Microbiology (2001), **147**, 43–51 Printed in Great Britain

Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance

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The authors previously described *srpABC*, an operon involved in proton-dependent solvent efflux in the solvent-tolerant *Pseudomonas putida* S12. Recently, it was shown that organic solvents and not antibiotics induce this operon. In the present study, the authors characterize a new efflux pump, designated ArpABC, on the basis of two isolated chloramphenicol-sensitive transposon mutants. The *arpABC* operon is involved in the active efflux of multiple antibiotics, such as tetracycline, chloramphenicol, carbenicillin, streptomycin, erythromycin and novobiocin. The deduced amino acid sequences encoded by the three genes involved show a striking resemblance to proteins of the resistance/nodulation/cell division family, which are involved in both organic solvent and multiple drug efflux. These findings demonstrate that ArpABC is highly homologous to the MepABC and TtgABC efflux systems for organic solvents and multiple antibiotics. However, ArpABC does not contribute to organic solvent tolerance in *P. putida* S12 but is solely involved in multidrug resistance.

Keywords: solvent tolerance, multidrug resistance, efflux

INTRODUCTION

It is well known that several strains within the family *Pseudomonadaceae* show significant intrinsic resistance to a wide variety of structurally unrelated compounds, such as antimicrobial agents and organic solvents (for reviews see Nikaido, 1996; Paulsen *et al*., 1996; Isken & de Bont, 1998; Kieboom *et al*., 2000). In the case of *Pseudomonas aeruginosa* it was thought that a very low, non-specific permeability was the main cause of resistance to antibiotics (Nikaido, 1994). In the case of *Pseudomonas putida* several changes at the level of the membrane contribute to organic solvent tolerance (Weber & de Bont, 1996; Isken & de Bont 1998). Nevertheless, the exceptional resistance of *Pseudomonadaceae* cannot be explained solely by the low permeability of the outer membrane and by changes at the level of the membrane. Not surprisingly, efflux systems have been identified that contribute to the exceptional resistance of these strains. These efflux systems, which belong to the resistance/nodulation/cell division (RND) family of transporters, consist of three components: the inner membrane transporter protein (RND protein), which is attached via a membrane fusion protein (MFP) to an outer membrane protein (OMP) (Saier *et al*., 1994). The OMP is thought to be an outer membrane channel by which to circumvent the outer membrane barrier, allowing the pumped molecule to be released into the medium (Dinh *et al*., 1994).

In the solvent-tolerant *P. putida* strain S12 it was shown that the energy-dependent efflux of organic solvents was the key factor in organic solvent tolerance via the organic solvent transporter SrpABC (Kieboom *et al*., 1998a). Similar efflux systems for the active removal of organic solvents have been found in other *P. putida* strains. These RND-type efflux systems, encoded by *ttgABC* (Ramos *et al*., 1998), *ttgDEF* (Mosqueda & Ramos, 2000) and *mepABC* (Fukumori *et al*., 1998), are involved in the active efflux of toxic compounds such as toluene, *p*-xylene and styrene. In the case of the multidrug-resistant *P. aeruginosa* four efflux systems have been described: MexAB-OprM (Poole *et al*., 1993), MexCD-OprJ (Poole *et al*., 1996), MexEF-OprM (Kohler *et al*., 1997) and AmrAB (Westbrock-Wadman *et al*., 1999). These systems contribute to the energy-dependent efflux of a wide variety of antimicrobial agents such as β-lactams, tetracycline, fluoroquinolones

Abbreviation: RND family, resistance/nodulation/cell division family.

The GenBank accession number for the *arp* sequence is AF183959.

and chloramphenicol. The interesting question now arises whether the RND-type transporters are able to export both antibiotics and solvents. Indications so far confirm that these pumps have dual pumping capacity. The *mex*-encoded efflux systems have recently been shown to be involved in the efflux of organic solvent in *P. aeruginosa* (Li *et al.*, 1998). Moreover, solvent-sensitive mutants of *P. putida* DOT-T1E and *P. putida* KT2442 became more sensitive to antibiotics such as tetracycline, chloramphenicol and ampicillin, suggesting the active removal of multiple antibiotics by these efflux systems (Ramos *et al.*, 1998; Fukumori *et al.*, 1998).

We have now studied this aspect at the molecular level and have found a new efflux system in *P. putida* S12 that is involved in the intrinsic resistance of this strain to a wide variety of structurally unrelated antibiotics. Sequence analysis showed that this efflux system is highly homologous to the solvent/antibiotic transporters MepABC and TtgABC. Moreover, we demonstrate that ArpABC in *P. putida* S12 is only involved in multidrug resistance and not in tolerance towards organic solvents.

METHODS

Bacterial strains, plasmids and growth of the strains. *P. putida* S12 (ATCC 700801) (Hartmans *et al.*, 1989), a wild-type strain capable of growth at supersaturated solvent concentrations (Weber *et al.*, 1993), was the object of the present investigation. Mutant strains of *P. putida* S12 generated in this study and those generated previously are shown in Table 1. *Escherichia coli* DH5α (Yanisch-Perron *et al.*, 1985) was utilized as host strain for all recombinant plasmids. The cloning vectors used and the plasmids generated in this study are shown in Table 1. Luria–Bertani (LB) broth (Sambrook *et al.*, 1989) was used as complete medium. For electroporation, cells were grown in 10 g tryptone l^{-1} and 5 g yeast-extract l^{-1}. Solid media contained 2 % agar. Ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and tetracycline (50 μg ml^{-1}) were added to the medium to maintain recombinant plasmids in *E. coli* and *P. putida*. The *E. coli* strains were routinely cultured at 37 °C and *P. putida* strains were grown at 30 °C.

Generation and screening of Tn*Mod*-KmO insertion mutants. The plasposon Tn*Mod*-KmO was introduced into *P. putida* S12 by electroporation (Dennis & Sokol, 1995). Kanamycin-resistant transposon mutants were tested for their ability to grow on LB agar plates supplemented with 100 μg chloramphenicol ml^{-1} and 50 μg kanamycin ml^{-1}. Mutants were incubated at 30 °C for 24 h. Mutants unable to grow in the presence of 100 μg chloramphenicol ml^{-1} were selected.

DNA techniques. Total genomic DNA from *P. putida* strains was prepared by the CTAB procedure (Ausubel *et al.*, 1991). Plasmid DNA was isolated by the alkaline SDS lysis method of Birnboim & Doly (1979). DNA was digested with restriction enzymes and ligated with T4 ligase as recommended by the supplier (Life Technologies). DNA restriction fragment and

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Source or reference
Strains		
P. putida S12	Ap^r Cm^r *srpABC*$^+$ *arpABC*$^+$	Hartmans *et al.* (1989)
P. putida JK1	Ap^r Cm^r *srpB*∇Tn*Mod*-KmO	Kieboom *et al.* (1998a)
P. putida CM1	Ap^r Cm^s *arpB*∇Tn*Mod*-KmO	This study
P. putida CM2	Ap^r Cm^s *arpC*∇Tn*Mod*-KmO	This study
P. putida DM1	Ap^r Cm^s *srpB*∇Tn*Mod*-KmO *arpA*::*tetA-oriR*::*arpB*	This study
E. coli DH5α	*recA1*	Yanisch-Perron *et al.* (1985)
Plasmids		
pTn*Mod*-KmO	Km^r, pMB1 replicon	Dennis & Zylstra (1998)
pUC18	Ap^r, cloning vector	Life Technologies
pUC19	Ap^r, cloning vector	Life Technologies
pBBR1MCS	Cm^r, cloning vector, REP replicon	Kovach *et al.* (1994)
pUCP22	Ap^r Gm^r, cloning vector, pRO1600 replicon	West *et al.* (1994)
pKRZ-1	Km^r, promoter probe cloning vector	Rothmel *et al.* (1991)
pACYC184	Tc^r, cloning vector	Calgene
pJD105	pUCP22 containing *srpABC*	Kieboom *et al.* (1998a)
pCM1P	Km^r *arpBC* from chromosomal DNA of *P. putida* CM1	This study
pCM2P	Km^r *arpBC* from chromosomal DNA of *P. putida* CM2	This study
pCM1B	Km^r *arpABC* from chromosomal DNA of *P. putida* CM1	This study
pCMPE	pUC18 containing *arpRA*	This study
pCMC1	pUCP22 containing *arpAB*	This study
pCMC2	pUCP22 containing *arpABC*	This study
pKRZ-arp	pKRZ-1 containing P_{arp} *lacZ* fusion	This study
pTO1	Tc^r Cm^s pBBR1MCS containing *tetA-oriR*	This study
pSC1	Tc^r *arpA*::*tetA-oriR*::*arpB*, pMB1 replicon	This study

*Ap, Cm, Km, Gm and Tc represent resistance (r) or sensitivity (s) to ampicillin, chloramphenicol, kanamycin, gentamicin and tetracycline, respectively.

PCR products were visualized by 0·8% agarose gel electrophoresis in 45 mM Tris/borate, 1 mM EDTA buffer. DNA from agarose gels was isolated using the method of Vogelstein & Gillespie (1979). Southern analyses of chromosomal DNA and colony hybridizations were carried out according to Maniatis *et al.* (1982). Probes were labelled with DIG-dUTP using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim) with the appropriate primers. Southern blot hybridizations were carried out by chemiluminescent detection under high-stringency conditions as described by the supplier (Boehringer Mannheim). Plasmid DNA was introduced into either *E. coli* DH5α or *P. putida* cells by electroporation (Dennis & Sokol, 1995) using a Gene Pulser (Bio-Rad).

DNA sequences of both strands of the *arpABC* operon were determined by a combination of subcloning and primer walking. All sequencing and PCR reactions were performed using a Gene Amp PCR System 9700 (Perkin-Elmer). Nucleotide sequencing reactions were performed with purified double-strand plasmid DNA using AmpliTaq FS DNA polymerase fluorescent dye terminator reactions (Perkin-Elmer) as recommended by the supplier. Sequencing products were detected using an Applied Biosystems 373A stretch automated DNA sequencer. Nucleotide sequence analysis was performed either with the Lasergene analysis package (DNASTAR) or with the National Centre for Biotechnology Information BLAST server (Altschul *et al.*, 1990).

Construction of pCMC2 for complementation. For the complementation experiments we reconstructed the *arpABC* operon since the clones obtained from *P. putida* CM1 and CM2 contain the transposon-mutagenized DNA. Therefore, a 2·2 kb *Bam*HI–*Pst*I fragment from pCM1B was cloned into the broad-host-range vector pUCP22; the resulting plasmid was designated pCMC1. A 2·3 kb *Pst*I–*Eco*RI fragment from pCM2P and a 2·0 kb *Eco*RI–*Pst*I fragment from pCM1P were ligated into *Pst*I-digested and alkaline-dephosphatase-treated pCMC1. The resulting plasmid, pCMC2, contained the *arpABC* genes in the same orientation as the *lac* promoter and was transferred to the mutant strains CM1 and CM2.

Construction of suicide plasmid pSC1. We amplified a 1289 bp fragment containing the tetracycline-resistance gene *tetA* from pACYC184. The primers 5′-CGGAATTCTCATGTTTGACAGCT-3′ and 5′-GCGGTACCTCAGGTCGAGGTGG-3′ contain added recognition sites for *Eco*RI and *Kpn*I, respectively. The reaction mixture (50 µl) was treated for 10 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 2 min at 55 °C and 1 min at 72 °C before finishing for 10 min at 72 °C. This 1·3 kb *Eco*RI–*Kpn*I fragment and a 0·7 kb *Kpn*I–*Sst*I fragment from Tn*Mod*-KmO, containing the ColE1 origin of replication, were ligated in *Eco*RI- and *Sst*I-digested pBBR1MCS, resulting in pTO1. The suicide plasmid pSC1 was constructed by ligating a 2·0 kb *Eco*RI–*Sst*I fragment from pTO1, a 3·0 kb *Pst*I–*Sst*I fragment from pCM1P and a 3·0 kb *Pst*I–*Eco*RI fragment from pCMPE.

Construction of pKRZ-arp and determination of β-galactosidase activity. PCR reactions for amplifying the region of genomic *P. putida* S12 DNA containing the *arp* promoter were performed using Super *Taq* DNA polymerase (SphearoQ). The reaction mixture (50 µl) was treated for 10 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C before finishing for 10 min at 72 °C. Primers for this reaction were 5′-CCGCTCGAGTACAACCTCATCTGGCCC-3′ and 5′-CGCTCTAGAATTGCATGAGGATCCTCG-3′, which amplified a 276 bp fragment corresponding to the region immediately upstream of the *arpABC* genes. The primers contain added recognition sites for *Xho*I and *Xba*I, respectively. β-Galactosidase activity in *P. putida* S12(pKRZ-arp) was determined by growing the cells to late-exponential phase at 30 °C in 10 ml LB broth supplemented with inducer. The β-galactosidase activity in these experiments was determined in triplicate by the method of Miller (1972) using chloroform and SDS to permeabilize the cells.

Determination of solvent tolerance and the MIC of antibiotics. Solvent tolerance of the *P. putida* strains was determined in duplicate by growing the cells in 10 ml liquid LB medium in 100 ml flasks supplemented with 1 mM magnesium chloride. Toluene and *p*-xylene (1% final concentration) were separately added to identical subcultures in LB/$MgCl_2$ medium during the early exponential growth phase. The maximal aqueous benzene concentration was determined by adding increasing amounts of benzene. Growth of the cultures was measured 24 h after solvent addition with no continued growth indicating solvent sensitivity. The MIC for various antibiotics was determined in duplicate by threefold serial dilution in LB broth in microtitre plates. The inoculum was 1% of an overnight culture and growth was determined by measuring the OD_{600} after 36 h at 30 °C.

RESULTS

Isolation of chloramphenicol-sensitive mutants

P. putida S12 is able to grow in the presence of a wide variety of normally toxic solvents and is relatively resistant to various antibiotics. Chloramphenicol-sensitive mutants were obtained by electroporating the transposon Tn*Mod*-KmO into *P. putida* S12. Over 4000 kanamycin-resistant transposon mutants were constructed, with an overall transformation efficiency for Tn*Mod*-KmO of $1{\cdot}5 \times 10^3$ per µg DNA. These mutants were tested for growth on solid LB medium supplemented with 100 µg chloramphenicol ml^{-1}. Two chloramphenicol-sensitive mutants were isolated and were designated strains CM1 and CM2, respectively.

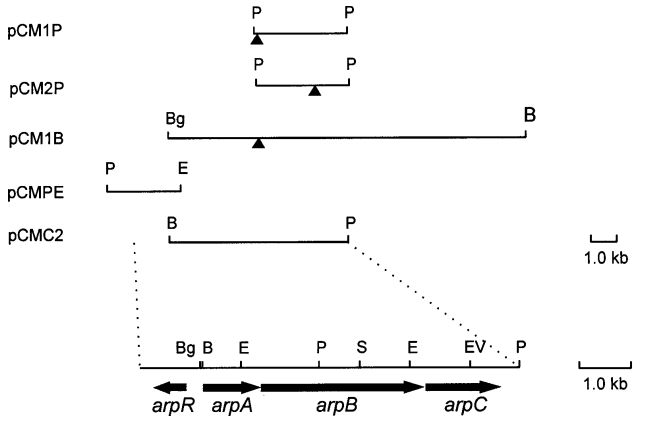

Fig. 1. Restriction maps of the clones derived from the transposon mutants CM1 and CM2. A diagram of the *arp* nucleotide sequence is shown at the bottom, in proportion to the restriction maps and showing the positions of the genes relative to the restriction enzyme cutting sites. The triangles in the restriction maps indicate the location of the inserted plasposon Tn*Mod*-KmO in the cloned genomic DNA.

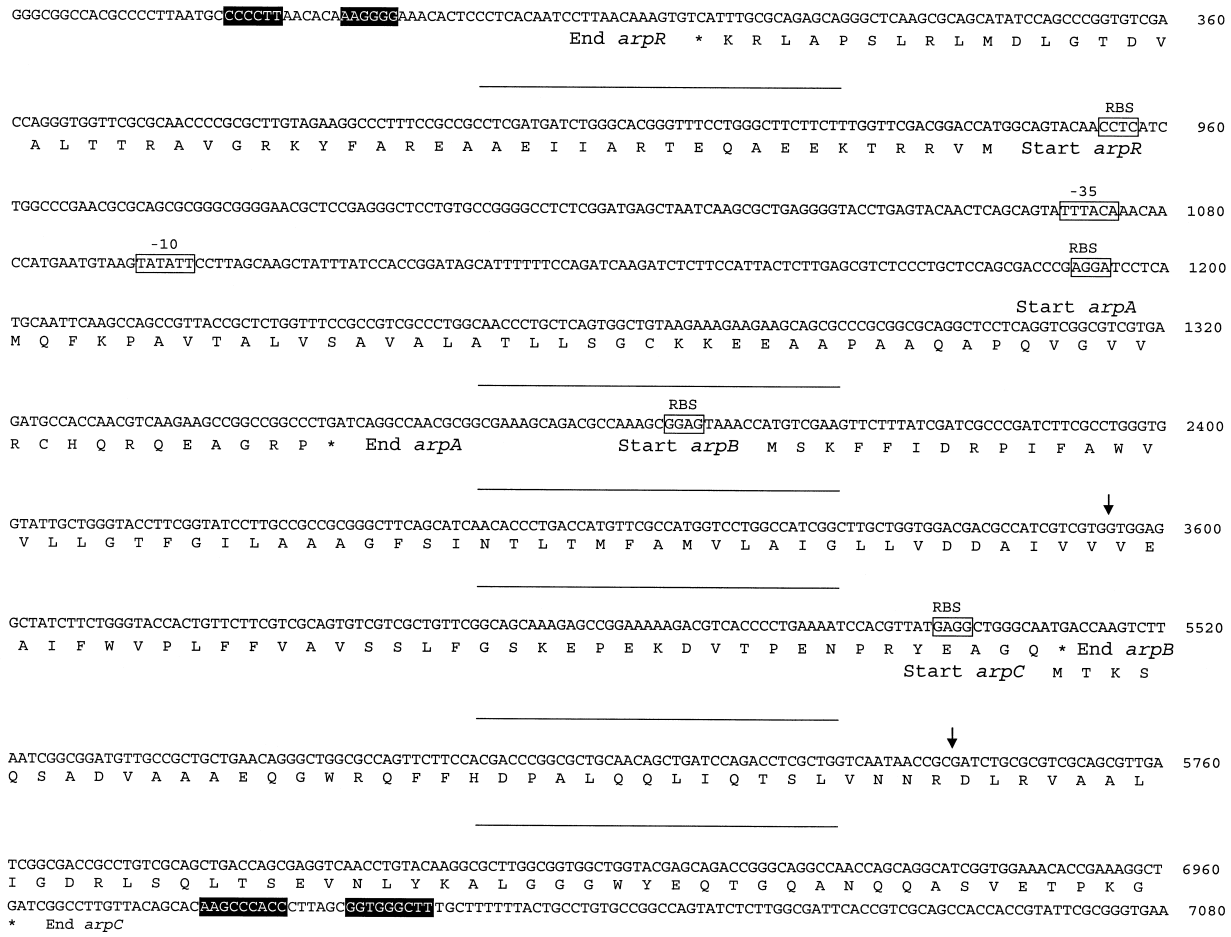

Fig. 2. Nucleotide sequence of the relevant portions of a 7313 bp fragment containing the *arp* genes. The deduced amino acid sequences of the encoded proteins are shown beneath the nucleotide sequence. Termination codons are indicated with an asterisk and putative ribosome-binding sites and a putative σ^{70}-dependent promoter motif that resembles the *E. coli* consensus motif 5′-TTGACA-$N_{(15–19)}$-TATAAT-3′ have been marked with boxes. Inverted repeat sequences are indicated in black boxes. An arrow indicates the position of Tn*Mod*-KmO in *P. putida* CM1 and *P. putida* CM2 (position 3594 and 5738 of the *arpABC* sequence, respectively). The GenBank accession number for the *arp* sequence is AF183959.

Cloning and analysis of the genes for chloramphenicol resistance

To characterize the genes for chloramphenicol resistance in *P. putida* S12, the regions of the genome containing the transposon insertion in mutants CM1 and CM2 (both 5·8 kb) were cloned. From both mutants a clone was obtained from *Pst*I-digested chromosomal DNA; the clones were designated pCM1P and pCM2P, respectively. Genomic DNA cut with *Bam*HI from strain CM1 was used to construct the 14·0 kb plasmid pCM1B. Plasmid pCM1B was shown to contain a complete operon, including the inserted Tn*Mod*-KmO (Fig. 1). The nucleotide sequence of the operon was determined and screening for similar nucleotide sequences in the GenBank database revealed a significant match with genes encoding multidrug and solvent export pumps. We labelled the genes *arp*, for antibiotic resistance pump. A diagram of the nucleotide sequence obtained is presented in Fig. 1 to show the relationship of the open reading frames with the three clones pCM1P, pCM2P and pCM1B. The deduced nucleotide sequences of *arpA*, *arpB* and *arpC* encode proteins of 371, 1050 and 484 amino acid residues with calculated molecular masses of 40·3, 112·8 and 52·8 kDa, respectively. Putative ribosome-binding sites precede the *arpABC* genes and a stable stem-loop structure was found downstream of *arpC* that may function as transcriptional terminator (Fig. 2).

Construction of an *arpABC* mutant of *P. putida* JK1

The presence of the solvent efflux pump SrpABC in *P. putida* CM1 and CM2 could mask the possible efflux of organic solvent by ArpABC. Therefore, to determine the role of ArpABC in solvent tolerance we decided to inactivate the *arpABC* gene cluster by site-specific

Table 2. Determination of the MIC of various antibiotics for the *P. putida* strains

The MIC for antibiotics was determined in duplicate by threefold serial dilution in LB broth in microtitre plates. The inoculum was 1% of an overnight culture and growth was determined by measuring the OD_{600} after 36 h at 30 °C. ND, Not determined.

Antibiotic	MIC (μg ml^{-1}) for strain:				
	S12	CM1	CM2	CM1(pCMC2)	CM2(pCMC2)
Tetracycline	16	1	2	16	16
Chloramphenicol	64	16	16	128	128
Ampicillin	256	64	128	ND	ND
Carbenicillin	1024	4	4	ND	ND
Streptomycin	32	8	8	64	64
Erythromycin	128	8	4	128	256
Rifampicin	64	32	32	32	32
Polymyxim B	16	8	8	8	8
Nalidixic acid	256	128	128	256	256
Novobiocin	1024	64	128	1024	1024
Penicillin G	> 4094	2048	4094	ND	ND

reciprocal recombination in the solvent-sensitive transposon mutant *P. putida* JK1, creating a *srp-arp* double mutant. We electroporated *P. putida* JK1 with the suicide plasmid pSC1 (Table 1) replacing part of *arpA* and *arpB* with the ColE1 origin of replication and tetracycline resistance gene, resulting in the *srp-arp* double mutant *P. putida* DM1. Recombination was confirmed by hybridization of *P. putida* DM1 chromosomal DNA with a probe for the *tetA* gene (data not shown).

Complementation of *P. putida* mutants

Complementation experiments were performed to prove that the Tn*Mod*-KmO-inserted open reading frames detected in *P. putida* CM1 and CM2 are actually responsible for chloramphenicol resistance. These complemented strains regained chloramphenicol resistance similar to the wild-type levels (Table 2). These results are consistent with the chloramphenicol-resistance phenotype being dependent on expression of the *arpABC* genes. We tested a variety of structurally unrelated antibiotics, which are known substrates for homologous efflux systems of the RND family of transporters (Nikaido, 1996; Paulsen *et al.*, 1996). Mutants CM1 and CM2 were not only sensitive to chloramphenicol but also to a number of other antibiotics (Table 2), demonstrating that a single genetic trait in *P. putida* S12 is responsible for resistance to the antibiotics tested.

Solvent resistance of the *P. putida* strains

P. putida S12 is resistant to organic solvents mainly through the presence of a solvent efflux system (Isken & de Bont, 1996; Kieboom *et al.*, 1998a). Therefore, it was determined whether the ArpABC efflux pump was involved in solvent resistance in *P. putida* S12. The antibiotic-sensitive mutant strains CM1 and CM2 were as resistant as the wild-type strain to benzene, toluene and *p*-xylene, whereas the *srp-arp* double mutant DM1 was as sensitive to organic solvents as the solvent-sensitive JK1 strain (Table 3). These results indicate that the solvent-resistance phenotype of *P. putida* S12 is not dependent on expression of *arpABC* genes.

Table 3. Solvent tolerance of the *P. putida* strains

Solvent tolerance of the *P. putida* strains was determined in LB broth supplemented with 1 mM magnesium chloride. A plus indicates growth with 1% organic solvent after 24 h at 30 °C.

Solvent	Strain:				
	S12	CM1	CM2	JK1	DM1
Benzene	10 mM	10 mM	10 mM	6 mM	6 mM
Toluene	+	+	+	−	−
p-Xylene	+	+	+	−	−

Cloning and induction of the *arpABC* promoter

To clone and sequence the promoter region and possible regulatory genes of the *arp* operon, we examined the region upstream of the *Bam*HI site of pCM1B. Therefore, we isolated approximately 3·0 kb *P. putida* S12 chromosomal DNA fragments digested with *Pst*I and *Eco*RI. These fragments were cloned into pUC18 cut with *Pst*I and *Eco*RI. We were able to isolate the plasmid from one colony out of 1200 by colony hybridization

Table 4. Amino acid sequence identity between ArpABC and multidrug and solvent efflux proteins

Organisms	Efflux system proteins*:							
	MFP	Identity (%)	RND	Identity (%)	OMP	Identity (%)	RP	Identity (%)
P. putida	ArpA	100	ArpB	100	ArpC	100	ArpR	100
	TtgA	99·7	TtgB	99·9	TtgC	99·6	–	–
	MepA	99·2	MepB	99·9	MepC	99·8	MepR	98·6
	TtgD	58·0	TtgE	62·2	TtgF	56·6	–	–
	SrpA	57·1	SrpB	63·4	SrpC	57·3	SrpR	30·9
							SrpS	27·3
P. aeruginosa	MexA	67·6	MexB	78·1	OprM	69·4	MexR	37·5
	AmrA	37·4	AmrB	48·7	–	–	AmrR	32·2
	MexC	43·1	MexD	48·4	OprJ	41·6	–	–
	MexE	29·6	MexF	41·4	OprN	28·4	MexT	22·0
E. coli	AcrA	53·8	AcrB	65·4	TolC	19·3	AcrR	37·6
	AcrE	50·6	AcrF	64·4	–	–	EnvR	30·3
N. gonorrhoeae	MtrC	43·3	MtrD	46·6	MtrE	39·7	MtrR	33·0

* MFP, membrane fusion protein; RND, efflux protein; OMP, outer membrane protein; RP, regulatory protein. Identity (matches over matches, mismatches and gaps) was calculated with the Lipman–Pearson algorithm using the deduced amino acid sequences of ArpA, ArpB, ArpC and ArpR (GenBank accession no. AF183959); TtgA, TtgB and TtgC (AF031417); MepA, MepB, MepC and MepR (AB008909); TtgD, TtgE and TtgF (PPY19106); SrpA, SrpB and SrpC (AF029405) : SrpR and SrpS (AF061937); MexA and MexB (L11616); OprM (L23839); MexR (U23763); AmrA, AmrB and AmrR (AF147719); MexC, MexD and OprJ (U57969); MexE, MexF and OprN (X99514); MexT (AJ007825); AcrA and AcrB (U00734); TolC (X00016); AcrR (U00734); AcrE and AcrF (AE000405); EnvR (X57648); MtrC (U14993); MtrD (U60099); MtrC (X95635) and MtrR (Z25797).

with a 0·7 kb *Bam*HI–*Eco*RI fragment from pCM1B. This plasmid, pCMPE, contained the complete promoter region and an ORF (*arpR*) that was transcribed in the opposite direction to that of *arpABC* (Fig. 1). The *arpR* gene product (ArpR) was most similar to MepR (98·6% amino acid identity), which is a putative regulator of the MepABC organic solvent transporter (Fukumori *et al*., 1998) (Table 4). The deduced sequence of *arpR* encodes a protein of 210 amino acid residues with a calculated molecular mass of 23·8 kDa. Sequence analysis of the ArpR revealed a TetR family signature motif. A putative ribosome-binding site preceded the *arpR* gene and a stable stem–loop structure was found downstream of *arpR* and may function as transcriptional terminator (Fig. 2). Moreover, a putative σ^{70}-dependent promoter consensus motif was found upstream of the start of *arpA*.

The *P. putida* S12 solvent transporter is solely induced by organic solvents (Kieboom *et al*., 1998b). In this light it was interesting to examine the induction of the multidrug transporter ArpABC. Therefore, the putative promoter region of the *arpABC* operon was amplified by PCR and cloned in the *lacZ* reporter plasmid pKRZ-1 (Rothmel *et al*., 1991) cut with *Xba*I and *Sal*I. The resulting plasmid, designated pKRZ-arp, was electroporated into *P. putida* S12. *P. putida* S12(pKRZ-arp) was grown in LB broth in the absence and presence of the antibiotics chloramphenicol, carbenicillin and erythromycin and the solvents toluene, *p*-xylene and hexane. β-Galactosidase activity was subsequently assayed in late exponential phase (OD_{600} 1·0). No significant induction was observed by any of these compounds.

DISCUSSION

In this report we describe the isolation and characterization of chloramphenicol-sensitive mutants from the solvent-tolerant and multidrug-resistant bacterium *P. putida* S12. Two chloramphenicol-sensitive mutants were isolated and sequence analysis showed that both mutants were disrupted in the same operon. Multidrug efflux systems play an important role in the intrinsic resistance of members of the family of *Pseudomonadaceae* to a variety of antibiotics. At present four antibiotic efflux systems have been described in *P. aeruginosa*: MexAB-OprM (Poole *et al*., 1993), MexCD-OprJ (Poole *et al*., 1996), MexEF-OprM (Kohler *et al*., 1997) and AmrAB (Westbrock-Wadman *et al*., 1999). These systems are involved in the proton-dependent efflux of antimicrobial agents such as β-lactams, tetracyclines, fluoroquinolones and chloramphenicol. In *P. putida* TtgABC (Ramos *et al*., 1998), MepABC (Fukumori *et al*., 1998) and the ArpABC efflux system described in this report are involved in tolerance towards antibiotics such as tetracycline, chloramphenicol, carbenicillin, streptomycin, erythromycin and novobiocin. Our findings demonstrate that the ArpABC efflux system is a key mechanism in multidrug resistance in *P. putida* S12.

Proton-dependent efflux systems also play an important role in organic solvent tolerance in *P. putida* strains

(Kieboom *et al*., 1998a; Ramos *et al*., 1998; Fukumori *et al*., 1998). The first RND-type efflux system for toluene was isolated by Kieboom *et al*. (1998) and was shown to be responsible for organic solvent tolerance in *P. putida* S12 (Kieboom *et al*., 1998a). The involvement of efflux systems in solvent tolerance was confirmed with the isolation of the *ttgABC*, *ttgDEF* genes from *P. putida* DOT-T1E (Ramos *et al*., 1998; Mosqueda & Ramos, 2000) and the *mepABC* genes from *P. putida* KT2442 (Fukumori *et al*., 1998). Moreover, Kim *et al*. (1998) reported that a transposon insertion in a protein of the RND family resulted in a *P. putida* mutant with a solvent-sensitive phenotype. Surprisingly, other studies suggested that the isolated solvent transporter in *P. putida* was also involved in the active efflux of multiple antibiotics (Ramos *et al*., 1998; Fukumori *et al*., 1998). A toluene-sensitive *mepB*∇Tn5 mutant of a *P. putida* KT2442 was also sensitive to ampicillin, penicillin G, erythromycin, novobiocin and tetracycline (Fukumori *et al*., 1998); and a toluene-sensitive *ttgB*∇ mini-Tn*5′phoA*-Km^r mutant of *P. putida* DOT-T1E was sensitive to chloramphenicol, ampicillin and tetracycline (Ramos *et al*., 1998). With the construction of a *srp-arp* double mutant we were able to demonstrate that the ArpABC efflux system was not involved in organic solvent tolerance in *P. putida* S12.

An interesting, but as yet not completely clear, picture is now emerging from studies on the efflux pumps of pseudomonads with regard to both their substrate recognition and their induction patterns. The inherent problem in studying these aspects is that responses to doses of antibiotics or organic solvents must be monitored at the whole-cell level. Both wild-type cells and mutants may or may not contain additional, and often unknown, pumps. Moreover, the rate of influx of compounds will depend on their chemical structure, while the cell may alter the composition of its cell envelope, thus further obscuring the explanation of results. Nevertheless, on the basis of this type of experimentation it is now possible to distinguish three types of proton-dependent efflux systems in pseudomonads on the basis of compounds expelled from the cell. To date two efflux pumps have been described that are solely involved in the efflux of solvents and do not appear to be able to export antibiotics. This type of efflux pump includes SrpABC in *P. putida* S12 and TtgDEF in *P. putida* DOT-T1E and was shown to be induced by organic solvents (Kieboom *et al*., 1998a; Mosqueda & Ramos, 2000). Most of the efflux systems characterized in pseudomonads, however, export both antibiotics and solvents. These systems include the constitutive TtgABC pump in *P. putida* DOT-T1E (Ramos *et al*., 1998), the MepABC pump in *P. putida* KT2442 (Fukumori *et al*., 1998) and the Mex efflux systems in *P. aeruginosa* (Li *et al*., 1998). The third type is the ArpABC system reported here, which is involved in antibiotic resistance but not in solvent tolerance as shown by the phenotype of the *srp-arp* double mutant. However, a note of caution in identifying ArpABC as an antibiotic-removing pump and not as a solvent-removing system is appropriate. A solvent pump has to operate at a higher speed than an antibiotic pump due to the considerably higher influx of hydrophobic solvents compared to the influx of antibiotics. Toluene may be present at 5 mM in the aqueous phase, while antibiotics usually remain below 1 mM and it is to be expected that antibiotics will diffuse more slowly into the cell than do uncharged, small solvent molecules. Consequently, solvent efflux pumps may have to generate an efflux that is probably 10–100 times higher than the efflux created by the antibiotic pumps. If the expression level is not affected by the presence of solvent as substrate, as is the case for the ArpABC system, then the solvent-pumping ability may not be registered at the whole-cell level. We have tried to overcome this pitfall by using benzene as test solvent and by employing it at different concentrations. This method should be relatively suitable for detecting minor contributions to solvent pumping, but nevertheless, no effect of the presence of ArpABC on benzene sensitivity was observed.

The grouping of the known *Pseudomonas* efflux pumps according to their pumping activity for either solvent and/or antibiotics is not supported by the molecular structure of these pumps. The ArpB, TtgB and MepB proteins are almost identical at the amino acid level, which is quite surprising because they have been identified in strains isolated from three very different locations. Also, their A and C components are almost identical. ArpB differs only in one amino acid from TtgB (Ala-544 to Gly-544), while compared to MepB it differs in three amino acids (Ala-533 to Thr-533, Ala-544 to Gly-544 and Glu-692 to Ala-692). On the basis of the MexB membrane topology (Guan *et al*., 1999) it is expected that Ala-544 is located in a putative transmembrane segment, whereas Ala-544 and Glu-692 are periplasmic. These observations would lead to the conclusion that Gly-544 might be a key amino acid in interactions with antibiotics. However, as yet the substrate-binding domain of RND-type proteins has not been identified.

Alternatively, it might be argued that MepABC and TtgABC are present at higher levels in their respective hosts. For *P. putida* KT2442 it was anticipated that the *mep* operon was overexpressed in the toluene-tolerant variant TOL. Possibly a similar adaptation phenomenon has taken place already during the isolation of strain *P. putida* DOT-T1E. Therefore, in both strains, a high transcription level of the efflux operons may result in a high solvent efflux, required for the cell to survive in the presence of toluene. Similar results were reported for the *mexAB-oprM* efflux system in *P. aeruginosa*. In this strain a single substitution in the *mexR* regulator resulted in the overexpression of MexAB-OprM resulting in an increased multidrug resistance and organic solvent tolerance (Poole *et al*., 1996; Li *et al*., 1998; Li & Poole, 1999).

In summary, the intrinsic resistance of *P. putida* S12 to multiple antibiotics is due to efflux of these components by ArpABC. Whether ArpABC in *P. putida* S12 is unable

to transport solvent due to an amino acid substitution or due to the lack of overexpression, the extrusion of organic solvents by this efflux system is too low to prevent their influx but sufficiently high to combat the antibiotic influx. *P. putida* S12 does not have to rely on ArpABC-mediated solvent efflux for its solvent-tolerant phenotype, since it possesses the SrpABC efflux system that is induced under solvent stress.

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Received 17 May 2000; revised 25 August 2000; accepted 19 September 2000.