NOTES

Emergence of the Trimethoprim Resistance Gene *dfrD* in *Listeria monocytogenes* BM4293

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The sequence of the trimethoprim resistance gene of the 3.7-kb plasmid (pIP823) that confers high-level resistance (MIC, 1,024 μ g/ml) to *Listeria monocytogenes* BM4293 was determined. The gene was identical to *dfrD* recently detected in *Staphylococcus haemolyticus* MUR313. The corresponding protein, S2DHFR, represents the second class of high-level trimethoprim-resistant dihydrofolate reductase identified in gram-positive bacteria. We propose that trimethoprim resistance in *L. monocytogenes* BM4293 could originate in staphylococci.

Listeria monocytogenes is a gram-positive pathogen responsible for severe food-borne infections which can lead to spontaneous abortion in pregnant women and to meningitis, meningoencephalitis, and septicemia primarily in newborns, immunocompromised patients, and elderly people (12). First-choice treatment of listeriosis generally consists of ampicillin combined with gentamicin or of co-trimoxazole (7, 11). Since 1988, more than 80 strains of *L. monocytogenes* resistant to one or more antibiotics have been isolated from food, the environment, or patients with sporadic cases of listeriosis (2).

Dihydrofolate reductase (DHFR) is a key enzyme in the tetrahydrofolic pathway, in which it catalyzes the NADPHdependent reduction of dihydrofolate to tetrahydrofolate (10). Due to structural analogy with dihydrofolate, trimethoprim is a competitive inhibitor of this enzyme in bacteria (10). The most common mechanism of resistance to trimethoprim is plasmidmediated production of an additional trimethoprim-resistant DHFR which can function in place of the susceptible host chromosomal enzyme. In gram-negative bacteria, a minimum of 17 different plasmid-encoded DHFRs have been described (10). By contrast, only two types of DHFR that confer resistance to trimethoprim have been described in gram-positive bacteria. The type S1 enzyme encoded by the dfrA gene located in transposon Tn4003 has been found in Staphylococcus aureus, Staphylococcus haemolyticus, Staphylococcus epidermidis, and Staphylococcus hominis (3, 13), and recently, the type S2 DHFR encoded by dfrD carried by plasmid pABU17 has been detected in S. haemolyticus MUR313 (4).

In a previous study, we described the first strain of *L. mono-cytogenes* (strain BM4293) resistant to high levels of trimethoprim (MIC, 1,024 μ g/ml). This strain, isolated from the environment in France, harbors plasmid pIP823, of 3.7 kb, which is responsible for the resistance (2). Since the resistance

determinant did not hybridize with a *dfrA*-specific probe (2), we decided to clone and sequence this new gene.

(Part of this work was presented at the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy [1].)

Plasmid pIP823 DNA partially digested with Sau3A and pUC18 DNA digested with BamHI were mixed, ligated, and introduced by transformation into Escherichia coli DH5a (Gibco BRL, Eragny, France). Cloning was performed with restriction endonucleases (Pharmacia Biotech, Saclay, France), T4 DNA ligase (Pharmacia), and alkaline phosphatase (Pharmacia) by standard methods (14). Transformants were selected on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) containing 100 µg of ampicillin (Laboratories Panpharma, Fougères, France) per ml and 5 µg of trimethoprim (Roche, Fontenay-sous-Bois, France) per ml and were screened for their plasmid contents by agarose gel electrophoresis of crude bacterial lysates. The smallest recombinant plasmid, pAT460, was found to contain a 1.1-kb insert. E. *coli* LH18 *thyA* Δ *fol::kan*, from which the gene for DHFR has been deleted, is a mutant auxotroph for thymine which is thus unable to grow on Mueller-Hinton agar (9). This strain, after acquisition of pAT460, was able to grow on Mueller-Hinton plates, whereas the same host containing pUC18 was not. This trans-complementation assay indicated that pAT460, which conferred resistance to the new host, encodes a functional DHFR.

The nucleotide sequence of both strands of the pAT460 insert was determined by the dideoxynucleotide chain-termination method (15) with T7-modified DNA polymerase (Sequenase; United States Biochemicals, Cleveland, Ohio), $[\alpha$ -³⁵S]dATP (Amersham France, Les Ulis, France), and oligonucleotides complementary to the sequence synthesized by the methoxy phosphoamidite method (Unité de Chimie Organique, Institut Pasteur, Paris, France). This sequence has been submitted to the GenBank database (accession number U43152). A search for stop codons in the three reading frames of each DNA strand revealed the presence of a single open reading frame. Two putative translational initiation codons, ATG at coordinate 548 and TTG at coordinate 560, preceded by a typical Shine-Dalgarno sequence were identified. The

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	NN	N		N	N					N	
	TTT .	NN .TN	TT.T		TT T	. NNN		0071100		TNN N	100
type S2	LKISLIVAMD.H	KKRVIGKDNDIPWR.	ISSDWEYVKNTTKGH	AIILGRKNL	QSIG.RALP	DRRNILLIR	. DKNFNFK . D	CEIAHS	. IEAAF.KL.CEN.EE.EV.	FIFGGLQ	100
type S1	MTLSIIVAHD.H	KQRVIGYQNQLPWH.	LPNDLKHIKQLTTGN	TLVMARKTF	NSIG.KPLP	NRRNVVLTN	QASFHELG	VDVINS	.LDEIK.EL.SGHV	TVPCOCE	
type Ia	MKLSLMVAIS.E	KNGVIGNGPDIPWS.	AKGEQLLFKAITYNQ	WLLVGRKTF.	ESMGALP	NRKYAVVTR	CONTROLED N	VLIFPS	. IKDAL. TN. LKKITD. HV.	TVSGGGE	
type Ib	LKVSLIAAKA.	KNGVIGCGPDIPWS.	AKGEQLLEKALTYNQ	CLLVGRRTF.	ESMGALP	ODDMBR/OD	NDOWONE O	VVVfQ5	. IEEAMDRL.AEF.TG.HV.	MITCOCO	
type IIIa	MLISLIAALA.	HNNLIGKDNLIPWH.	LPADERHFRAVTEGR	PVVMGRRTF.	ESIG. RPLP	GRENVVVSR	UNPOWOAE.G	UTUEDO	. LDAAL.AL.LTD.CE.EA.	TVECCCE	
type V	MKVSLMAAKA.	KNGVIGCGPHIPWS.	AKGEQLLFKALTYNQ	WLLVGRKTF.	ESMG. ALP	NRKIAVVIR	. SAWIADNDN	WINEDS	. IEEAM. YG. LAELTD. HV.	VVBCCCCO	
type VII	LKISLISATS.	ENGVIGNGPDIPWS.	AKGEQLLFKALTINQ	WILLVGRKTF	DSMGVLP	ODDOTIMOD	ON LELDON		, IEIAL.QE.LSKITD.HL. NVSDAM.RF.AQEESVGDVA		
type VIII/IIIc	MIELHAILAAT./	ANGCIGKDNALPWPF	DESCRIPTION OF A CONTRACT OF A	VVIMGRETT.	ESLEVK.LE	GRICIVMIR	TUPPTONTC	OWWGAIT W	AVRTAS.LL.VDKPEYSQI	FUTCOKS	
type IX	MASLNMIVAVN.I	KTGGIGFENQIPWH.	EPEDLKHFKAVIMNS	VLINGRATE	ASLP.KVLP	GRENVVVSK	TTTELEVIN	NNTOTEKSEF	SFLEAF.RD.TTKPIN	VIGGNG	
type X	MNISLIFANEL	TTRAFGNQGKLPWQF	TREDMORFORITENS.	VVVMGLNIW.	RSDPARAAL	NDUPIVICD	ONNYDAT O	CULTRAL	.LSHAI.AL.ASELGN.EL	VVACCAE	
type XII	MNSESVRIYLVAAMG.	ANRVIGNGPNIPWK.	I PGEQKIFREITEGK	VVVMGRAIF.	ESIG. NPLF	NERTINE	ADDOFFO C	CTUNES	.LKDVL.DI.CSG.PE.EC	FVTGGAO	
B. subtilis	MISFIFAMD.A	ANRLIGKDNDLPWH.	I DNDLAIFARIISGE	SIINGRAIF.	ESIG.RPLP	NDDMAILTO	DISENVE C	VDWTHS	.IEDIY.QL.PGHV	FIRGOT	
S. aureus	ILSIGVAND.	LORVIGPENQUEWN.	I DNDI KHVKKDSIGH	TUMORICIT	MGTO VDLE	NEENAULTN	OASENNE G	VDVINS	.LDEIK.EL.SGHV	FIRGOT	
S. epidermidis	MTRATISTIVAND.	RURVIGIUNULPWH.	I DARLOURVETTINU	ATL MORATE	DOMORRILE	KRETLITE	NPEEKID.G	VATEOD	.VQSVL.DW.YQD.QEKNL	YIIGGKO	
S. pneumoniae	MIKKIVAIWAQD.I	EEGVIGKENKLEWR.	I DAROUNEVETTMNO	VTI MODETE	FOMNERVIE	CRISILTR	DETYOSE N	EKVLIMHS	. PKEVL. DWYYKQ. DK. DL	FTTGGAE	
L. lactis	METCHNACD	EVGLIGEADAMEWS.	LONDWOLDSTIMUA	TIMORATY	EGMGK1.SLE	VENTIVLTT	OKDEKVEKN	AEVLHS	.IDELL.AY.AKDIPE.DI	YVSGGSR	
E. faecium	MTATIWAQD	NIGLIGADGELFWA.	L'DDDLHVERAOTVCK	TMAAGRATY	ESEPKEPLE	ERTNVVLTH	OEDYOAO.G	AVVVHD	.VAAVF.AY.AKQHPDQEL	VIAGGAO	
L. casei E. coli	MIAF DWAQD.	UDDUT CMENN MDWNI	LDADIAWERDNTINK	DVIMORHTW	ESTG RPLE	GRENTTLSS	OPGTD. D. R		.VDEAL.AA.CGD.VP.EI	MVIGGGR	
E. com E. aerogenes	MISLIANLA	VDRVICMENAMPWD	LPADLAWFKRNTLNK	PVVMGRLTW	ESTG.RPLE	GRKNIVISS	.KPGSD.D.R	VOWVKS	.VDEAI.AA.CGD.AE.EI	MVIGGGR	
N. gonorrhoeae	MLKTTTTAACA	ENLCIGAGNAMPWH	TPEDFAFFKVYTLGK	PVTMGRKTW	ESLPVKPLF	GRRNIVISR	.OADYCAA.G	AETVAS	.LEVAL.AL.CAG.AE.EA	.VIMGGAQ	
	NN . T	. NN									
type S2	NN . T IYVMFLPYVEKMYVTK		V VNFDDWKEVS	VEKGIKDEK	NPY DYYFH	TYERIR*		162			
type S1	LYEAMIDQVDDMYITV	ID2 KEOGDTFFF	P. YTEENWEVES	SVEGOLDEK	NTT.PHTFI	HLVRRKGK*					
type Ia	IYKSLIDQVDTLHIST	IDT EPEGDVYFP.	E. TP. SNERPVE	TODFASN	I.NYSYC	IWOKG*					
type Ib	ITRETLPMASTLHLST	IDI. EPEGDVEEP.	SIPNTFEVV	FEOHFTSNI	NYCYC	IWKKG*					
type IIIa	LYAEALPRADRLYLTY	IDA. OLNGDTHFP.	D.YLSLGWOELE	RSTHPADDK	NSY.ACEFV	TLSRQR*					
type V	IYRETLPMASTLHIST	IDIEPEGDVFFP.	N. IP. NTFEVVF	EQHFSSN	I.NYCYC	IWQKG*					
type VII	IYNSLIEKADIIHLST	VHVEVEGDINFP.	K.IP.ENFNLVF	EQFFLSN	I.NYTYC	IWKKG*					
type VIII/IIIc	IFKRLALMITQIELTF	VKRLYEGDTYV	DLAEMVK	DYEQNGMEE	HD.LHTYFI	YRKKELTE*					
type IX	AYENLAAYVDKLYLTR	VQLNTQQDTELD.	L.SLFKSWKLVS	EVPTITENK	TKL.IFQIW	INPNPISEE	PTC*				
type X	LLSEAIEHASTVYMSS										
type XII	IYTLALPHAHGVFLSE										
B. subtilis	L Y TDLFPYADRLYMTK										
S. aureus	LFEEMIDKVDDMYITV										
S. epidermidis	LFEAMIDQVDDMYITV										
S. pneumoniae	IFQAFEPYLDEVIVTH										
L. lactis	ILALFESELELLYRTV										
E. faecium	IFQALLPETKIIWRTL										
L. casei	IFTAFKDDVDTLLVTR										
E. coli	VYEQFLPKAQKLYLTH										
E. aerogenes	VYEQFLPKAHKLYLTH	IDA. EVEGDIHFP.	D.YDPDEWESVF	SEFRUAUAQ	NSH. SICH	TDEKK					
N. gonorrhoeae	IYGQAMPLATDLRITE	VDLSVEGDAFFP	E.IDRTHWREAE	KI EKKVSSK		n1LGK".,.					

FIG. 1. Alignment of the deduced amino acid sequences of S2DHFR from *L. monocytogenes* BM4293, additional bacterial trimethoprim-resistant DHFRs (types S1 to XII), and prokaryotic chromosomal DHFRs. The sequence numbering is based on that of S2DHFR. The stop codons are indicated by asterisks. The amino acids at positions 32, 96, and 102 in S2DHFR are indicated in boldface type. The amino acid positions involved in the binding of trimethoprim (T) and NADPH cofactor (N), based on studies of the *E. coli* K-12 enzyme (3, 4), are indicated. The Swissprot and GenBank accession numbers of the additional and chromosomal DHFRs are as follows: type S1, P13355; type Ia, P00382; type IIIa, P12833; type V, P11731; type VII, P27422; *Bacillus subtilis*, P11045; *S. aureus*, P10167; *Enterobacter aerogenes*, P31074; and *Neisseria gonorhoeae*, P04174 in the SwissProt database and type Ib, Z50805; type III, U10186; type IX, X57730; type X, L06418; type XII, Z21672; *S. epidermidis*, Z48233; *Streptococcus pneumoniae*, Z74778; and *Lactococcus lactis*, X60681 in the GenBank database.

486-bp sequence from the TTG codon at coordinate 560 to the TAA codon at coordinate 1046, designated dfrD, could code for a protein of 162 amino acid residues with a calculated molecular mass of 19,273 Da, designated S2DHFR. The G+C content of dfrD (31.5%) was more similar to that of Staphylococcus DNA (34%) than to that of DNA from L. monocytogenes (38%) and Enterococcus (38%). The deduced amino acid sequence of type S2 DHFR encoded by pIP823 was aligned with those of known DHFRs in the SwissProt and GenBank databases by using the Genetics Computer Group program (Fig. 1) (5, 8). This sequence was found to be identical to that of the recently described S2DHFR encoded by dfrD of pABU17 from S. haemolyticus MUR313 (4). The two enzymes differ by their number of amino acid residues since the ATG translational initiation codon was chosen for the S2DHFR from S. haemolyticus. On the basis of the structural and kinetic properties, the active site of S2DHFR is very closely related to that of the resistant and susceptible DHFRs already described in staphylococci (3, 4, 13). The S2DHFR was more distantly related to the S1DHFR from S. aureus, with 38.1% amino acid identity, and it showed less similarity with trimethoprim-resistant DHFRs from gram-negative bacteria (from 22.2% with type X DHFR to 37.7% with type IIIa DHFR).

In contrast to plasmid pABU17 (3.8 kb) from *S. haemolyticus*, plasmid pIP823 (3.7 kb) from *L. monocytogenes* does not

possess a *Bam*HI site, and the three *Eco*RI sites present in the two plasmids yield restriction fragments of different sizes (Fig. 2). These plasmids therefore appear to be different. However, the 81-bp sequence upstream and the 203-bp sequence downstream from *dfrD* are identical in pIP823 and pABU17 (data not shown). Plasmid pIP823 is able to replicate and is stable in *E. coli* HB101 and *S. aureus* RN4220, where it confers highlevel trimethoprim resistance (2; data not shown). It is therefore likely that pIP823 belongs to the family of rolling-circle replicating plasmids, common in staphylococci, that possess a broad host spectrum including gram-positive and gram-negative bacteria (2, 6).



FIG. 2. Map of 3.7-kb plasmid pIP823 from *L. monocytogenes* BM4293. Only relevant restriction sites are indicated. The open arrow represents the direction and extent of translation of *dfrD*. The bar at the bottom represents 500 bp.

Our results suggest that the trimethoprim resistance gene *dfrD* from *L. monocytogenes* BM4293 could originate in the genus *Staphylococcus*. However, it would be interesting to characterize the trimethoprim-susceptible chromosomal *dfr* gene of *L. monocytogenes* to obtain additional information on the origin of *dfrD* and on the divergence and the evolution of DHFRs from gram-positive bacteria. The emergence of trimethoprim resistance in *L. monocytogenes* is of particular interest since the trimethoprim-sulfamethoxazole combination is a successful alternative treatment for human listeriosis (7). Dissemination of plasmid pIP823 from BM4293 to other strains of *L. monocytogenes* and to other species of *Listeria* is likely. Acquisition by *L. monocytogenes* of other resistance genes carried by this family of plasmids can also be anticipated.

Nucleotide sequence accession number. The nucleotide sequence of the pAT460 insert has been submitted to the Gen-Bank database and has been given accession number U43152.

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