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Improvement of organic solvent tolerance by disruption of the *lon* gene in *Escherichia coli*

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The Lon ATP-dependent protease plays an important role in regulating many biological processes in bacteria. In this study, we examined the organic solvent tolerance of a Δ*lon* mutant of *Escherichia coli* K-12 and found that the mutant showed remarkably higher organic solvent tolerance than the parent strain. Δ*lon* mutants are known to overproduce capsular polysaccharide, resulting in the formation of mucoid colonies. We considered that this increase in capsular polysaccharide production might be involved in the organic solvent tolerance in *E. coli*. However, a Δ*lon*Δ*wcaJ* double-gene mutant displaying a nonmucoid phenotype was as tolerant to organic solvents as the Δ*lon* mutant, suggesting that capsular polysaccharide is not involved in organic solvent tolerance. On the other hand, the Lon protease is known to exhibit proteolytic activity against the transcriptional activators MarA and SoxS, which can enhance the expression level of the AcrAB-TolC efflux pump. We found that the Δ*lon* mutant showed a higher expression level of AcrB than the parent strain. In addition, the Δ*lon*Δ*acrB* double-gene mutant showed a significant decrease in organic solvent tolerance. Thus, it was shown that organic solvent tolerance in the Δ*lon* mutant depends on the AcrAB-TolC pump but not capsular polysaccharide. *E. coli* strain JA300 *acrR*IS *marR* overexpresses the AcrAB-TolC pump and exhibits high-level solvent tolerance. In an attempt to further improve the solvent tolerance of JA300 *acrR*IS *marR*, a *lon* gene disruptant of this strain was constructed. However, the resulting mutant JA300 *acrR*IS *marR* Δ*lon* showed lower solvent tolerance than JA300 *acrR*IS *marR*.

[**Key words:** *Escherichia coli*; Organic solvent tolerance; *lon*; Capsular polysaccharide; AcrAB-TolC; Efflux pump]

Application of biotechnology to chemical synthesis can reduce the amounts of raw materials and energy required for the manufacture of various chemicals. A number of attempts have been made to produce valuable compounds through the bioconversion of hydrophobic and/or toxic organic compounds in two-phase systems consisting of a hydrophobic organic solvent and an aqueous medium (1–4). In addition, increasing attention has been paid to microbial production of relatively hydrophobic biofuels such as long-chain alcohols and fatty acid-based fuels from renewable biomass resources (5–7). In a two-phase culture system containing a large amount of a hydrophobic organic solvent, the extent of bacterial growth inhibition is inversely correlated with the log P_{OW} of the solvent (8). Organic solvents with a log P_{OW} of 2–5 intercalate into the cell membrane and disrupt the integrity of the membrane, thereby affecting several vital functions, such as membrane transport and energy generation (9,10).

Various mechanisms related to microbial tolerance and response to solvents have been proposed for *Pseudomonas* species and *Escherichia coli* strains (1,11–21). In *E. coli*, the AcrAB-TolC efflux pump belonging to the resistance/nodulation/cell division (RND) family has been shown to provide intrinsic tolerance to organic solvents (22). *acrAB* and *tolC* are *marA*/*soxS*/*rob* regulon genes (23). The *acrAB* and *tolC* expression is activated by transcriptional activators such as MarA and SoxS proteins (24). Transcriptions of the *marA* and *soxS* genes are repressed by MarR and SoxR, respectively. In addition, *acrAB* expression is modulated locally by the repressor AcrR (25). We previously reported that an *E. coli* strain could acquire high-level organic solvent tolerance via mutations in only the two regulatory genes *marR* and *acrR* (19).

The Lon protease plays an essential role in protein quality control by destroying unfolded proteins (26). The *E. coli* Lon protease has been shown to be involved in a number of biological processes, such as SOS response, capsule synthesis, DNA methylation, motility, defense against chemicals, methionine biosynthesis, acid tolerance, and nutrient stress (26,27). The Lon protease causes a rapid turnover of MarA and SoxS (28). Therefore, MarA and SoxS are unstable in the presence of Lon protease. *lon* mutants increase the expression level of the AcrAB-TolC pump (29). In addition, *lon* mutants enhance the production of a capsular polysaccharide, colanic acid, and this leads to a mucoid phenotype (30). The enhanced polysaccharide biosynthesis has been thought to cause increased antibiotic resistance via decreased permeability (31). In addition, extracellular polysaccharide is suggested to play an important role in organic solvent tolerance in several bacteria (32–35). Thus, we expected that overproduction of capsule polysaccharide would contribute to organic solvent tolerance in *E. coli*. The colanic acid biosynthesis requires 19 genes located on the same cluster, denoted

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TABLE 1. Bacterial strains used.

E. coli strain	JW ID[a]	Genotype	Reference
BW25113		*lacI*^{q} *rrnB*$_{T14}$ lacZ$_{WJ16}$ *hsdR*514 *araBAD*$_{AH33}$ *rhaBAD*$_{LD78}$	38
BW25113Δ*lon*	JW0429	Same as BW25113, but with *lon*::Km^{R}	38
BW25113Δ*acrB*	JW0451	Same as BW25113, but with *acrB*::Km^{R}	38
BW25113Δ*wcaJ*	JW2032	Same as BW25113, but with *wcaJ*::Km^{R}	38
BW25113Δ*lon*Δ*acrB*		Same as BW25113, but with Δ*acrB* and *lon*::Km^{R}	This study
BW25113Δ*lon*Δ*wcaJ*		Same as BW25113, but with Δ*wcaJ* and *lon*::Km^{R}	This study
JA300		F^{-} *thr leuB6 trpC1117 thi rpsL20 hsdS*	37
JA300 *acr*RIS *marR*		Same as JA300, but with *acrR*::IS*5* and *marR109*	19
JA300 *acr*RIS *marR* Δ*lon*		Same as JA300, but with *lon*::Km^{R}, *acrR*::IS*5* and *marR109*	This study

[a] JW ID of the Keio collection by the National Bio-Resource Project (NIG, Mishima, Japan): *E. coli* (38).

wca and formerly called *cps* (36). WcaJ is predicted to initiate the synthesis of colanic acid by transferring α-D-glucose-1-phosphate to undecaprenyl phosphate (Und-P). WcaJ is required for capsule polysaccharide synthesis, and the Δ*wcaJ* mutant forms nonmucoid colonies.

In this study, we examined the organic solvent tolerance of an *E. coli* Δ*lon* mutant and found that the mutant showed higher organic solvent tolerance than the parent strain. In addition, we clarified the contribution of the capsular polysaccharide and the AcrAB-TolC pump to organic solvent tolerance in the Δ*lon* mutant.

MATERIALS AND METHODS

Media, culture conditions and materials The organisms were grown aerobically at 30°C in LBG medium consisting of 1% Bacto Tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, and 0.1% glucose. The same medium supplemented with 10 mM $MgSO_4$ (LBGMg medium) (37) was also used. The LBGMg medium was solidified with 1.5% (wt/vol) agar. Ampicillin (50 μg/ml) or kanamycin (50 μg/ml) was added to the medium when necessary. The organic solvents used were of the highest quality available (Wako Pure Chemical Industries, Osaka, Japan). The hydrophobic solvents used and their log P_{ow} values were as follows: *n*-nonane (log P_{ow}, 5.5), *n*-hexane (log P_{ow}, 3.9), cyclohexane (log P_{ow}, 3.4) and *p*-xylene (log P_{ow}, 3.1).

Bacterial strains and plasmids The *E. coli* K-12 derivatives used to evaluate solvent tolerance are summarized in Table 1. Strain BW25113 and its single-gene knockout mutants were supplied by the National Bio-Resource Project (NIG, Mishima, Japan): *E. coli* (38). The plasmid pCP20 was also obtained from NIG, Japan.

Disruption of *lon*, *acrB*, and *wcaJ* in *E. coli* strains The plasmid pCP20 shows temperature-sensitive replication and thermal induction of FLP synthesis (39). The Km^{R} cassettes in BW25113Δ*acrB* and BW25113Δ*wcaJ* were eliminated with pCP20. These strains were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which several were colony-purified at 43°C and then tested for loss of kanamycin-resistance. Elimination of the Km^{R} cassette was confirmed by PCR analysis using chromosomal DNA. The combination of primers for BW25113Δ*acrB* was acrB-S and acrB-AS, and that for BW25113Δ*wcaJ* was cpsGwcaJ-S and wcaJ-AS (Table 2). The sizes of the amplified products from BW25113Δ*acrB* and BW25113Δ*wcaJ* were estimated to be 1.7 kb and 3.1 kb, respectively. Consistent with the expected values, the sizes of the amplified products from the Km^{R} cassette-eliminated strains BW25113Δ*acrB* and BW25113Δ*wcaJ* were 0.2 kb and 1.7 kb, respectively. BW25113Δ*lon*Δ*acrB* and BW25113Δ*lon*Δ*wcaJ* were constructed from the Km^{R} cassette-eliminated mutants BW25113Δ*acrB* and BW25113Δ*wcaJ*, respectively, by P1 transduction of kanamycin-resistance with BW25113Δ*lon* as the donor. Disruption of the *lon* gene was confirmed by PCR analysis using chromosomal DNA prepared from BW25113Δ*lon*Δ*acrB* or BW25113Δ*lon*Δ*wcaJ* as the template with the combination of primers lon-S and lon2-AS (Table 2). JA300Δ*lon* and JA300 *acr*RIS *marR* Δ*lon* mutants were also constructed from JA300 and JA300 *acr*RIS *marR*, respectively, by P1 transduction of kanamycin-resistance with BW25113Δ*lon* as the donor. Disruption of the *lon* gene was confirmed by PCR analysis with primers lon-S and lon2-AS. Corresponding to the expected values, the sizes of the amplified products from both BW25113 and JA300 were 2.4 kb and those of the products from the Km^{R} cassette-eliminated strains (BW25113Δ*lon*Δ*acrB*, BW25113Δ*lon*Δ*wcaJ*, JA300Δ*lon*, and JA300 *acr*RIS *marR* Δ*lon*) were all approximately 1.5 kb.

TABLE 2. Primers used in this study.

Primer	Sequence (5′–3′)	Positions
acrB-S	CGCTGATAATAACCAGCAAG	61–80 bp upstream of the initiation codon of *acrB*
acrB-AS	GTTGGTGGTTCAATTACTCC	64–83 bp downstream of the stop codon of *acrB*
cpsGwcaJ-S	AGGCAATGAATGCGAAACCG	75–94 bp upstream of the initiation codon of *cpsG*
wcaJ-AS	TGATGATCACCGTGGCAATC	46–65 bp downstream of the stop codon of *wcaJ*
lon-S	CATCTGATTACCTGGCGGAA	22–41 bp upstream of the initiation codon of *lon*
lon2-AS	CCTGCCAGCCCTGTTTTTAT	23–42 bp downstream of the stop codon of *lon*

Measurement of organic solvent tolerance of *E. coli* Cultures of *E. coli* strains in LBGMg medium after 16 h of incubation (optical density at 660 nm [OD_{660}], 4–5) were diluted with 0.8% saline by serial 10-fold dilutions. Each suspension was plated on LBGMg agar. The agar surface was overlaid with a 3-mm-thick layer of an organic solvent. The approximate frequency at which the cells formed colonies on the agar was estimated after 48 h of incubation at 25°C.

Antibodies against AcrB Antibodies against AcrB were obtained as described previously (19).

Immunoblotting analysis *E. coli* cells grown in LBGMg medium to an OD_{660} of about 0.6 were harvested by centrifugation (4400 ×*g* for 10 min at 4°C), suspended in 10 mM Tris–HCl buffer (pH 8.0), and then sonicated on ice. Ten micrograms of total cell lysate protein in the supernatant was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% separating gel (40). The gel was then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) by the application of 50 V for 30 min in Tris-glycine-methanol buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol [pH 8.3]). The membrane was blocked overnight at room temperature in Tris-buffered saline (TBS; 0.15% NaCl, 10 mM Tris–HCl, pH 7.4) containing 3% gelatin, washed twice in wash buffer (0.05% Tween 20 in TBS), and hybridized at room temperature with anti-AcrB antibody. The membrane was then probed for 1 h with goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The bands were visualized by use of an alkaline phosphatase color development kit (Bio-Rad). The AcrB expression levels were quantified by gel analysis software UN-SCAN-IT (Silk Scientific, Orem, UT, USA).

Protein content The protein concentration was determined using the method of Bradford (41) and bovine serum albumin as the standard.

Antibiotic susceptibility The minimum inhibitory concentrations (MICs) of various antibiotics were determined by a sequential dilution method (42). LBG medium liquid cultures containing different concentrations of antibiotic and freshly grown 10^3 cells of the tested *E. coli* strain were incubated at 37°C for 16 h. The lowest concentration of antibiotic which completely inhibited growth was defined as the MIC (42).

RESULTS

Organic solvent tolerances of strain BW25113 and its gene-knockout mutants The organic solvent tolerances of the BW25113-based Δ*lon*, Δ*acrB*, Δ*wcaJ*, Δ*lon*Δ*acrB* and Δ*lon*Δ*wcaJ* mutants were investigated by measuring the colony-forming efficiency on an agar plate overlaid with pure *n*-nonane, pure *n*-hexane, a mixture of *n*-hexane and cyclohexane (9:1 vol/vol mixture), and pure cyclohexane (Fig. 1). All strains formed colonies in all spots on the plate without any solvent. The *lon* gene disruptants, BW25113Δ*lon* and BW25113Δ*lon*Δ*acrB*, formed mucoid colonies. The capsular polysaccharide synthesis leading to the mucoid colonies formation was partially suppressed by the addition of organic solvents. BW25113Δ*lon* exhibited 10^2- and 10^3-fold higher colony-forming efficiencies than the parent strain BW25113 in the presence of *n*-hexane and the solvent mixture, respectively. To clarify the involvement of the AcrAB-TolC efflux pump and/or capsular polysaccharide in the organic solvent tolerance in BW25113Δ*lon*, we examined the organic solvent tolerances of BW25113Δ*lon*Δ*acrB* and BW25113Δ*lon*Δ*wcaJ* together with BW25113Δ*acrB* and BW25113Δ*wcaJ*. Unlike BW25113Δ*lon*, BW25113Δ*lon*Δ*wcaJ* formed nonmucoidal colonies. The organic

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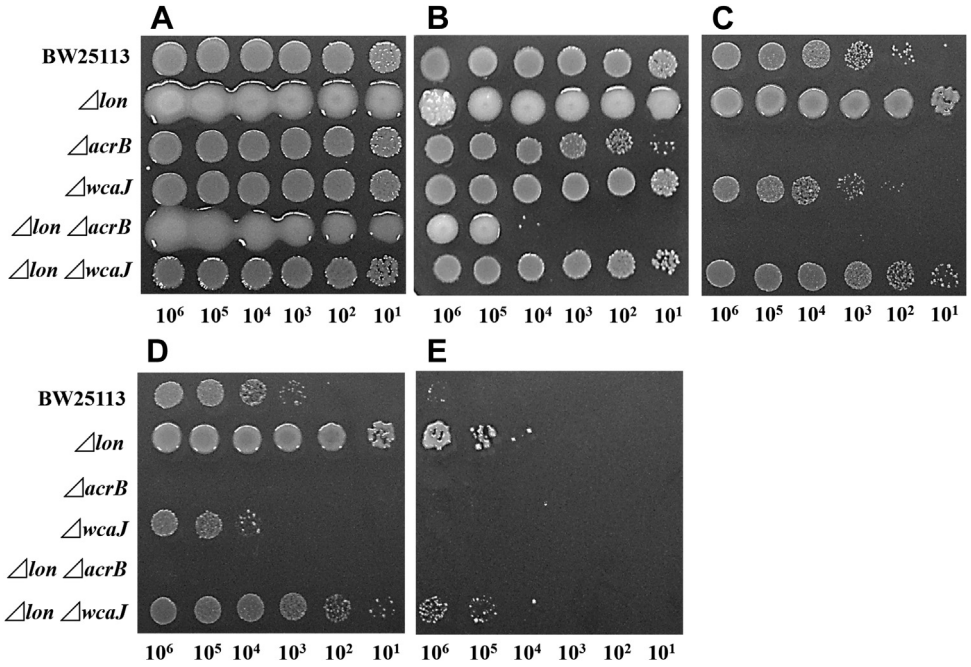

FIG. 1. Colony-forming efficiency of *E. coli* BW25113 and its mutants on LBGMg agar medium in the absence of an organic solvent (A) and in the presence of *n*-nonane (B), *n*-hexane (C), *n*-hexane and cyclohexane (9:1 vol/vol mixture) (D), or cyclohexane (E). Each strain was spotted at a tenfold dilution and incubated at 25°C for 48 h. There was no significant difference between three independent experiments. Typical results are shown in the figure.

solvent tolerances of BW25113Δ*wcaJ* and BW25113Δ*lon*Δ*wcaJ* were similar to those of the BW25113 and BW25113Δ*lon* strains, respectively. Thus, the increased capsular polysaccharide synthesis was not involved in the organic solvent tolerance in the Δ*lon* mutant. On the other hand, both BW25113Δ*lon*Δ*acrB* and BW25113Δ*acrB* were highly sensitive to organic solvents. Therefore, the efflux pump was shown to significantly contribute to organic solvent tolerance in the *lon* mutant.

Growth of the Δ*lon* mutant in liquid medium in the presence of organic solvents The cell growth of BW25113 and BW25113Δ*lon* in the LBGMg liquid medium in the presence of a hydrophobic solvent mixture of *n*-hexane and cyclohexane (9:1 vol/vol) and a hydrophilic solvent (ethanol or *n*-butanol) was examined by measuring turbidity (Fig. 2). No significant difference in growth was found among these strains in the absence of organic solvents. The growth of BW25113 was highly suppressed by the addition of the hydrophobic solvent. In contrast, BW25113Δ*lon* was able to grow in the presence of the hydrophobic solvent, although the growth rate and yield of the mutant were partially reduced compared to those in the absence of solvent. On the other hand, the growth of BW25113Δ*lon* was somewhat suppressed compared to the growth of BW25113 in the presence of ethanol and *n*-butanol.

Organic solvent tolerances of strain JA300 *acrR*IS *marR* and its Δ*lon* mutant We previously constructed JA300 *acrR*IS *marR*, which had a nonsense mutation in *marR* and an insertion of IS5 in *acrR*, and this mutant displayed high-level organic solvent tolerance (19). *E. coli* strains in general cannot form colonies on an agar medium that is overlaid with organic solvents with log P_{ow} values less than 3.9. However, JA300 *acrR*IS *marR* can form colonies on an agar plate overlaid with a mixed solvent of cyclohexane (log P_{ow}, 3.4) and *p*-xylene (log P_{ow}, 3.1). In an attempt to further improve the organic solvent tolerance of JA300 *acrR*IS *marR*, we constructed JA300 *acrR*IS *marR* Δ*lon* and compared its organic solvent tolerance with that of JA300 *acrR*IS *marR* (Fig. 3). Contrary to our expectation, JA300 *acrR*IS *marR* Δ*lon* was more susceptible

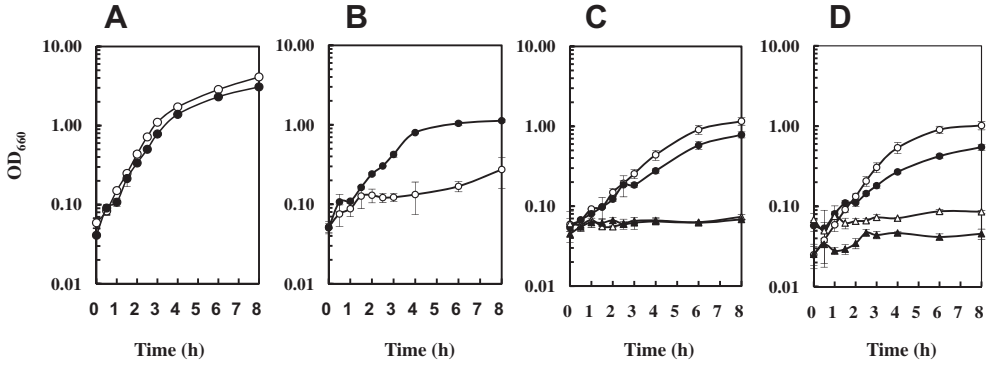

FIG. 2. Growth of *E. coli* BW25113 and BW25113Δ*lon* in LBGMg liquid medium in the absence of an organic solvent (A) and in the presence of a 10% (vol/vol) *n*-hexane and cyclohexane mixture (9:1 vol/vol) (B), 5% or 10% (vol/vol) ethanol (C) and 1% or 2% (vol/vol) *n*-butanol (D). A 100-μl culture of overnight-grown *E. coli* strain was inoculated into 10 ml of fresh LBGMg liquid medium containing an organic solvent. This culture was incubated at 30°C. Growth was monitored by measuring turbidity (OD_{660}). Values indicate the means of results and standard deviations of results from three independent experiments. Open and filled symbols represent BW25113 and BW25113Δ*lon*, respectively. (C) Symbols: circles, 5% (vol/vol) ethanol; triangles, 10% (vol/vol) ethanol. (D) Symbols: circles, 1% (vol/vol) *n*-butanol; triangles, 2% (vol/vol) *n*-butanol.

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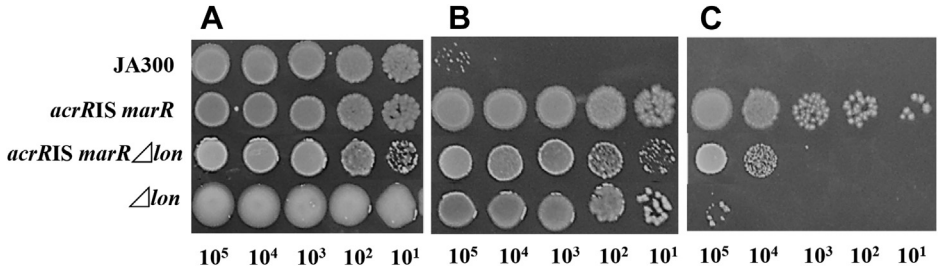

FIG. 3. Colony-forming efficiency of JA300, JA300 *acrR*IS *marR* and JA300 *acrR*IS *marR* Δ*lon* on LBGMg agar medium in the absence of an organic solvent (A) and in the presence of *n*-hexane (B), and cyclohexane (C). Each strain was spotted at a tenfold dilution and incubated at 25°C for 48 h. There was no significant difference between three independent experiments. Typical results are shown in the figure.

to organic solvents than JA300 *acrR*IS *marR*, although the tolerance level in JA300 *acrR*IS *marR* Δ*lon* was higher than that in JA300 and slightly higher than that in JA300Δ*lon*.

AcrB levels in *E. coli* Δ*lon* mutants Mutations in *lon* can increase the expression level of the AcrAB-TolC efflux pump (29). We investigated that AcrB levels in Δ*lon* mutants by immunoblotting analysis (Fig. 4). The AcrB levels in BW25113Δ*lon* and BW25113Δ*lon*Δ*wcaJ* were about twofold higher than that in the parent strain BW25113 (Fig. 4A). The AcrB level in BW25113Δ*lon* was similar to that in BW25113Δ*lon*Δ*wcaJ*, indicating that disruption of the *wcaJ* gene did not affect the expression level of AcrB. JA300 *acrR*IS *marR* significantly increased the expression level of AcrB (Fig. 4B). The AcrB level in JA300 *acrR*IS *marR* Δ*lon* was about half of that in JA300 *acrR*IS *marR*, but was about twofold higher than that in strain JA300.

Antibiotic susceptibilities of the Δ*lon* mutants Organic solvent-tolerant *E. coli* mutants are known to exhibit resistance to a variety of antibiotics (42,43). The antibiotic susceptibilities of strains BW25113, BW25113Δ*lon* and BW25113Δ*lon*Δ*wcaJ* to each of chloramphenicol, novobiocin, and ofloxacin were investigated by assessing the MICs. It has previously been shown that *E. coli* strains overexpressing the AcrAB-TolC pump become resistant to these antibiotics (20). We found that the MICs of strain BW25113 to these antibiotics were as follows: chloramphenicol, 6 μg/ml; novobiocin, 1600 μg/ml; ofloxacin, 0.4 μg/ml. On the other hand, the MICs of BW25113Δ*lon* and BW25113Δ*lon*Δ*wcaJ* to these antibiotics were identical and as follows: chloramphenicol, 12 μg/ml; novobiocin, 1600 μg/ml; ofloxacin, 0.4 μg/ml. Although BW25113Δ*lon* and BW25113Δ*lon*Δ*wcaJ* exhibited higher chloramphenicol resistance than BW25113, the levels of resistance of these mutants to novobiocin and ofloxacin were similar to those of BW25113.

DISCUSSION

The Lon ATP-dependent protease contributes to protein quality control and cellular homeostasis by eliminating abnormal proteins and participating in rapid turnover of several regulatory proteins (26,27). Although the Lon protease is involved in the regulation of numerous pathways, it is a nonessential enzyme under normal growth conditions in many bacteria (27). *E. coli lon* mutants become sensitive to DNA-damaging agents and accumulate abnormal proteins. In this study, we showed that disruption of the *lon* gene was able to improve organic solvent tolerance in *E. coli*. Colonies of *E. coli lon* mutants present a mucoid phenotype, because the *lon* mutants overproduce the capsular polysaccharide due to the stabilization of the transcriptional activator RcsA (30). Extracellular polysaccharide has been reported to be involved in organic solvent tolerance in several bacteria as follows. *Rhodococcus* sp. 33 efficiently degrades benzene and produces a large quantity of extracellular polysaccharide which plays an important role in the benzene tolerance (32). *Rhodococcus rhodochrous* (a mucoidal strain) producing extracellular polysaccharide is resistant to *n*-hexadecane, while its nonmucoidal derivatives are sensitive to this solvent (33). *Staphylococcus* sp. ZZ1 produced an unusual extracellular capsule in response to toluene, suggesting that the hydrophilic carbohydrate capsule repels organic solvents and prevents them from reaching the cell membrane (35). *Acinetobacter venetianus* Rag-1 increased production of the capsular polymer emulsan in the presence of organic solvents (34). These findings in capsule polysaccharide production in bacteria are indicative of common survival strategies to withstand organic solvent stress. Thus, it was expected that the overproduction of capsule polysaccharide might improve organic solvent tolerance also in *E. coli*. However, this study showed that the organic solvent tolerance of nonmucoidal BW25113Δ*lon*Δ*wcaJ* was similar to that of BW25113Δ*lon*. This suggested that the overproduction of capsular polysaccharide is not involved in the improvement of organic solvent tolerance in *E. coli*. On the other hand, it has been reported that disruption of the *lon* gene can enhance the expression level of the AcrAB-TolC pump (29). The present study also showed that the expression level of AcrB protein in BW25113Δ*lon* was higher than that in BW25113. To clarify the involvement of the AcrAB-TolC pump in organic solvent tolerance in BW25113Δ*lon*, we examined the organic solvent tolerance of BW25113Δ*lon*Δ*acrB*. The results

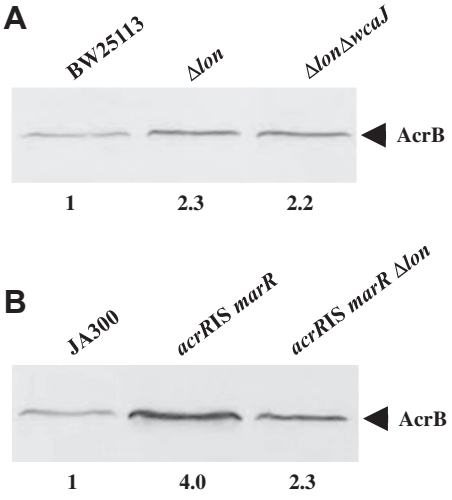

FIG. 4. Western blot analysis of AcrB expression in BW25113 mutants (A) and JA300 mutants (B). Total cell lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with polyclonal anti-AcrB antibodies. The expression ratio compared to the AcrB level of the parent strain is shown below each lane. (A) The strains were assigned to the following lanes: BW25113 (lane 1), BW25113Δ*lon* (lane 2), and BW25113Δ*lon*Δ*wcaJ* (lane 3). (B) The strains were assigned to the following lanes: JA300 (lane 1), JA300 *acrR*IS *marR* (lane 2), and JA300 *acrR*IS *marR* Δ*lon* (lane 3). There was no significant difference between three independent experiments. Typical results are shown in the figure.

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showed that BW25113Δ*lon*Δ*acrB* was highly sensitive to organic solvents. Thus the increase in the AcrAB-TolC efflux pump was the main cause of the improved organic solvent tolerance in the Δ*lon* mutant. BW25113Δ*lon*Δ*acrB* became more sensitive to *n*-nonane than BW25113Δ*acrB*. Thus, disruption of the *lon* gene seemed to further decrease the solvent tolerance of a mutant lacking AcrAB-TolC pump activity. BW25113Δ*lon* also exhibited higher organic solvent tolerance in the LBGMg liquid medium than the parent strain. However, the tolerance of BW25113Δ*lon* against hydrophilic solvents such as ethanol and *n*-butanol was not improved but partially lowered. Since hydrophilic solvents are thought to have higher affinity to hydrophilic capsular polysaccharide than hydrophobic solvents, the *lon* mutant might be sensitive to hydrophilic solvents rather than hydrophobic solvents. On the other hand, the AcrAB-TolC pump has been reported to be not involved in the tolerance against short-chain alcohols (44).

The JA300 *acrR*IS *marR* overexpressing the AcrAB-TolC pump exhibited the highest organic solvent tolerance among about 100 cyclohexane-tolerant *E. coli* mutants (19). Mutations in *marR* can enhance the expression of MarA, and mutations in *lon* can increase the stability of MarA. Therefore, mutations both in *marR* and *lon* were assumed to significantly increase the expression level of the AcrAB-TolC pump and concomitantly enhance the organic solvent tolerance level in *E. coli*. In the present study, we attempted to construct an *E. coli* mutant with high-level organic solvent tolerance by disrupting the *lon* gene in JA300 *acrR*IS *marR*. However, the resulting strain JA300 *acrR*IS *marR* Δ*lon* became more sensitive to organic solvents than JA300 *acrR*IS *marR*. In addition, the AcrB expression level in JA300 *acrR*IS *marR* Δ*lon* was lower than that in JA300 *acrR*IS *marR*. Accumulation of these mutations seemed to decrease the level of AcrAB-TolC pump and thereby reduce the solvent tolerance level in *E. coli*. BW25113Δ*lon*Δ*acrB* was more sensitive to organic solvents than BW25113Δ*acrB* (Fig. 1). This result suggests that the *lon* gene is involved in organic solvent tolerance to some extent. Therefore, the decreased tolerance in JA300 *acrR*IS *marR* Δ*lon* is considered to be due to cumulative effects of deletion of Lon and decreased expression of AcrAB-TolC.

It has been reported that *lon* mutants of *E. coli* did not produce a significant increase in multidrug resistance, despite their overproduction of the AcrAB-TolC efflux pump (29). These mechanisms are not yet fully understood. We also found that the Δ*lon* mutant did not exhibit a remarkable multidrug resistance-phenotype. However, the present study showed that *lon* disruption can significantly enhance the tolerance of *E. coli* against hydrophobic organic solvents, unlike in the case of multidrug resistance.

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