ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2004, p. 1416–1418 Vol. 48, No. 4
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.4.1416–1418.2004

Identification of the *Haemophilus influenzae tolC* Gene by Susceptibility Profiles of Insertionally Inactivated Efflux Pump Mutants

Catherine M. Trepod* and John E. Mott

Antibacterials, Pfizer, Groton, Connecticut 06340

Received 2 April 2003/Returned for modification 3 June 2003/Accepted 21 November 2003

Isogenic strains containing insertional disruptions of 10 *Haemophilus influenzae* Rd genes were investigated for their effects on the susceptibility of the organism to various classes of antimicrobial compounds. MIC results show that HI1462, which encodes an *Escherichia coli* TolC homolog, is the third component of the *H. influenzae* AcrAB pump.

Efflux pumps are of interest to the medical community because of their contributions to clinical antibiotic resistance, and they are of interest to the pharmaceutical industry as targets for the development of inhibitors that enhance antibiotic potency and spectrum. This study was undertaken to inactivate putative *Haemophilus influenzae* efflux pump homologs and determine their susceptibility profiles with selected antibacterial compounds. The availability of complete genome sequence data has allowed the systematic prediction of efflux pumps based on sequence homology. Paulsen et al. (8) performed a phylogenetic analysis of membrane transport systems across 36 sequenced organisms and have provided a compiled list of transporters categorized by protein family and transporter type that can be accessed at the Genomic Comparison of Membrane Transport Systems database (http://www.66.93.129.133/transporter/wb/index2.html). According to this database, *H. influenzae* contains six predicted multidrug efflux pumps: the major facilitator superfamily pumps encoded by HI0135 (*ydeA*), HI0852 (*yieO*), HI0897 (*emrB*), and HI1242 (*bcr*); a multidrug and toxic compound extrusion family pump encoded by HI1612 (*norM*); and a resistance nodulation-cell division superfamily (RND) pump encoded by HI0895 (*acrB*). HI0897 has been shown to be essential in *H. influenzae* and was not included in this study (1). Additional pumps studied included the membrane fusion protein HI0894 (*acrA*) (9) and the *emrA* homolog HI0898 (5). The contributions of the putative repressor of the *acrAB* genes encoded by HI0893 (*acrR*) (7) and HI0251 (*tonB*), which encodes a homolog of the *tonB* gene that has been shown to influence drug efflux pumps in *Pseudomonas aeruginosa* (12), were also investigated. It was thought that the inactivation of the *acrR* gene may increase resistance to those drugs to which the *acrA*- and *acrB*-inactivated strains have increased sensitivity.

The RND efflux pumps are tripartite pumps facilitating the efflux of compounds directly into the external medium rather than into the periplasm. In *Escherichia coli*, the AcrAB efflux pump is composed of the inner membrane transporter AcrB, the membrane fusion protein AcrA, and the outer membrane channel protein TolC (4, 6). The *H. influenzae* homologs of AcrA (HI0894) and AcrB (HI0895) have been shown by Sanchez et al. (9) to function as efflux genes. In that work, these investigators describe the disruption of the *H. influenzae* *acrA* and *acrB* genes and show the increased susceptibilities of these mutants to several antimicrobial compounds. However, no information demonstrating that the third component of the AcrAB pump exists in *H. influenzae* has been published to date. A putative TolC homolog (HI1462) sharing 21% sequence identity and 36% protein similarity with *E. coli* TolC has been identified in *H. influenzae* both by our own homology search and by a homology search published in Sharff et al. (10). In total, 10 genes were subjected to transposon mutagenesis for insertional inactivation, and the resulting strains were then evaluated for their susceptibilities to different classes of antimicrobial compounds.

A 3-kb region containing a targeted efflux gene and its flanking sequences was amplified from *H. influenzae* Rd genomic DNA (ATCC 51907) with primers designed to hybridize approximately 1,000 bp upstream and downstream of the target gene (Table 1). Following PCR amplification with BRL Platinum *Taq* (94°C for 5 min [1 cycle]; 94°C for 30 s, 55°C for 1 min, and 72°C for 3 min [30 cycles]; and 72°C for 5 min [1 cycle]), the resulting PCR product was subjected to in vitro transposon mutagenesis with the Epicentre EZ::TN <KAN-2> insertion kit and transformed directly into M-IV chemically competent cells of *H. influenzae* as described by Barcak et al. (2). Recombinant colonies were selected on Difco brain heart infusion medium supplemented with 5% Fildes enrichment (sBHI) and 30 μg of kanamycin/ml. Individual colonies were then screened by PCR for the presence of a transposon inserted within the first 25% of the target gene. DNA sequencing (Applied Biosystems) confirmed each insertion site. The susceptibility profiles of the 10 isogenic *H. influenzae* strains were determined by using 24 antimicrobials, dyes, and detergents that have been reported to be efflux pump substrates (11). MICs were determined by serial twofold dilution in sBHI with 5×10^4 cells/well as the inoculum. The plates were incubated overnight at 37°C with 5% CO_2 and scored for growth or no growth at 24 h (Table 2).

Inactivation of the RND transporters AcrA and AcrB, as well as that of the putative TolC, increased the susceptibility of *H. influenzae* to 16 of the 24 compounds tested. In all cases, the susceptibility profile of the putative *tolC*::*kan* strain matched those of the *acrA*::*kan* and *acrB*::*kan* strains. The chromosomal location of HI1462 indicates that it is not part of an operon, since adjacent genes are transcribed in the opposite direction

* Corresponding author. Mailing address: Antibacterials, MS/8220-2343, Pfizer Inc., Groton, CT 06340. Phone: (860) 686-6810. Fax: (860) 715-4693. E-mail: catherine.m.trepod@pfizer.com.

TABLE 1. Oligonucleotides used to construct *H. influenzae* Rd strains

Gene	Amplification primers[a]	Screening primers[b]
HI0135	5′ CACACAAAATCCTGCCAATG 3′ GCAACGGATGAGCACCAAC	5′ ATGGCGCGTGCTTATTATGG 3′ GCCAGTACCGCAATAATGCC
HI0251	5′ GGTAAGCGTGGCACATTATTC 3′ TATCAGCGTAGCAACCAGTG	5′ AACGTTCGCTATTGGGTTTG 3′ GGCTTTCCTTTTGGTTTTCC
HI0852	5′ TTGCTTATGCTTGCCTTGTG 3′ ACAAGAGTTGCAGCGGCGAG	5′ CCTATCGCGGTTTAGCTTGG 3′ ACTGGCGTGAATGACTAACC
HI0893	5′ GATGAATGGCCTGAACGCTG 3′ CCTGCTTTGCCATCAAATGG	5′ GACAGATCGTTTAATGGCAAG 3′ CCACATTTGTCGATACTGTTC
HI0894	5′ GTGCCACCGCAAGGACATAC 3′ GGCAGACATCACTGTTGGAC	5′ GCGTAATGATAAGCCGAGCC 3′ TTCACAATACCTGCTTTGCC
HI0895	5′ AAGGCGCAATGCTCAGTACAC 3′ GTTCTACGCGTTCTTCCATCC	5′ TCGTCGTCCTGTTTTAGCAG 3′ GCAGACATCACTGTTGGCAC
HI0898	5′ TGACCAACCGCTGCAAGCAG 3′ GCGACAGCACCTTGAATGAC	5′ GCAACTGAAAACCCATCAAC 3′ GATTCTTTATCAACGCACCC
HI1242	5′ CTCAAGCAACAAAAGCGATCC 3′ TACCGAGCTTCCTGCGAGTG	5′ CCCTCGGTATTCTCTCTATG 3′ ATGCAGCGGCTAATAATCCC
HI1462	5′ ACCTTCAACCTTATTTGCCC 3′ TGTAGCATCAGTCGCATCAG	5′ CACGCTTGCTTTGTTGATGTC 3′ AGCCACGCCTTGCACTAAAC
HI1612	5′ CTTTACGCCGCCCCATTAAG 3′ TTGCGAGTTGAGTGAGTTCC	5′ ATCGCACAAAACTCAATGGG 3′ CCCAGTTCACAATAGCTCTG

[a] Primers used to amplify the target gene and flanking sequences from the *H. influenzae* genome.
[b] Primers used to screen for the presence of a transposon insert.

TABLE 2. Susceptibilities of *H. influenzae* Rd mutant strains to toxic compounds[a]

Compound	MIC (μg/ml) for[b]:										
	Rd	HI0135 (*ydeA*) (227)	HI0251 (*tonB*) (331)	HI0852 (*yieO*) (269)	HI0893 (*acrR*) (61)	HI0894 (*acrA*) (112)	HI0895 (*acrB*) (382)	HI0898 (*emrA*) (309)	HI1242 (*bcr*) (72)	HI1462 (*tolC*) (135)	HI1612 (*norM*) (107)
Erythromycin	8	8	8	8	8	**0.25**	**0.25**	8	8	**0.25**	8
Puromycin	8	8	8	8	8	**2**	**2**	8	8	**2**	8
Tetracycline	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Chloramphenicol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Linezolid	8	8	8	8	8	**4**	**4**	8	8	**4**	8
Fusidic acid	1	1	1	1	1	**<0.06**	**<0.06**	1	1	**<0.06**	1
Ampicillin	4	4	4	4	4	**1**	**1**	4	4	**1**	4
Carbenicillin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Rifampin	0.25	0.25	0.25	0.25	0.25	**0.12**	**0.12**	0.25	0.25	**0.12**	0.25
Naldixic acid	0.5	0.5	**1**	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Norfloxacin	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032
Novobiocin	0.25	0.25	0.25	0.25	0.25	**<0.06**	**<0.06**	0.25	0.25	**<0.06**	0.25
Trimethoprim	2	2	2	2	2	**1**	**1**	2	2	**0.5**	**1**
EtBr[c]	4	4	4	4	4	**0.5**	**0.5**	4	4	**0.5**	**2**
Acriflavin	4	4	4	4	4	**2**	**2**	4	4	**2**	**2**
Proflavin	16	16	16	16	16	16	16	16	16	16	16
Crystal violet	4	4	**8**	4	4	**0.5**	**0.5**	4	4	**0.5**	4
SDS[d]	64	64	64	64	64	**16**	**16**	64	64	**16**	64
Na-cholate	>2,000	>2,000	>2,000	>2,000	>2,000	**250**	**250**	>2,000	>2,000	**250**	>2,000
Triton X-100	>10,000	>10,000	>10,000	>10,000	>10,000	**38**	**38**	>10,000	>10,000	**38**	>10,000
Rhodamine 6G	64	64	64	64	64	**2**	**2**	64	64	**2**	64
Plumbagin	4	4	4	4	4	4	4	4	4	4	4
CTAB[e]	16	16	16	16	16	**8**	**8**	16	16	**8**	16

[a] Boldface values differ from the Rd control value.
[b] Numbers appearing in parentheses with strain designators indicate the nucleotide position from the start codon of the inserted transposon as determined by DNA sequencing.
[c] EtBr, ethidium bromide.
[d] SDS, sodium dodecyl sulfate.
[e] CTAB, hexadecyltrimethylammonium bromide.

(3). This finding eliminates the possibility of polar effects of the *tolC*::*kan* insertion on downstream cotranscribed genes. The identical drug susceptibilities of the *acrA*::*kan*, *acrB*::*kan*, and *tolC*::*kan* strains provide strong evidence that these proteins are components of a single pump and that HI1462 is the *H. influenzae* TolC equivalent. Very minor effects were observed with only two other inactivated proteins, NorM (trimethoprim, ethidium bromide, and acriflavin) and TonB (nalidixic acid and crystal violet). We were unable to identify any substrates for the remaining pumps among the 24 compounds tested.

The data reported here for the *acrA* and *acrB* mutants agree with those of Sanchez et al. (9) except for those for fusidic acid susceptibility, for which Sanchez et al. found no effect. The fusidic acid results presented in Table 2 are consistent between the two mutant stains, as well as with the putative *tolC* mutant. The reasons for this difference are unknown.

Interestingly, the mutations in the *H. influenzae* AcrAB/TolC pump do not confer sensitivity to chloramphenicol, tetracycline, or fluoroquinolones, all substrates of *E. coli* AcrAB/TolC. Sanchez et al. (9) attributed this finding to a difference in permeability due to the wider porin channel in *H. influenzae*. These differences underscore the need to examine efflux pumps from one organism in other bacterial species.

Of the efflux pumps examined in this study, the AcrAB/TolC efflux pump is the primary *H. influenzae* efflux pump. These findings are in accordance with reports that other obligate organisms generally contain a limited number of multidrug efflux pumps (8). However, additional regulatory and environmental factors may contribute to the ability of other efflux pumps to modulate drug resistance in *H. influenzae*. These factors include varying growth conditions and the presence of other substrates that may activate these or other unidentified pumps.

We thank Cheryl Quinn and Alita Miller for their critical reading of the manuscript.

REFERENCES

1. **Akerley, B. J., E. J. Rubin, V. L. Novick, K. Amaya, N. Judson, and J. J. Mekalanos.** 2002. A genome-scale analysis for identification of genes required for growth or survival of *Haemophilus influenzae*. Proc. Natl. Acad. Sci. USA **99:**966–971.
2. **Barcak, G. J., M. S. Chandler, R. J. Redfield, and J.-F. Tomb.** 1991. Genetic systems in *Haemophilus influenzae*. Methods Enzymol. **204:**321–342.
3. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. Fitzhugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
4. **Fralick, J. A.** 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178:**5803–5805.
5. **Lomovskaya, O., and K. Lewis.** 1992. Emr, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
6. **Ma, D., D. N. Ciik, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
7. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
8. **Paulsen, I. T., L. Nguyen, M. K. Sliwinski, R. Rabus, and M. H. Saier.** 2000. Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. J. Mol. Biol. **301:**75–100.
9. **Sanchez, L., W. Pan, M. Vinas, and H. Nikaido.** 1997. The *acrAB* homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. J. Bacteriol. **179:**6855–6857.
10. **Sharff, A., C. Fanutti, J. Shi, C. Calladine, and B. Luisi.** 2001. The role of the TolC family in protein transport and multidrug efflux. Eur. J. Biochem. **268:**5011–5026.
11. **Sulavik, M. C., C. Houseweart, C. Cramer, N. Jiwani, N. Murgolo, J. Greene, B. DiDomenico, K. J. Shaw, G. H. Miller, R. Hare, and G. Shimer.** 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob. Agents Chemother. **45:**1126–1136.
12. **Zhao, Q., and K. Poole.** 2002. Differential effects of mutations in *tonB1* on intrinsic multidrug resistance and iron acquisition in *Pseudomonas aeruginosa*. J. Bacteriol. **184:**2045–2049.