materials in the cytoplasm (Fig. 6c). Furthermore, some organisms had lost their cytoplasmic contents through an advanced lytic point and became ghost cells (Fig. 6d). Under immunoelectron microscopy, the immune complexes were mainly observed in the cell wall, and a little in the cytoplasm (Fig. 6b).

In contrast to AL072, the protein synthesis inhibitors, erythromycin or rifampicin, induced some elongated, filamentous organisms with numerous vesicles on their surfaces. In rifampicin-treated organisms, the ribosomes of these cells were enlarged, electron dense, and associated with areas of cytoplasmic clearing. However, spheroplasts and minicells were not observed [9, 19]. The DNA synthesis inhibitor, ciprofloxacin, exhibited the formation



(a) (b)

Fig. 5. Electromicrographs of untreated *L. pneumophila* ATCC 33152.
(a) The structure of the cell wall. Wavy and ruffled cell surface is a typical appearance of *Legionella*. Negative staining. (magnitude of 32,700). Bar represents 0.2 μm. (b) The cells contained some vacuoles in the cytoplasm and had a vacant space between the cell and cytoplasmic membranes. Uranium and lead staining. (magnitude of 18,800). Bar represents 0.5 μm.



(a) (b)

(c) (d)

Fig. 6. Electromicrographs of *L. pneumophila* ATCC 33152 treated with AL072.
(a) Note the extruding cytoplasmic contents due to deterioration of the cell membrane and formation of filaments. Uranium and lead staining. (magnitude of 23,300). Bar represents 0.5 μm. (b) Note the increase in size and number of vacuoles (long arrows), blebbings in the cell wall (short arrows), ghost cells (arrow head), clear cytoplasm and electron-dense globular organisms. Small black dots at the cell wall represent gold particles. Uranium and lead staining. (magnitude of 20,000). Bar represents 0.5 μm. (c) Note the spheroplast indicating lack of a cell wall. Uranium and lead staining. (magnitude of 50,000). Bar represents 0.2 μm. (d) Note the ghost cell where the cytoplasmic contents were lost. Uranium and lead staining. (magnitude of 73,000). Bar represents 0.2 μm.

of abnormally elongated filaments, each of which possessed centrally located "pinched zones", suggestive of arrested division. The inner membranes were intermittently lysed and separated from the remaining cell wall. The intracellular contents of the cells were also more densely packed due to an increase in the size of the individual ribosomes [9, 19]. However, *L. pneumophila* treated with AL072 exhibited cytoplasmic extrusion and blebbing of the cell wall.

Furthermore, some of them changed to spheroplasts. Particularly, the cytoplasmic clearing seen in the cases of the protein and DNA synthesis inhibitors was not observed. Moreover, under immunoelectron microscopical observation, the particles of gold conjugated protein A attached mainly to the cell wall of *L. pneumophila* were observed. Accordingly, it appears that AL072 affects the cellular outer coat of the organisms. Furthermore, some spheroplasts were found when treated with methicillin, an inhibitor of the cell wall synthesis [17]. In conclusion, electron microscopic studies have shown that AL072, like the inhibitors of cell wall synthesis, selectively inhibited bacterial growth by acting on the cell wall of *L. pneumophila*.

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