Transformation of *Escherichia coli* with a Large Plasmid of *Acidiphilium multivorum* AIU 301 Encoding Arsenic Resistance

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*Acidiphilium multivorum* AIU 301 isolated from acid mineral water had strong arsenic resistance. This bacterium harbored a number of plasmids with different molecular sizes. A plasmid of 56 kbp, named pKW301, was isolated from *A. multivorum* AIU 301. When pKW301 was transferred into *Escherichia coli* JM109 by electroporation, an *E. coli* transformant carrying pKW301 exhibited resistance to sodium arsenite, sodium arsenate, and mercuric(II) chloride.

The population of acidophilic bacteria in acid mine drainage, coal refuse drainage, and coal mine drainage containing heavy metals is 100 times higher than that in typical municipal drainage. The predominant bacteria found in such acid mineral environments are *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Thiobacillus acidophilus*, bacteria with well-characterized physiology and genetics. Inoue et al. (10, 11) identified the mer operon in the chromosome of *T. ferrooxidans* and determined the nucleotide sequence of a gene encoding merccuric reductase in the operon. Besides these obligately autotrophic acidophiles, many acidophilic, mesophilic, and chemoorganotrophic bacteria were also isolated from acid mine drainage (14, 26), coal refuse, and coal mine drainage (16, 24). In addition, acidophilic chemoorganotrophs were isolated from contaminating bacteria of the *T. ferrooxidans* isolates (4, 6–8, 12, 15, 28). Harrison (5) studied these additional bacteria systematically and proposed the designation of the genus *Acidiphilium* for them. The genus *Acidiphilium* consists of five species, *A. cryptum*, *A. organovorum*, *A. angustum*, *A. facilis*, and *A. rubrum*, in Bergey's Manual of Systematic Bacteriology (22). Recently, we have found a sixth species in acid mine drainage of the Matsuo sulfur-pyrite mine area, Iwate Prefecture, Japan, and identified it as *Acidiphilium multivorum* (25). *A. multivorum* utilizes a wide variety of organic compounds for growth but does not use ferrous iron, elemental sulfur, or thiosulfate ion as the sole energy source. This bacterium oxidizes arsenite to arsenate, while the other *Acidiphilium* species do not (25), suggesting that it has resistance to arsenic and other heavy metals.

In this study, therefore, we investigated the relationship between a large plasmid from *A. multivorum* AIU 301 and arsenic resistance. *A. multivorum* AIU 301 was cultivated in basal salts medium (pH 3.5) containing (NH₄)₂SO₄ (0.2%, wt/vol), KCl (0.2%, wt/vol), KH₂PO₄ (0.01%, wt/vol), MgSO₄ ⋅ 7H₂O (0.01%, wt/vol), Ca(SO₄)₂ (0.001%, wt/vol), polyethylene (0.2%, wt/vol); Daigo Eiyo Co., Osaka, Japan), Trypticase soy (0.5%, wt/vol; BBL Microbiology Systems, Cockeysville, Md.), and glucose (0.5%, wt/vol) at pH 3.5 and 30°C with vigorous shaking (25). When required, sodium arsenite (5 to 15 mM) was added to the growth medium. For assaying the arsenite resistance of *A. multivorum* AIU 301, the fresh media containing various concentrations of sodium arsenite were inoculated with 1% inoculum of the bacterium previously grown for 2 days in the absence of sodium arsenite and incubated at 30°C with vigorous shaking for 60 h. Growth was monitored by measuring the A₆₆₀ (Fig. 1A). *A. multivorum* AIU 301 resisted and grew in relatively high concentrations of sodium arsenite in comparison with *Escherichia coli*; i.e., *A. multivorum* AIU 301 grew well in the presence of 15 mM sodium arsenite (Fig. 1A), while 5 mM of the same salt prevented *E. coli* JM109 from growing (Table 1). *A. multivorum* AIU 301 could grow in medium containing 30 mM sodium arsenite but at a low rate (data not shown). Many microorganisms that resist high concentrations of heavy metal salts are known to harbor plasmids that govern their resistances to various toxic heavy metals (17, 23). Plasmid-determined bacterial resistances to arsenic and antimony have been known since the work of Novick and Roth (17) and Hedges and Baumberg (9). Arsenic resistance genes (ars operon) were located on large plasmids such as the *Saprophycoccus aureus* plasmid pZ58 (21), the *Saprophycoccus xylosus* plasmid pSX267 (3), and the *E. coli* plasmid R773 (18). Therefore, we also attempted to isolate large plasmids from *A. multivorum* AIU 301 by the method described by Yano and Nishi (27). Plasmid samples were analyzed on 0.7% (wt/vol) agarose gels with Tris-borate-EDTA buffer (pH 8.3) (19). A few plasmids were detected in a position corresponding to a size larger than the 23 kbp of the standard DNA on the agarose gel (Fig. 2A, lane 2). These large plasmids could be isolated from this bacterium by the method of Yano and Nishi (27) but not by the method of Kado and Liu (13) or the alkaline sodium dodecyl sulfate method routinely used for *E. coli* plasmids (19) (data not shown). Some plasmids were also observed at the positions corresponding to 9.4 kbp and smaller. We presumed that at least one of these large plasmids encoded the arsenic resistance genes. The plasmid whose band showed the strongest intensity on the agarose gel was recovered from a low-melting-point (SeaPlaque GTG agarose; Takara Shuzo Co., Kyoto, Japan) gels with Tris-borate-EDTA buffer (pH 8.3) (19). A few plasmids were detected in a position corresponding to a size larger than the 23 kbp of the standard DNA on the agarose gel (Fig. 2A, lane 2). These large plasmids could be isolated from this bacterium by the method of Yano and Nishi (27) but not by the method of Kado and Liu (13) or the alkaline sodium dodecyl sulfate method routinely used for *E. coli* plasmids (19) (data not shown). Some plasmids were also observed at the positions corresponding to 9.4 kbp and smaller. We presumed that at least one of these large plasmids encoded the arsenic resistance genes. The plasmid whose band showed the strongest intensity on the agarose gel was recovered from a low-melting-point (SeaPlaque GTG agarose; Takara Shuzo Co.) agarose gel and was named pKW301.

Plasmid pKW301 was introduced into *E. coli* JM109 [recA1 supE44 endA1 hsdR17 gyrA96 relA1 Δ(lac-proAB) thi] by electroporation. Electroporation was carried out with the Electrocell Manipulator 600 (BTX Inc., San Diego, Calif.) as follows. Electrocompetent cells of *E. coli* JM109 (40 µl) prepared as the growth medium. For assaying the arsenite resistance of *A. multivorum* AIU 301, the fresh media containing various concentrations of sodium arsenite were inoculated with a 1% inoculum of the bacterium previously grown for 2 days in the absence of sodium arsenite and incubated at 30°C with vigorous shaking for 60 h. Growth was monitored by measuring the A₆₆₀ (Fig. 1A). *A. multivorum* AIU 301 resisted and grew in relatively high concentrations of sodium arsenite in comparison with *Escherichia coli*; i.e., *A. multivorum* AIU 301 grew well in the presence of 15 mM sodium arsenite (Fig. 1A), while 5 mM of the same salt prevented *E. coli* JM109 from growing (Table 1). *A. multivorum* AIU 301 could grow in medium containing 30 mM sodium arsenite but at a low rate (data not shown). Many microorganisms that resist high concentrations of heavy metal salts are known to harbor plasmids that govern their resistances to various toxic heavy metals (17, 23). Plasmid-determined bacterial resistances to arsenic and antimony have been known since the work of Novick and Roth (17) and Hedges and Baumberg (9). Arsenic resistance genes (ars operon) were located on large plasmids such as the *Saprophycoccus aureus* plasmid pZ58 (21), the *Saprophycoccus xylosus* plasmid pSX267 (3), and the *E. coli* plasmid R773 (18). Therefore, we also attempted to isolate large plasmids from *A. multivorum* AIU 301 by the method described by Yano and Nishi (27). Plasmid samples were analyzed on 0.7% (wt/vol) agarose gels with Tris-borate-EDTA buffer (pH 8.3) (19). A few plasmids were detected in a position corresponding to a size larger than the 23 kbp of the standard DNA on the agarose gel (Fig. 2A, lane 2). These large plasmids could be isolated from this bacterium by the method of Yano and Nishi (27) but not by the method of Kado and Liu (13) or the alkaline sodium dodecyl sulfate method routinely used for *E. coli* plasmids (19) (data not shown). Some plasmids were also observed at the positions corresponding to 9.4 kbp and smaller. We presumed that at least one of these large plasmids encoded the arsenic resistance genes. The plasmid whose band showed the strongest intensity on the agarose gel was recovered from a low-melting-point (SeaPlaque GTG agarose; Takara Shuzo Co.) agarose gel and was named pKW301.

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described in the BTX protocol (1) and the plasmid DNA (1 µl) were added to a cuvette with a 1-mm gap between the electrodes (BTX) previously kept on ice. Immediately after electroporation at 1.40 kV and 129 V, 960 µl of SOC medium (19) was added to the cuvette and the cell suspension was transferred to a sterilized polypropylene tube and incubated at 30°C for 60 min with shaking. E. coli cells were spread on YT agar plates containing 15 mM sodium arsenite and cultivated at 30°C. E. coli transformants with resistance to sodium arsenite were successfully obtained while the efficiency of transformation was low, which was 6.6\times10^2 CFU/µg of pKW301 DNA. The efficiency of transformation with pKW301 might depend on the host strain of E. coli used as suggested by Sheng et al. (20). A large plasmid was recovered from a transformant of E. coli JM109 by the Yano and Nishi method (27), as shown in Fig. 2A. The origin of this plasmid was examined by Southern blot hybridization. Plasmids extracted from A. multivorum AIU 301 and the E. coli transformant were separated on a 0.7% agarose gel and blotted onto a positively charged nylon membrane (Boehringer GmbH, Mannheim, Germany). Hybridization was performed by the standard protocol (19) with the second largest fragment of pKW301-digested EcoRI as a hybridization probe (see Fig. 3). The probe DNA was labeled with digoxigenin-labeled dUTP with a digoxigenin labeling kit (Boehringer), and hybridized fragments were detected with a digoxigenin luminescent detection kit (Boehringer). As shown in Fig. 2B, two DNA bands were detected in the plasmid sample from A. multivorum by Southern blot hybridization, i.e., one corresponding to the supercoiled form of the major plasmid pKW301 and another corresponding to the open circular form of pKW301. A similar pattern was obtained for the plasmid preparation from the E. coli JM109 transformant (Fig. 2B), indicating that the large plasmid harbored in the transformant originated from pKW301 of A. multivorum. The lineage of this transformant was clearly confirmed by phenotypic examination, i.e., thiamine requirement, malidixic acid resistance due to gyrA96, and increased sensitivity to UV irradiation due to recA1. Furthermore, we succeeded in introducing pKW301 into E. coli DH5α. To our knowledge, this is the first successful transformation of E. coli with such a large plasmid from an acidophilic bacterium of the genus Acidiphilium, while conjugal transfer of R factors, the large plasmids, is well known between E. coli and Pseudomonas aeruginosa (2).

**TABLE 1.** Effect of sodium arsenite, sodium arsenate, and mercuric chloride on growth of E. coli JM109 and E. coli JM109(pKW301)\(^a\)

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>MIC (mM)</th>
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<tbody>
<tr>
<td></td>
<td>JM109</td>
</tr>
<tr>
<td>NaAsO(_2)</td>
<td>5</td>
</tr>
<tr>
<td>Na(_2)HAsO(_4)</td>
<td>10</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>0.3</td>
</tr>
</tbody>
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\(^a\) MICs were determined from growth of cells in 5 ml of YT medium containing various concentrations of heavy metal ions for 22 h.

**FIG. 1.** Growth of A. multivorum AIU 301 (A) and E. coli JM109(pKW301) (B) in the presence of various concentrations of sodium arsenite. A. multivorum AIU 301 and E. coli JM109(pKW301) were cultivated in the appropriate media containing 0 (○), 10 (■), or 15 (▲) mM sodium arsenite at 30°C, and the \(A_{660}\) was measured periodically. E. coli JM109 was also cultivated in the presence of 0 (●), 10 (■), or 15 (▲) mM sodium arsenite.

**FIG. 2.** Agarose gel (0.7%) electrophoresis (A) and Southern blot hybridization analysis (B) of plasmids isolated from A. multivorum AIU 301 and the transformant of E. coli JM109. (A) Lanes: 1, DNA digested by HindIII; 2, plasmid preparation from A. multivorum AIU 301; 3, plasmid preparation from transformed E. coli JM109(pKW301). (B) Southern blot hybridization analysis was done with the second largest EcoRI fragment of pKW301 visible in Fig. 3 from the E. coli JM109 transformant as a probe. Lanes: 1, A. multivorum AIU 301; 2, E. coli JM109(pKW301).
pKW301 was around 56 kbp. This size is extremely large compared with other plasmids tested here cleaved pKW301 into three or four fragments.

For determining the level of arsenic resistance of the transformed E. coli, overnight cultures (0.05 ml) of the transformant were diluted 100-fold into the fresh TY medium (5 ml) (19). For determining the size of pKW301, pKW301 was digested with NotI, 4, pKW301 digested by NheI; 5, pKW301 digested by SpeI; 6, pKW301 digested by BamHI; 7, pKW301 digested by EcoRI.

To determine the size of pKW301, pKW301 was digested with various restriction endonucleases and the resultant DNA fragments were analyzed by electrophoresis on 0.3% (wt/vol) agarose gel. Lanes: 1, T4dcDNA and T4dcDNA digested by BglII; 2, intact pKW301; 3, pKW301 digested by NotI; 4, pKW301 digested by NheI; 5, pKW301 digested by SpeI; 6, pKW301 digested by BamHI; 7, pKW301 digested by EcoRI.

FIG. 3. Agarose gel electrophoresis of DNA fragments of pKW301 digested with some restriction endonucleases. pKW301 was prepared from the E. coli transformant. DNA fragments were analyzed on a 0.3% agarose gel. Lanes: 1, T4dcDNA and T4dcDNA digested by BglII; 2, intact pKW301; 3, pKW301 digested by NotI; 4, pKW301 digested by NheI; 5, pKW301 digested by SpeI; 6, pKW301 digested by BamHI; 7, pKW301 digested by EcoRI.

In conclusion, the large plasmid pKW301 of 56 kbp from A. multivorum carries the genes responsible for resistance to arsenic and mercuric chloride and these genes are expressed in E. coli.

REFERENCES