APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2004, p. 4424–4431 Vol. 70, No. 8
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.8.4424–4431.2004

Salt-Inducible Multidrug Efflux Pump Protein in the Moderately Halophilic Bacterium *Chromohalobacter* sp.

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Received 2 October 2003/Accepted 11 March 2004

It has been known that halophilic bacteria often show natural resistance to antibiotics, dyes, and toxic metal ions, but the mechanism and regulation of this resistance have remained unexplained. We have addressed this question by identifying the gene responsible for multidrug resistance. A spontaneous ofloxacin-resistant mutant derived from the moderately halophilic bacterium *Chromohalobacter* sp. strain 160 showed a two- to fourfold increased resistance to structurally diverse compounds, such as tetracycline, cefsulodin, chloramphenicol, and ethidium bromide (EtBr), and tolerance to organic solvents, e.g., hexane and heptane. The mutant produced an elevated level of the 58-kDa outer membrane protein. This mutant (160R) accumulated about one-third the level of EtBr that the parent cells did. An uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, caused a severalfold increase in the intracellular accumulation of EtBr, with the wild-type and mutant cells accumulating nearly equal amounts. The *hrdC* gene encoding the 58-kDa outer membrane protein has been cloned. Disruption of this gene rendered the cells hypersusceptible to antibiotics and EtBr and led to a high level of accumulation of intracellular EtBr. The primary structure of HrdC has a weak similarity to that of *Escherichia coli* TolC. Interestingly, both drug resistance and the expression of HrdC were markedly increased in the presence of a high salt concentration in the growth medium, but this was not observed in *hrdC*-disrupted cells. These results indicate that HrdC is the outer membrane component of the putative efflux pump assembly and that it plays a major role in the observed induction of drug resistance by salt in this bacterium.

Halophilic microorganisms living in high-salt environments may be classified into two main groups: extremely halophilic archaea and moderately halophilic bacteria. Extremely halophilic archaea are adapted to survive in environments with extremely high concentrations of salt, such as the Great Salt Lake, while moderately halophilic bacteria can survive over a wide range of salt concentrations, between 3 and 15% (12, 13).

These bacteria may be exposed to naturally occurring xenobiotics. To cope with such potential hazards, many bacteria are equipped with a xenobiotic efflux pump, which exports stereochemically unrelated compounds at the expense of cellular energy. Miyauchi et al. reported the presence of a doxorubicin efflux pump in the archaeon *Haloferax volcanii* (10, 19). This finding was made in an extremely halophilic archaeon, and the study of such efflux pumps in moderately halophilic bacteria has been limited (20). In addition, it has been observed that moderately halophilic bacteria exhibit natural resistance to structurally and functionally diverse compounds (35). This observation and its connection to high-salt environments remained to be examined. We therefore undertook a study by selecting a multiantibiotic-resistant mutant of a moderately halophilic bacterium which showed an enhanced activity of the putative multidrug efflux pump. We report here the cloning of the gene responsible for multidrug resistance and the effects of gene disruption and a high salt concentration on the resistance and expression of the corresponding protein.

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MATERIALS AND METHODS

Bacterial strains, growth conditions, and isolation of ofloxacin-resistant mutant. The halophilic bacterium used was strain 160, which was isolated from a seashore specimen (Matsushima, Japan). The physiological properties and nucleotide sequence of the 16S rRNA gene (accession number AB105069) suggested that this strain belongs to the genus *Chromohalobacter*, and it was found to be identical to strain 560, which was used previously (29). Cells were grown at 37°C in nutrient broth (NB; 1% beef extract and 1% polypeptone, pH 7.0) supplemented with 2 M NaCl. SW medium (32) was used for the conjugation of *Chromohalobacter* and *Escherichia coli*. *E. coli* JM109 and S17-1 were used for DNA manipulations. An ofloxacin-resistant mutant was isolated by plating ca. 5×10^8 cells on a nutrient agar containing 2 M NaCl and 0.4 μg of ofloxacin/ml.

Determination of MICs of antibiotics and assay of organic solvent tolerance. Antibiotic susceptibility was measured by the twofold agar dilution method with Mueller-Hinton agar (5) supplemented with 2 M NaCl. Antibiotics, ethidium bromide (EtBr), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were chemically stable in the presence of 2 M NaCl. For assays of organic solvent tolerance, cell cultures grown to the early log phase (~10^8 cells/ml) were mixed with test solvents (final concentration, 3%) and incubated for 15 min at 37°C. The mixtures were diluted immediately and plated on NB–2 M NaCl agar, and the viable cells were counted. The experiment was done in triplicate.

Determination of intracellular accumulation of EtBr. Logarithmically growing cells in NB–2 M NaCl (A_{600} = 0.4) were harvested by centrifugation at 7,000 × *g* for 15 min, washed once with 50 mM sodium phosphate buffer, pH 7.0, containing 2 M NaCl, and suspended in the same buffer at an A_{600} of 0.5 to 1.0. One milliliter of cell suspension was mixed with EtBr to a final concentration of 37.5 μM and the fluorescence intensity was recorded for 5 min, with excitation and emission wavelengths of 520 and 590 nm, respectively. To dissipate the membrane potential, we added CCCP to 100 μM, and fluorescence monitoring was then continued. Experiments were performed within 2 h after cell preparation.

Membrane isolation, protein analysis, and lipopolysaccharide (LPS) extraction. Inner and outer membranes were separated as described before (21). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (14).

LPS was extracted according to the method of Westphal and Jann (36). The water phase was dialyzed against a large excess of distilled water for 5 days by frequent changing of the dialysis water. LPS was quantified by the method of Karkhanis et al. (9) and analyzed by electrophoresis as described by Tsai and Frasch (31).

Determination of NH_2-terminal and internal amino acid sequences. The HrdC protein band in the SDS-PAGE gel was transferred to a ProBlott (Applied Biosystems) membrane by electroblotting, and the stained band was cut out for NH_2-terminal sequence analysis. For internal sequence determination, the gel pieces of the first SDS-PAGE, containing the HrdC protein, were excised and digested with lysyl endopeptidase (Wako) or endoproteinase Glu-C (Roche Diagnostics) in a second gel buffer according to the method of Ikezono et al. (7). The resulting peptide bands in the SDS-PAGE gel were transferred to a ProBlott membrane and subjected to amino acid sequencing.

DNA manipulations. DNA manipulations were performed according to standard procedures as described by Sambrook et al. (28). The chromosomal DNA of *Chromohalobacter* sp. strain 160 was isolated by the procedure of Ausubel et al. (2). Southern and colony hybridization were performed by using an ECL Direct or AlkPhos Direct labeling and detection kit according to the manufacturer's instructions (Amersham Biosciences). The DNA sequence was determined by the dideoxy chain termination method with a BigDye terminator cycle sequencing kit (Applied Biosystems).

Cloning of *hrdC* gene and construction of a HrdC expression vector. To amplify a DNA fragment containing a part of the *hrdC* gene, we designed several mixed primers based on the NH_2-terminal and internal amino acid sequences. A PCR with the forward primer 5′-TGGACSATYACSCARGAYGC (encoding the 4th to 10th amino acid residues from the NH_2 terminus) and the reverse primer 5′-TTRAAYTGYTCYTGSGCYTGRTC (encoding DQAQEQFN in the internal sequence) amplified a 476-bp fragment encoding part of the *hrdC* gene from the *Chromohalobacter* sp. strain 160R chromosomal DNA. This fragment was cloned into the EcoRV site of pBR322 (designated pBR-476). The chromosomal DNA from strain 160R was digested with several restriction enzymes, and the DNA fragments were transferred to a nylon membrane (Hybond-N^+; Amersham Biosciences) after agarose gel electrophoresis. The membrane was probed with a peroxidase-labeled 476-bp fragment, and HindIII fragments of chromosomal DNA (~7 kb) were subcloned into the HindIII site of pBR322. A few colonies harboring the above fragments were positive by colony hybridization with a peroxidase-labeled 210-bp probe encoding the 11th to 80th residues of the mature HrdC protein. One of the plasmids isolated from these positive colonies, pBR-H, contained the whole *hrdC* gene. An online database search was performed by using the GenomeNet Database Service operated by the Institute for Chemical Research, Kyoto University (http://www.genome.ad.jp/), with the program FASTA (26).

The HrdC expression vector, pHS-*hrdC* (14.1 kb), was constructed as follows. The cloned *hrdC* gene was amplified by PCR as two fragments: one fragment was amplified by use of a forward primer (5′-GGGGGTACCCGCGCACCAGG CAT) containing a KpnI site (underlined) and a reverse primer (5′-GGTCGC ATCGATTTGCGGCAA) containing a ClaI site (underlined) in the *hrdC* gene, and the other was amplified by use of a forward primer (5′-GCAAATCGATG CGACCGCGC) containing the same ClaI site (underlined) and a reverse primer (5′-GGGGTCGACCTATTCTACCTCG) containing a SalI site (underlined). The 518- and 1,373-bp fragments that were thus amplified were digested with KpnI and ClaI and with ClaI and SalI, respectively, and were ligated to a KpnI- and SalI-digested *Halomonas-E. coli* shuttle vector pHS15 (33) to make pHS-*hrdC*.

Disruption of *hrdC* gene. The plasmid pHS-Δ*hrdC*-Δ*rep* (12.4 kb) was constructed for gene disruption as follows. The plasmid pHS-*hrdC* digested with HindIII was self-ligated to make pHS-*hrdC*-ΔSm^r (12.1 kb). For amplification of the 2.7-kb fragment containing the XbaI site in the multicloning site and the *oriT* (origin of transfer) region of pHS-*hrdC*-ΔSm^r, a PCR was carried out with a forward primer (5′-GGGTCTAGAACTAGTGGAT) containing an XbaI site (underlined) and a reverse primer (5′-GGGGAATTCCCAACTACATCG) containing the sequence up to *oriT* followed by an EcoRI site (underlined). The resulting 2.7-kb fragment was ligated to a 7.7-kb fragment of XbaI- and EcoRI-digested pHS-*hrdC* to make pHS-*hrdC*-ΔSm^r-Δ*rep* (10.4 kb). This plasmid was digested with Sse8387I, which cuts only a single site of the *hrdC* gene, at the middle. The 2-kb Sm^r cassette derived from HindIII digestion of pHS-*hrdC* was end filled and inserted at the blunt-ended Sse8387I site of plasmid pHS-*hrdC*-ΔSm^r-Δ*rep* to construct pHS-Δ*hrdC*-Δ*rep*. This plasmid was introduced into *Chromohalobacter* sp. strain 160, and Sm^r integrants were selected on SW-8 plates (32) containing 0.5 mg of streptomycin/ml (29).

For the screening of colonies with the correct gene replacement, chromosomal DNAs from the wild type and the disruptants were analyzed by Southern hybridization with probes X and Y. Fragment X (831 bp) was amplified by a PCR using the forward primer 5′-GCGCACCAGGCATGATGA and the reverse primer 5′-GAGCTGACGCTTGATAGCCTT. Fragment Y (335 bp) was amplified by a PCR using the forward primer 5′-TACGATTACGAGGCCCAG and the reverse primer 5′-ACGGTCTTTGTCTCCCGCAG.

Preparation of anti-HrdC antiserum. The DNA region encoding the mature HrdC protein was amplified by PCR using a forward primer, 5′-CCGGATCCG CCGATTTGTGGAC, which contained a BamHI site (underlined) followed by the coding sequence starting with the codon for Ala 31(NH_2 terminus of mature protein), and a reverse primer, 5′-CCGCGGCCGCTTCTACCTCGCCCA, which contained the sequence up to 92 bp downstream of the termination codon followed by a NotI site (underlined). The amplified fragment was digested with BamHI and NotI and was inserted into BamHI- and NotI-digested pGEX4T-1 carrying the glutathione *S*-transferase (GST) gene. The resulting plasmid, pGEX-*hrdC*, was introduced into *E. coli* JM109. The GST-HrdC hybrid protein was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the fusion protein thus expressed was purified by affinity chromatography with a glutathione-Sepharose column according to the manufacturer's instructions (Amersham Biosciences). The purified GST-HrdC fusion protein was digested with thrombin, and the NH_2-terminal amino acid sequence of the HrdC portion was confirmed to be identical to the predicted one encoded by pGEX-*hrdC*. Rabbits were immunized with the GST-HrdC fusion protein and an antiserum was obtained as described previously (30).

Chemicals. Ofloxacin and cefsulodin were provided by the Daiichi and Takeda pharmaceutical companies, respectively. All other chemicals used were of the highest commercially available grade.

Nucleotide sequence accession number. The nucleotide sequence of the *hrdC* region was deposited in the DDBJ/EMBL/GenBank database under accession number AB069976.

TABLE 1. MICs of antibiotics and EtBr for *Chromohalobacter* sp. strains 160, 160R, and 160Δ[a]

Strain	MIC (μg/ml)				
	OFLX	CFS	CP	TC	EtBr
160	0.19	3.12	1.56	1.56	62.5
160R	0.39	6.25	3.12	3.12	250
160Δ	0.045	0.78	0.39	0.78	0.122

[a] Abbreviations: OFLX; ofloxacin; CFS, cefsulodin; CP, chloramphenicol; TC, tetracycline; EtBr, ethidium bromide.

RESULTS

Isolation of mutant with elevated antibiotic resistance and solvent tolerance. A spontaneous ofloxacin-resistant mutant was isolated with a frequency of 10^{-7} to 10^{-8}. This ofloxacin-resistant mutant, strain 160R, was used for subsequent experiments. As shown in Table 1, the MICs of ofloxacin, cefsulodin, chloramphenicol, and tetracycline were twofold higher for 160R than for the parent strain. These data clearly demonstrate that the mutant had cross-resistance to nonselected antibiotics. This mutant also showed a fourfold higher resistance to EtBr than the parent strain (Table 1). We examined the

TABLE 2. Organic solvent tolerance of *Chromohalobacter* sp. strains 160 and 160R

Strain	No. of cells/ml in presence of solvent[a]					
	Control	Decane (6.0)	Octane (4.9)	Heptane (4.4)	Hexane (3.9)	*p*-Xylene (3.1)
160	1.9×10^8	2.1×10^8	2.0×10^8	1.4×10^7	5.8×10^2	0
160R	1.8×10^8	1.9×10^8	1.8×10^8	1.5×10^8	1.0×10^4	0

[a] log P values (8) for the solvents are shown in parentheses.

possibility that the mutant might also be resistant to organic solvents. Octane and decane had no detectable effect on cell survival, suggesting that the cell membrane may not be permeable for these solvents (Table 2). When 160R cells were incubated in the presence of 3% hexane and heptane, more cells survived (17- and 10.7-fold more cells) than when the parent 160 cells were exposed to these solvents. On the other hand, neither the mutant nor the wild-type cells survived in the presence of 3% *p*-xylene. Attempts were made to determine the effect of pentane on cell survival, but without success, probably because of pentane's volatile nature. These results demonstrate that the ofloxacin-resistant mutant gained the capability of tolerating short-chain hydrocarbon solvents.

Energy-dependent extrusion of EtBr. To investigate the cause of the multidrug resistance of the mutant, we examined the intracellular accumulation of EtBr. As shown in Fig. 1A, 160R cells accumulated about one-third the level of EtBr that cells of the parent strain did. The time course of fluorescence intensity is a reflection of the intracellular accumulation of EtBr, since the dye fluoresces strongly upon binding with double-stranded DNA. When CCCP, an uncoupler, was added to 100 μM to the assay mixture, the dye accumulated 13 and 5 times more in 160R and 160 cells, respectively, than in cells without CCCP (Fig. 1B). The eventual fluorescence intensity of EtBr in both strains of cells reached the same level in the presence of CCCP (Fig. 1B). We confirmed that CCCP treatment under our conditions did not affect cell viability (data not shown). These results suggest that both parent and mutant cells pump out EtBr in an energy-dependent manner and that the mutant excretes the dye more efficiently than the parent.

Analysis of membrane proteins and LPSs. Figure 1C shows an SDS-polyacrylamide gel electrophoretogram of the inner and outer membrane proteins of both the parent and the resistant mutant. The mutant cells overproduced an outer membrane protein with an apparent molecular mass of 58 kDa (lanes 1 and 2 and Fig. 2). Two other proteins, of 34 and 20 kDa, were also slightly overproduced, but they were not studied further here because of the limited amount of proteins in the preparations. No significant alteration of the inner membrane protein profile was noticed within the resolution of our gel (lanes 3 and 4).

Since this observation could be explained by the increased barrier function of the outer membrane, we compared the LPS of the mutant cells with that of the wild-type cells. The parent and mutant cells produced indistinguishable quantities of LPS, at 3.3% of the dry cell weight. Furthermore, the electrophoretic profile of the mutant LPS appeared to be indistinguishable from that of the wild type (data not shown). It is less

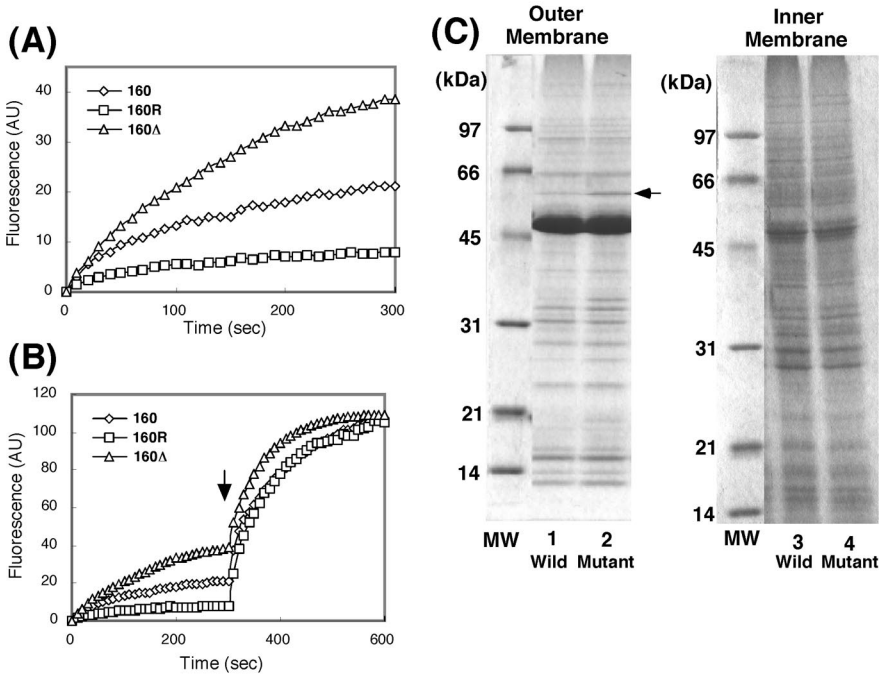

FIG. 1. Determination of cell-associated EtBr and analysis of membrane proteins. (A) Time course accumulation of cell-associated EtBr. The experiment was done as described in Materials and Methods. AU, arbitrary units. Symbols: diamonds, strain 160 (wild type); squares, strain 160R (ofloxacin-resistant mutant); triangles, strain 160Δ (disruptant). (B) Time course accumulation of EtBr in the presence of CCCP. CCCP was added to 100 μM at the time indicated by the arrow. (C) SDS-PAGE profiles of membrane proteins. Membrane fractions containing 20 μg of protein were mixed with an equal amount of Laemmli solubilizer and heated at 95°C for 5 min. Lanes 1 and 3, strain 160; lanes 2 and 4, strain 160R. An arrow indicates the position of the 58-kDa protein.

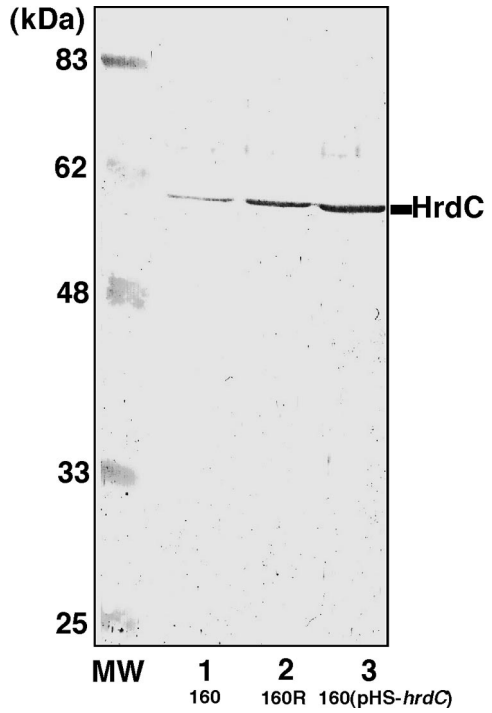

FIG. 2. Overproduction of HrdC protein in 160(pHS-*hrdC*) cells. Crude homogenates (20 μg of protein) were analyzed by Western blotting with an anti-GST-HrdC antiserum. Lane 1, strain 160; lane 2, strain 160R; lane 3, strain 160(pHS-*hrdC*).

likely, therefore, that the increased multidrug resistance in the mutant is attributable to a change in the LPS.

Cloning and nucleotide sequencing of *hrdC* gene encoding the 58-kDa protein and its overproduction. The NH_2-terminal amino acid sequence of the 58-kDa outer membrane protein was determined to be ADLWTITQDALQNNSTLGASRST FQ. We obtained 35- and 19-kDa peptides by lysyl endopeptidase and endoproteinase Glu-C treatment, respectively, of the 58-kDa protein. The NH_2-terminal sequences of the 35- and 19-kDa peptides were determined to be AIKRQLDQA QEQFNVGLVATTDVNEAQ and ATQYDYEAQRRDTT QQVRSLFTQVMNDV, respectively. Using two mixed primers designed as described in Materials and Methods, we cloned the gene coding for the 58-kDa protein, *hrdC* (halophilic bacterial resistance to multiple drugs), by PCR amplification followed by Southern hybridization. The partial amino acid sequences determined above were found in the deduced amino acid sequence of the *hrdC* gene.

By performing a database search (FASTA in GenomeNet), we found that HrdC has a low-level similarity (~33% identity) with TolC homologues of *Erwinia chrysanthemi* (AF421372-1), *Serratia marcescens* (pirT48674), and *Salmonella enterica* serovar Typhimurium LT2 (AE008846-13), all three of which had high levels of similarity with each other (~72% identity). TolC is an outer membrane tunnel protein involved in the efflux of small toxic compounds and in type I secretion of proteins (11). No ATP-binding cassette motif was found in the HrdC sequence. The mature HrdC protein contains a significantly larger number of acidic amino acids (14.3%) than *Erwinia chrysanthemi* TolC (8.3%). On the other hand, the total content of basic amino acids differs slightly between HrdC (8.4%) and TolC (9.1%). The high content of acidic residues is a typical characteristic of halophilic proteins (16).

A putative −35/−10 promoter region (T_{-201}TGGTA/T_{-178}ACATT; numbering was done with the G in the upstream initiation codon of *hrdC* as −1) and terminator signals (ACGCCGCACC/GGTGCGGCGT) were found upstream and downstream of the *hrdC* gene, suggesting that this gene forms a monocistronic locus. A putative Shine-Dalgarno sequence (G_{-17}AAAGGAA) was also found. The GC content of the *hrdC* gene was 59.8%. We cloned the same DNA region from the parent 160 chromosomal DNA and found that the nucleotide sequences of both the parent and mutant genes were exactly identical, including the putative regulatory regions. This result indicates that the enhanced efflux pump activity and overproduction of the HrdC protein in the mutant most likely depend on an upward mutation of a gene regulator involved in the control of efflux pump expression. We found a similar nucleotide sequence for binding of MarA (17), a positive regulator of the *marA-soxS-rob* regulon in *E. coli*, upstream of the putative −35/−10 region of *hrdC* (G_{-254}GCTTGTCATGCCACCGTT).

In order to overproduce the HrdC protein, we constructed a HrdC expression vector, pHS-*hrdC*, by using a *Halomonas-E. coli* shuttle vector, pHS15, and transferred it into strain 160 cells by conjugation. The protein profile of the outer membrane of 160(pHS-*hrdC*), examined by Western blotting with an anti-GST-HrdC antiserum, revealed that the amount of HrdC protein in 160(pHS-*hrdC*) was approximately fourfold higher than that in 160 (Fig. 2). We then examined the antibiotic susceptibility of the cells that overproduced HrdC and found that the overproducer exhibited an antibiotic susceptibility profile that was indistinguishable from that of wild-type cells (data not shown). These results suggest that the outer membrane component HrdC is not a rate-limiting step of efflux pump activity. It is likely that an inner membrane protein(s) may also be overproduced in the 160R mutant.

Construction of *hrdC* knockout mutant. In order to investigate the role of the *hrdC* gene product, we tested the effect of a *hrdC* knockout on antibiotic susceptibility. The plasmid pHS-Δ*hrdC*-Δ*rep*, containing a *hrdC* gene that was destroyed by insertion of the Sm^r marker and lacking a replication origin (34), was transferred to strain 160 by conjugation. For the screening of transformants for the correct replacement of chromosomal *hrdC* with plasmid-borne Δ*hrdC*, Southern hybridization was performed with probes X (831 bp) and Y (335 bp), as shown in Fig. 3A and B. The XmnI-digested chromosomal DNAs of strains 160 and 160Δ showed a 9.6- and a 6.4-kb fragment, respectively, when probed with fragment X (Fig. 3B, lanes 1 and 2, respectively). When probed with fragment Y, the DNAs of the parent (lane 3) and the disruptant (lane 4) showed a 9.6- and a 5.2-kb fragment, respectively. Hybridization experiments using different restriction enzymes confirmed the above results (data not shown).

We then tested the level of HrdC by Western blotting. As shown in Fig. 3C, 160R cells produced a larger amount of HrdC (lane 2) than that produced in the wild-type cells (lane 1). In contrast, 160Δ cells produced no detectable protein band corresponding to HrdC (lane 3). The 160Δ cells exhibited about 512 and 2,048 times the hypersusceptibility to EtBr of

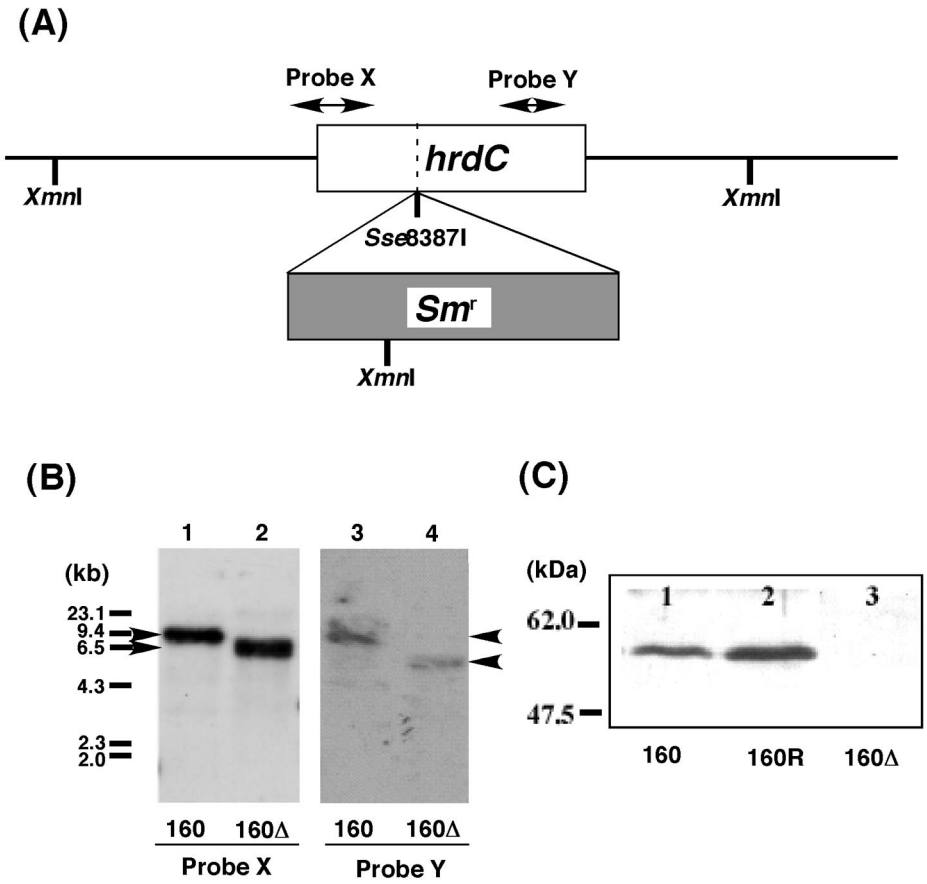

FIG. 3. Gene disruption of *hrdC*. (A) Schematic representation of disrupted *hrdC* gene on chromosome derived from the plasmid pHS-Δ*hrdC*-Δ*rep*. (B) Southern blotting analysis of chromosomal DNAs. DNAs (10 μg) were digested with XmnI and hybridized with probes X (lanes 1 and 2) and Y (lanes 3 and 4). Lanes 1 and 3, *Chromohalobacter* sp. strain 160 (wild type); lanes 2 and 4, *hrdC* gene disruptant (160Δ). (C) Western blotting analysis of HrdC protein. Cells were subjected to sonic oscillation, and 20 μg of protein was analyzed by SDS-PAGE. Lane 1, strain 160; lane 2, strain 160R; lane 3, strain 160Δ.

160 and 160R cells, respectively (Table 1). The intracellular accumulation of EtBr was significantly higher in 160Δ cells than in parent cells (Fig. 1A and B), suggesting that HrdC is an outer membrane component of the multidrug efflux pump. The disruptant was also hypersusceptible to other antibiotics (Table 1). However, HrdC seems not to be essential for the survival of *Chromohalobacter* sp. strain 160 cells under these conditions.

Induction of HrdC with increasing concentrations of salt. One of the characteristics of moderately halophilic bacteria is that they have the ability to grow over a wide range of salinities: they grow optimally at 0.5 to ~2.5 M salt, but sometimes they can even grow under conditions in which NaCl is close to saturated. We tested the influence of medium salt concentrations on the expression of HrdC, since it was reported that the susceptibility of halophilic bacteria to antimicrobial agents is influenced by salinity (4). The 160, 160R, and 160Δ strains grew well in NB medium containing 0.5 to ~2.5 M NaCl, and the growth curve for 160 cells is shown in Fig. 4A. The effects of salt concentration on the protein profiles of 160 and 160R cells by SDS-PAGE (Coomassie blue stain) are shown in Fig. 4B. The amounts of several proteins were affected: in 2.0 M NaCl, the amounts of proteins with apparent molecular masses of 77, 62, and 56 kDa were decreased (shown by bars), while the amounts of 54-, 43-, 41-, and 37-kDa proteins were increased (shown by dots). Since the profile of HrdC could not be detected by Coomassie blue staining because of its small amount (Fig. 4B), Western blotting of HrdC was performed with a specific anti-HrdC antiserum (Fig. 4C). Both 160 and 160R cells produced increased levels of the HrdC protein in high-salt medium. The effect of salt concentration on EtBr susceptibility was determined and showed that the MICs of EtBr for 160 and 160R cells were 256-fold higher in the presence of 2.0 M NaCl than those in 0.8 M NaCl (Table 3). In contrast, the *hrdC*-disrupted cells caused only a twofold increase in the MIC of EtBr at 2.0 M NaCl compared with that at 0.8 M NaCl (Table 3). Those cells also showed the same trend in response to ofloxacin when the salt concentration was increased (data not shown).

DISCUSSION

Although halophilic bacteria often show natural resistance to antibiotics and toxic metal ions (23, 24), the mechanism behind the resistance remains to be elucidated. We isolated an ofloxacin-resistant mutant from the moderately halophilic bacterium *Chromohalobacter* sp. strain 160 that showed markedly

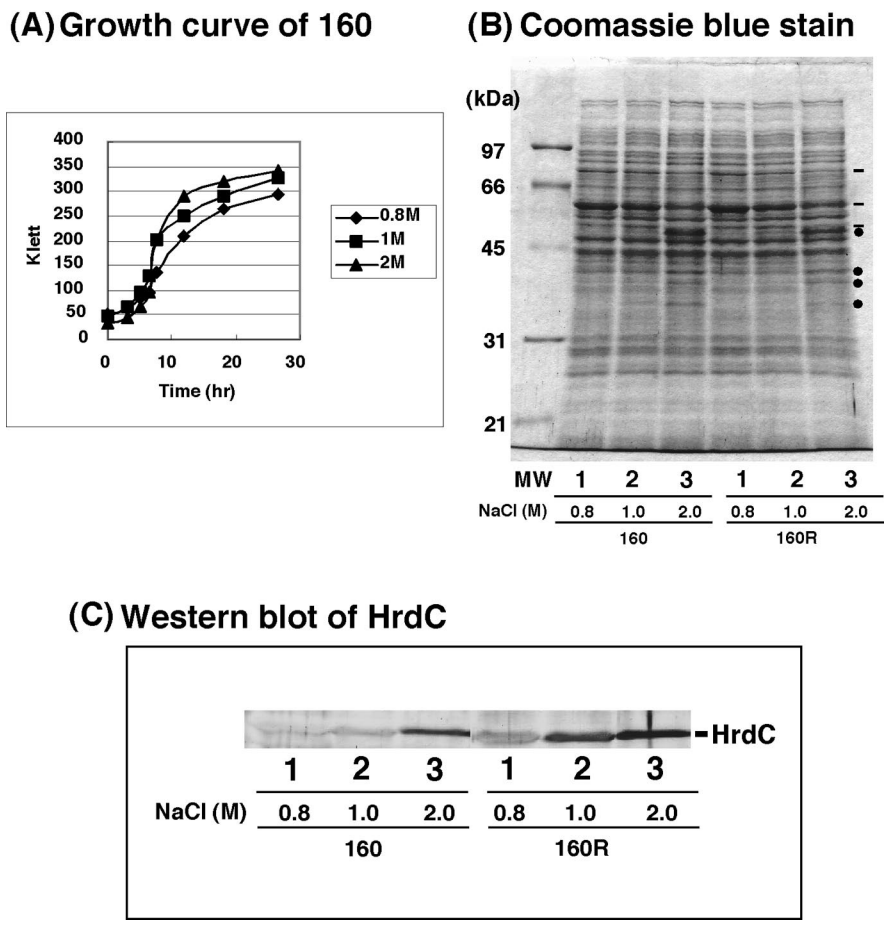

FIG. 4. Growth curves, SDS-polyacrylamide gel electrophoretogram, and Western blotting analysis of cells grown in medium containing different concentrations of NaCl. The cells were grown in the presence of the indicated NaCl concentration and subjected to sonic oscillation, and crude homogenates (20 μg of protein) were analyzed by SDS-PAGE. (A) Growth curve of strain 160 cells in the presence of 0.8 M (diamonds), 1.0 M (squares), and 2.0 M (triangles) NaCl. (B) SDS-polyacrylamide gel electrophoretogram with Coomassie blue stain. Samples from cells grown in medium containing 0.8 M (lanes 1), 1.0 M (lanes 2), and 2.0 M (lanes 3) NaCl were analyzed. 160, wild type; 160R, ofloxacin-resistant mutant. (C) The amounts of HrdC were examined by Western blotting. The samples were the same as those described for panel B.

elevated resistance to a wide variety of compounds, including antibiotics, EtBr, and short hydrocarbon organic solvents. Thus, moderately halophilic bacteria are equipped with some device which protects the cells from these potential hazards. The most likely candidates for such a device are RND-type efflux pumps, found in a wide variety of gram-negative bacteria (25, 27, 38), since the outer membrane protein seems to be a pump component. This assumption was supported by the facts that the intracellular accumulation of EtBr was significantly less in the mutant than in parent cells and that upon the addition of CCCP, EtBr accumulated to a level that was sev-

TABLE 3. Effect of NaCl on MIC of EtBr

Strain	EtBr MIC (μg/ml)		$MIC_{2.0M}/MIC_{0.8M}$
	0.8 M NaCl[a]	2.0 M NaCl[a]	
160	0.244	62.5	256
160R	0.97	250	256
160Δ	0.06	0.122	2

[a] NaCl concentration in the growth medium.

eralfold higher than that without CCCP. A low level of the efflux pump may be expressed in wild-type cells.

A 58-kDa outer membrane protein was found to be overproduced in this multidrug-resistant mutant, from which the gene *hrdC*, encoding the 58-kDa protein, was cloned. HrdC showed a local similarity to TolC, which constitutes an outer membrane tunnel of the *E. coli* three-component efflux pump, AcrAB/TolC.

Recently, the functional and structural characterization of this efflux pump, especially its three-dimensional structure, was extensively resolved (3, 11, 22, 37). The TolC structure comprises a repeat of amino- and carboxyl-proximal halves. The amino-proximal repeating unit consists of four α-helices (H) and two β-strands (S) in the order H1-H2-S1-S2-H3-H4 and the carboxyl-proximal half consists of H5-H6-S3-S4-H7-H8 (11). In spite of an overall low similarity between HrdC and *E. coli* TolC (29% identity), two regions of HrdC have a higher local similarity (~50% identity) to the region of TolC at the boundaries between H3 and H4 and between H7 and H8. These regions of TolC were assumed to be located proximal to the bottom of the TolC tunnel, which might interact with the inner membrane subunits.

The expression of *acrAB*/*tolC* is known to be regulated by the *mar* (multiple antibiotic resistant) locus encoding the repressor MarR, the activator MarA, and MarB, with an unknown function. Once the expression of the activator MarA is increased, it activates the expression of *acrAB*/*tolC* by binding to an upstream sequence, called the *mar* box, of these genes. We found a similar *mar* box consensus sequence upstream of the promoter region of *hrdC*. The expression of *hrdC* in halophilic bacteria might be regulated by a *mar*-like mechanism such as those found in *Enterobacteriaceae*. For *Pseudomonas aeruginosa*, mutant cells resistant to structurally diverse antibiotics have been isolated (15), and this type of mutant was shown to produce a derepressed level of an outer membrane protein with a molecular mass of 50 kDa (18). The 50-kDa protein was identified as an outer membrane subunit, OprM, of the MexAB/OprM efflux pump, which is responsible for multidrug resistance in this organism (21). This type of mutation, causing overexpression of the MexAB/OprM efflux pump, was found to be located in the *mexR* gene, one of the *marR* family of regulators, in *P. aeruginosa* (1). In this study, we found that in our 160R mutant, the nucleotide sequence of the *hrdC* locus, including the putative *mar* regulatory site through the terminator region, was identical to that in wild-type 160 cells. This is consistent with the idea that the *hrd* (halophilic resistance to drug) phenotype of the 160R mutant might be caused by the upward mutation of the *mar*-like regulatory locus of this bacteria.

The disruption of *hrdC* rendered the cells hypersusceptible not only to ofloxacin, but also to tetracycline, cefsulodin, chloramphenicol, and EtBr, and it led to a higher intracellular accumulation of EtBr, suggesting that HrdC is a part of the putative efflux pump assembly. The overproduction of HrdC alone did not confer elevated drug resistance, suggesting that pump assembly requires unidentified components such as energy-dependent transporter and auxiliary proteins like AcrAB of *E. coli* (22) and MexAB of *P. aeruginosa* (6). Further investigation may be necessary to elucidate the whole assembly of the Hrd efflux system.

The most unique characteristic of the HrdC protein discovered in this study is its salt-dependent inducibility. Although it was reported that *Chromohalobacter marismortui* exhibited enhanced antibiotic resistance with increasing salinities, the mechanism behind this observation was totally unclear (4). Here we report that *Chromohalobacter* sp. strain 160 and 160R cells showed a 256-fold increased EtBr resistance in the presence of 2.0 M NaCl compared with that in 0.8 M NaCl (Table 3). This observation is consistent with the fact that these cells produced an increased level of the HrdC protein in a high-salt medium (Fig. 4C). In contrast, we observed only a twofold increase in drug resistance in *hrdC*-disrupted cells (Table 3). These results indicate that the Hrd efflux pump is the major cellular component responsible for salt-inducible multidrug resistance.

The expression of the putative multidrug efflux system studied here seems to be regulated by both the mutation that occurred in strain 160R and the salt or osmolarity sensing system. Research on osmosensing in halophilic bacteria has not been explored. Studies on the expression and regulation of the Hrd efflux pump are now under way.

ACKNOWLEDGMENTS

This work was supported by research grants from The Salt Science Research Foundation.

We are grateful to Tsutomu Arakawa for critical reading of the manuscript.

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